Biochemical Pharmacology, Vol. 51, pp. 1489–1501, 1996.

ISSN 0006-2952/96/$15.00 + 0.00
PII S0006-2952(96)00090-1

Inhibition of Squalene Synthase *In Vitro* by 3-(Biphenyl-4-yl)-Quinuclidine

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ABSTRACT. Squalene synthase (SQS) catalyses a step following the final branch in the pathway of cholesterol biosynthesis. Inhibition of this enzyme, therefore, is an approach for the treatment of atherosclerosis with the potential for low side effects. We have characterised the inhibition of rat liver microsomal SQS by 3-(biphenyl-4-yl)quinuclidine (BPQ). BPQ follows slow binding kinetics in that the rate of accumulation of product decreases with time if the inhibitor is added when the assay is started. Preincubation of BPQ and SQS leads to a biphasic dose-response where accumulation of product is linear with time only for the sensitive phase. When the farnesyl pyrophosphate (FPP) substrate is present at 19.6 μM, approximately 77% of the SQS activity is sensitive to the inhibitor (v_{0s}) and the remainder is insensitive (v_{0i}). The apparent inhibition constants (K'_i values) are respectively K'_{is} = 4.5 nM and K'_{ii} = 1300 nM. Similar biphasic behaviour is exhibited by other inhibitors and in microsomes prepared from human and marmoset liver. As the concentration of FPP is reduced below 19.6 μM, there is a decrease in the relative contribution from v_{0i}. Conversely, the value of K'_{is} for BPQ remains constant when the FPP concentration is changed, showing noncompetitive kinetics with respect to this substrate. Possible causes of the observed kinetics are discussed. Inhibition by BPQ is said to follow tight binding kinetics because the value of K'_{is} is similar to the concentration of inhibitor binding sites. Thus, to avoid an artefactual variation in potency when the enzyme concentration is varied, it is necessary to allow for the effects of depletion of free inhibitor. Furthermore, estimates of potency that average activity across the two phases are influenced by the relative contributions of each phase. These contributions differ according to the FPP concentration and the species used as the source of microsomes. Thus, it is necessary to separate the phases to compare measurements made in different experiments. Our observations indicate that careful experimental design and data analysis are required to characterise the kinetics of SQS inhibitors. BIOCHEM PHARMACOL 51;11:1489–1501, 1996.

KEY WORDS. anticholesterolemic agents; atherosclerosis; enzyme inhibitors; hypercholesterolemia; quinuclidines; squalene synthase

Reduction of plasma cholesterol levels is associated with reduced mortality in patients with a history of coronary heart disease [1]. Inhibitors of cholesterol biosynthesis, such as the 3-hydroxy-3-methylglutaryl CoA reductase inhibitors, lovastatin and simvastatin, are effective cholesterol-lowering agents in man and animals [1, 2]. However, the results from clinical trials suggest that there is scope to reduce adverse effects and improve efficacy for cholesterol-lowering agents (for example, see Ref. 3).

The mammalian isoprenoid pathway not only produces sterols, but also generates dolichol, ubiquinone, the farnesyl group of haem A, the farnesyl and geranylgeranyl groups of prenylated proteins, and the isopentenyl side-chain of isopentenyl adenine. The pathways for the synthesis of these isoprenoids diverge from the synthesis of cholesterol either at, or before, the FPP† branch point [4]. Thus, SQS (EC 2.5.1.21), which catalyses the reductive dimerization of FPP to squalene *via* the intermediate presqualene pyrophosphate (Fig. 1, see Ref. 5), is the first committed step in sterol biosynthesis.

Reductions in the levels of ubiquinone and possibly other noncholesterol metabolites of mevalonate have been im-

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† *Abbreviations:* AA, diphenylmethyl 4,8,8-trimethyl-1-azaadamantane-4-carboxylate; BPQ, 3-(biphenyl-4-yl)-quinuclidine; BPQ-OH, 3-(biphenyl-4-yl)-3-hydroxyquinuclidine; FPP, farnesyl pyrophosphate; k, apparent rate constant for transition from initial to final velocity; k_{cat}, turnover number (moles product/mole enzyme/sec); K_a, product of the apparent dissociation constants for the first and second molecules of FPP to bind; K_b, apparent dissociation constant for the second molecule of FPP; K_i, inhibition constant; K'_i, apparent inhibition constant; K'_{ii} and K'_{is}, apparent inhibition constants for insensitive and sensitive phases, respectively; LK_a, log of K_a; LK'_i, log of K'_i, LK'_{ii}, and LK'_{is}, logs of K'_{ii} and K'_{is}, respectively; SQS, squalene synthase; V, rate at saturating substrate concentration; v_{0i} and v_{0s}, uninhibited rates for inhibitor-insensitive and sensitive phases respectively; v_1, initial rate; v_2, final rate; v_{ss}, steady-state rate.

Received 11 July 1995; accepted 30 January 1996.

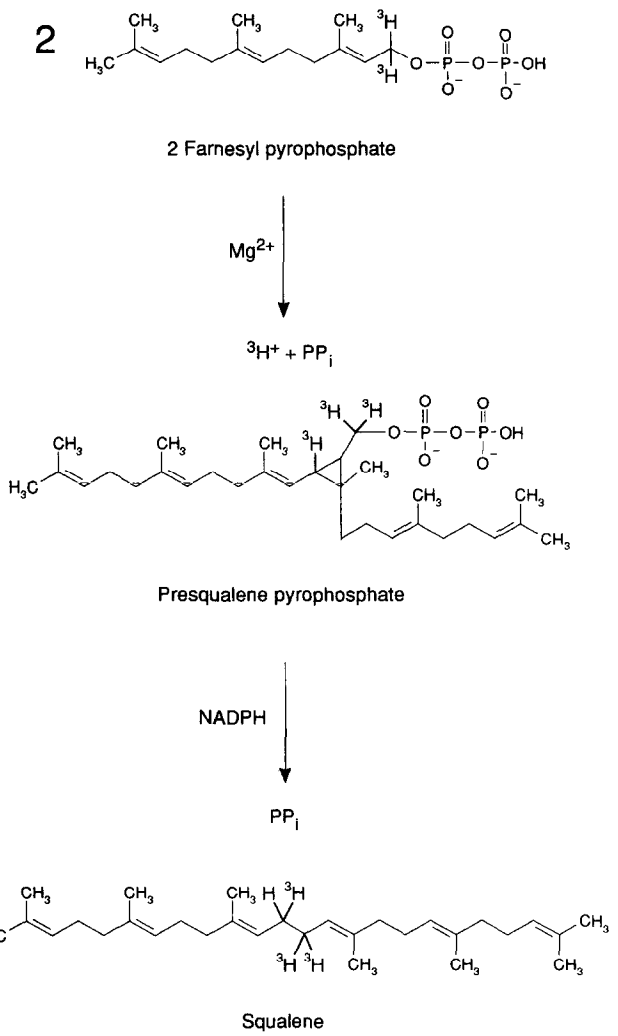

FIG. 1. The reaction catalysed by SQS. In the SQS assay, the FPP substrate is tritiated at C1. The label is uniformly distributed between the two prochiral hydrogens. Thus, one mole of tritium is released as protons for each 3 moles that are included in squalene.

plicated in generating some of the side effects of 3-hydroxy-3-methylglutaryl CoA reductase inhibitors [6, 7]. A specific inhibitor of SQS should inhibit the biosynthesis of squalene, avoid depletion of other isoprenoids, and possibly show reduced side effects. Furthermore, FPP, the substrate for SQS, is water soluble and may be readily metabolized [8], so that inhibition should not lead to excessive accumulation. The potential of SQS inhibitors is illustrated by experiments in rats, where significant inhibition of cholesterol biosynthesis by lovastatin occurs with concomitant inhibition of dolichol and coenzyme-Q_9 biosynthesis. In contrast, a bisphosphonate SQS inhibitor has no effect on these metabolites under conditions where cholesterol biosynthesis is inhibited >90% [9]. Similarly, the SQS inhibitor, squalestatin 1, blocks cholesterol synthesis and leads to increased production of ubiquinones in tissue culture [10].

SQS is a membrane-bound microsomal enzyme [11]. DNA encoding SQS from two species of yeast, rat, and man has been cloned [12–16]. Truncated forms have been produced by partial proteolysis and genetic engineering. These derivatives are soluble enzymes with similar kinetic properties to native microsomal SQS [17–20],* suggesting that SQS contains a soluble catalytic fragment and a C-terminal membrane anchor.

Pharmacological interest has focused on anionic SQS inhibitors: FPP analogues [21–23], bisphosphonates [9], and tricarboxylic acids (known as squalestatins or zaragozic acids) [24, 25]. These compounds are reported to follow competitive kinetics with respect to FPP. We, now, describe the first kinetic characterisation of a representative from a novel cationic series of SQS inhibitors, the 3-substituted quinuclidines. The activity of one of these compounds, BPQ-OH, has been characterised *in vivo* [26]. This compound inhibits the biosynthesis of cholesterol in rat liver at, and not before, SQS. It has no effect on the synthesis of dolichols or ubiquinone 9. The activity of a related compound, BPQ (Fig. 2), *in vitro* is characterised in the present work. We show that BPQ does not exhibit kinetic competition with respect to FPP, and we present the first demonstration of biphasic dose-response for inhibitors of SQS, with the two phases exhibiting a marked difference in affinity. Potent SQS inhibitors follow tight binding kinetics because the concentration of binding sites is similar to the concentration of compound added to the assay. We show how the occurrence of biphasic, tight binding kinetics makes it essential to use appropriate experimental design and rigorous data analysis to measure correctly the potency of SQS inhibitors.

* Using a truncated enzyme that contains residues 25 to 383 of human SQS, Ward WHJ, Holdgate GA, Davidson RG, Pioli D, Abbott M and Charles AD, unpublished data.

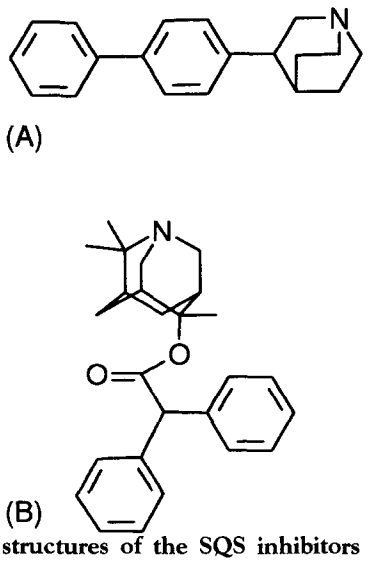

FIG. 2. The structures of the SQS inhibitors used in this study. (A) 3-(biphenyl-4-yl)-quinuclidine. (B) Diphenylmethyl 4,8,8-trimethyl-1-azaadamantane-4-carboxylate.

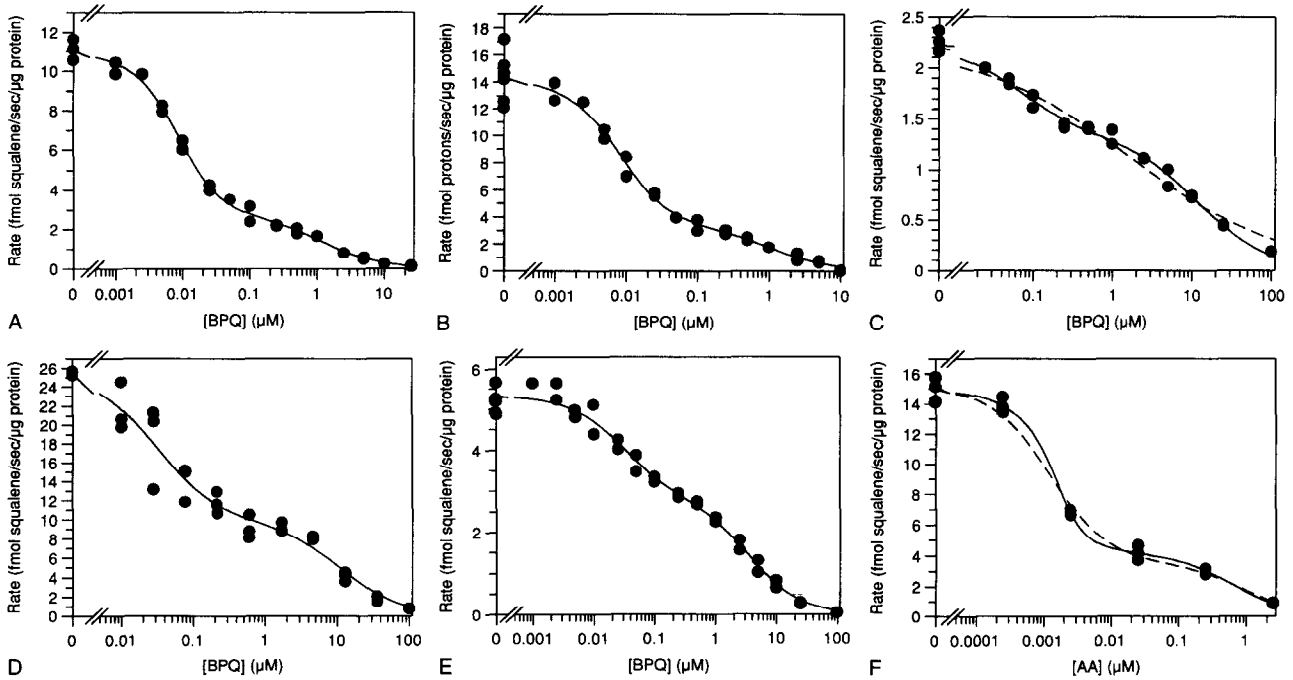

FIG. 3. Dose-response curves for inhibition of SQS. Graphs (A, and C to F) show inhibition of squalene production, whereas graph (B) shows inhibition of proton release. Rat liver microsomes were used in (A, B and F), human liver microsomes in (C), human recombinant SQS in (D) and marmoset liver microsomes in (E). The SQS inhibitor was BPQ in graphs (A to E), and AA in (F). These experiments were performed using the standard assay procedure (see Materials and Methods), except for (D) which was at 0.3 µM FPP. The solid lines were calculated using the best fit parameters in Table 1. The broken line in (C) was calculated by fitting Eqn (13), and that in (F) was calculated by fitting Eq (1) (see Discussion).

MATERIALS AND METHODS

Reagents were purchased from the Sigma Chemical Co. (Poole, U.K.), BDH (Lutterworth, U.K.), and NEN (Stevenage, U.K.). SQS inhibitors were synthesized at ZENECA Pharmaceuticals [27, 28]. Microsomes were prepared from rat and marmoset liver as described by McTaggart *et al.* [26]. Human liver microsomes were supplied by the Keystone Skin Bank, Exton, PA.

Assay of SQS Activity

The procedures are detailed by McTaggart *et al.* [26]. Briefly, in the standard assay, microsomes were preincubated for 15 min at 37° with the inhibitors (total volume 180 µL) prior to addition of 20 µL FPP to start the reaction. The assay was performed at 37° in 50 mM phosphate buffer, pH 7.4, and 19.6 µM [1-^{3}H]FPP (approximately 30 Ci/mol) and 0.9 mM NADPH in the presence of 4.95 mM $MgCl_2$ (essential for SQS activity) and 9.9 mM KF (to inhibit phosphatases). Inhibitors were dissolved in DMSO, which was added to a final concentration of 2.5% (v/v). After 15 min the reactions were stopped by addition of KOH to 0.8%. The mixtures were applied to C18 reverse phase columns. Fraction 1 was eluted using 0.1 M KOH, and fraction 2 was released using 10% (v/v) ethyl actetate in hexane. Fraction 1 contains the protons released in the first step of the SQS reaction (Fig. 1), and fraction 2 contains squalene and squalene epoxide. Assays were performed under initial rate conditions, following only the part of the time-course when substrate depletion is low (typically below 5%). The critical micelle concentration for FPP under the assay conditions was measured as 40 µM by using a fluorimeter to detect any dispersion of light by micelles at various concentrations of the substrate. This value is in agreement with that of 50 µM measured by Mookhtiar *et al.* [20] under similar conditions. Thus, FPP does not form micelles under any of the conditions described in the current work.

Analysis of Measured Rates

Equations were fitted to data by unweighted nonlinear regression using *GraFit*™ [29]. When substrate and inhibitor were varied in the same experiment, multivariate regression was used. Identification of the most suitable rate equation was assisted by an *F*-test [30] that was used to compare the residual sum of squares obtained after fitting the various relationships. The best rate equation was taken as that with the most fitting parameters, none of which was redundant. The *F*-test estimated the probability, *P*, that the improvement in fit associated with an extra parameter arose due to chance. Thus, this test indicated that the term was justified by the data if *P* was low (<1%), but not if it was high (>20%). The following additional criteria were used to help selection of the best rate equation: reasonable parameter values and SEs, and the residual differences between ob-

served and calculated rates following a random distribution [30].

Rate Equations

Biphasic inhibition kinetics were analysed using the following equation:

$$v = \{v_{0s}/(1 + [I]/10^{LK_{i's}})\} + \{v_{0i}/(1 + [I]/10^{LK_{i'i}})\} \quad (1)$$

where v is the measured rate, v_{0s} and insensitive phases, and LK'_{is} and LK'_{ii} are the logs of the apparent inhibition constants for the sensitive and insensitive phases. This relationship was obtained from the standard rate equation for reversible enzyme inhibition [31] by adding another component due to the second phase.

Log dissociation constants are widely used in the characterisation of receptors (see Ref. 32). The logarithmic procedure and the standard arithmetic method give the same parameter values and residual sum of squares. They differ in that one calculates the SE of log K'_i, whereas the other estimates the SE of K'_i. SE values can be used to estimate the confidence limits of parameter values (see Ref. 32). Direct calculation of K'_i gives confidence limits that are arithmetically distributed about the best fit value, whereas calculation of LK'_i leads to geometrically distributed confidence limits. The logarithmic fitting approach is more reasonable because K'_i values are more likely to be correct within a certain factor, rather than a certain arithmetic range. We also determined logarithmic values for the concentration of binding sites.

When the range of inhibitor concentrations is sufficient to inhibit only the sensitive phase, equation (1) becomes:

$$v = \{v_{0s}/(1 + [I]/10^{LK_{i's}})\} + v_{0i} \quad (2)$$

Potent compounds follow tight binding kinetics, where equation (1) changes to:

$$v = \{0.5v_{0s}(-b + \{b^2 + 4[10(10^{LK_{i's}-LSi})]^{0.5}\}) + \{v_{0i}/(1 + [I]/10^{LK_{i'i}})\} \quad (3)$$

where:

$$b = 10^{(LK_{i's}-LSi)} + [I]/10^{LSi} - 1 \quad (4)$$

and LSi corresponds to the log of the concentration of inhibitor binding sites. These relationships were obtained from the rate equation for tight binding inhibition [32].

RESULTS

Inhibition of SQS by BPQ Follows Biphasic Kinetics

The SQS assay used in this work generates two fractions. Fraction 1 contains the protons that are released in the first step of the reaction catalysed by SQS, and fraction 2 contains squalene that is formed in the second step (Fig. 1). The compositions of fractions 1 and 2 have been analysed by TLC, and HPLC followed by UV spectroscopy [26]. Following incubation of rat microsomes with 19.6 μM FPP, the total radioactivity in fraction 1 is around 40% of that in fraction 2. This is close to the value of 33% that is expected when utilising FPP that is uniformly tritiated at the C1 position (Fig. 1). In fraction 1, 80% of the radiolabel is

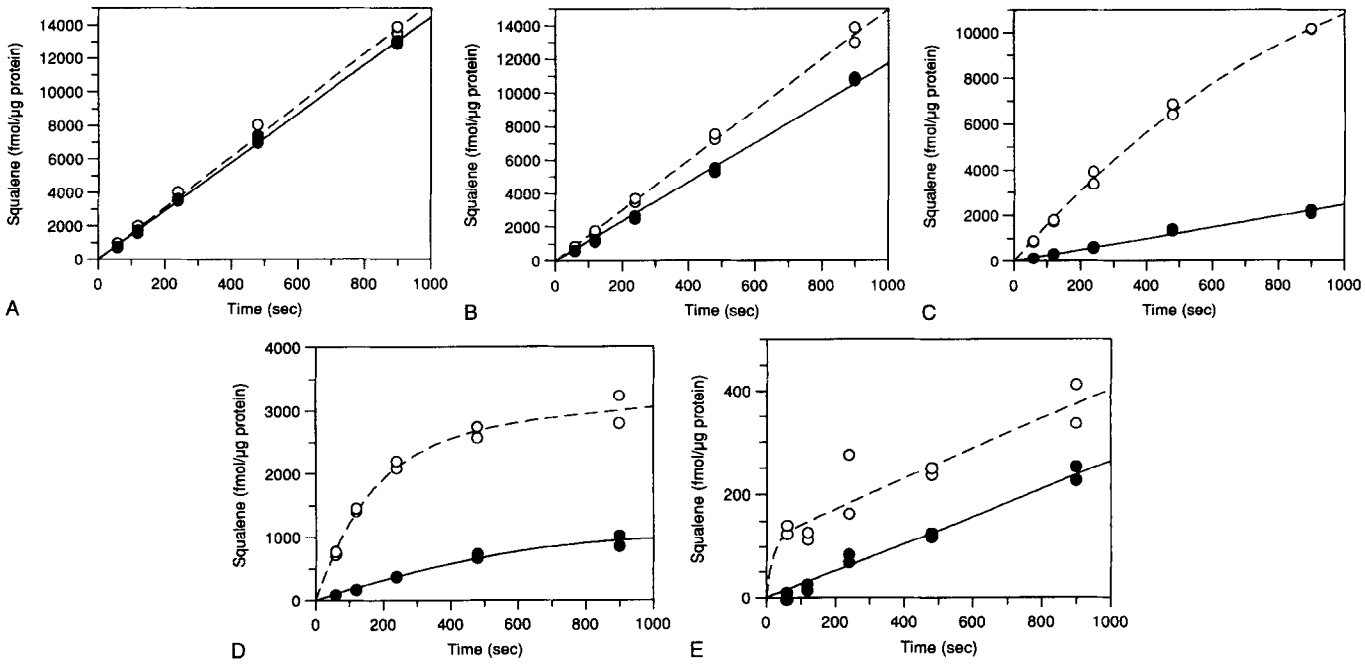

FIG. 4. Time-courses for inhibition of rat microsomal SQS. Assays were performed as described in Methods with (●, solid lines), or without (○, broken lines) a 15-min preincubation of SQS and BPQ. All lines were calculated using the best fit parameters in Table 2. Final concentrations of BPQ were as follows: A, 0 μM; B, 0.01 μM; C, 0.25 μM; D, 2.5 μM; E, 100 μM.

accounted for as protons, 10% comigrates with squalene, and the remainder is insoluble under the chromatography conditions. In fraction 2, 76% of the radioactivity corresponds to squalene, 23% squalene epoxide and <1% lanosterol [26]. This reflects the ability of microsomes to catalyse formation of the epoxide and lanosterol from squalene during cholesterol biosynthesis [25]. These steps involve no change in specific radioactivity. Thus, inclusion of squalene, squalene epoxide and lanosterol in fraction 2 makes a more accurate measure of SQS activity than following accumulation of only squalene. Inhibitors of squalene metabolism were not added because they are not necessary, and may bind to SQS.

Inhibition of rat microsomal SQS by BPQ clearly follows biphasic kinetics when FPP is present at 19.6 μM (Figs. 3A and B; Table 1). The following evidence shows that both phases reflect the activity of SQS and not other enzymes in the preparation. First, there is close agreement between fraction 1 and fraction 2 data, and second, after purification to homogeneity, a truncated human recombinant SQS follows biphasic kinetics (Table 1; Fig. 3D). Analysis by HPLC indicates that squalene is the only labelled organic molecule formed by this enzyme‡.

Preincubation of the inhibitor and enzyme for 15 min could load SQS with BPQ, which dissociates during the subsequent assay with FPP. This could invalidate conventional analysis that assumes approximation to a steady state where the concentration of inhibited enzyme is constant, leading to linear accumulation of product as a function of time [31, 34, 35]. The effects of preincubation on the time-course of product accumulation were investigated at various BPQ concentrations (Fig. 4; Table 2). Very similar results are obtained for fractions 1 and 2. The preincubation has no effect in the absence of inhibitor where the accumulation of product is linear (Fig. 4A). When there is sufficient BPQ to give inhibition without preincubation, there is downward curvature in the time course (Fig. 4C–E). This is characteristic of "slow binding inhibition" where the concentration of inhibited enzyme increases during the assay, invalidating the steady-state approximation [see 35]. The curvature is more marked at a higher inhibitor because of the concentration-dependence of the association between SQS and BPQ (Fig. 4B–E). Slow binding inhibition follows the equation [35]:

$$Pr = v_2 t + (v_1 - v_2)(1 - e^{-kt})/k \quad (5)$$

where Pr is product, t is time, v_1 and v_2 are the initial and final velocities, and k is the apparent rate constant for transition between v_1 and v_2. There is approximation to steady state when k is very low or high, or if $v_1 \approx v_2$, so that the relationship becomes:

$$Pr = v_{ss} t \quad (6)$$

where v_{ss} is the steady-state velocity. The fits obtained using Eq (5) and (6) were compared using an F-test (see Methods). The time-courses indicate either slow binding ($P < 0.01\%$), or steady state ($P > 20\%$) (Table 2). This analysis shows which data justify the additional v_2 and k terms for slow binding. Slow binding is seen when there is inhibition without preincubation (Fig. 4C–E). There is approximation to steady-state during the sensitive phase when there is a preincubation (BPQ at 0.01 and 0.25 μM, Fig. 4B, C). At 2.5 μM BPQ, there is slow binding even after preincubation (Fig. 4D). There is no gradual increase in rate due to dissociation of inhibitor after preincubation at any BPQ

‡ The truncated enzyme contains residues 25 to 383 of human SQS, Ward WHJ, Holdgate GA, Davidson RG, Pioli D, Abbott M and Charles AD, unpublished data.

TABLE 1. Values of kinetic parameters for the inhibition of squalene synthase

Inhibitor	Rat BPQ	Rat* BPQ	Human BPQ	Human† BPQ	Marmoset BPQ	Rat AA
K'_{is} (nM)	4.5 (2.3–8.6)	5.2 (1.1–24)	70 (46–110)	31 (18–53)	30 (17–59)	0.30 (0.03–3.0)
K'_{ii} (μM)	1.3 (0.67–2.5)	1.1 (0.20–5.8)	12 (9.5–16)	11 (5.5–22)	3.4 (2.3–5.1)	0.60 (0.25–1.6)
$K'_{ii}/K_{i's}$	290	210	170	350	110	2,000
[Si] (nM)	7.6 (4.0–15)	5.3 (0.048–58)	–	–	–	2.1 (0.7–6.5)
v_{0s}	8.5 (7.8–9.2)	11 (9.0–13)	0.91 (0.80–1.0)	16 (14–18)	2.5 (2.1–2.9)	11 (10–12)
v_{0i}	2.6 (2.0–3.1)	3.2 (1.2–5.1)	1.3 (1.2–1.4)	9.8 (8.0–12)	2.8 (2.5–3.2)	4.2 (3.3–5.0)
$v_{0s}SQ/v_{0s}H^+$	0.77		0.81	0.57	0.67	0.83
$v_{0i}SQ/v_{0i}H^+$	0.81		0.77	0.80	0.71	0.63
$v_{0s}/(v_{0s} + v_{0i})$ (%)	77	77	41	62	42	72
Rate equation	3	3	1	1	1	3

Experimental details, procedures for data analysis, and most symbol definitions are given in Methods. The remaining symbols for v_0 are as follows: SQ relates to squalene production, and H^+ relates to proton release; the subscripts $_s$ and $_i$ relate to sensitive and insensitive phases. [Si] is the concentration of inhibitor binding sites associated with the sensitive phase. Parameter values were calculated from the rate of squalene formation, except for * where proton release was monitored. Liver microsomes were the source of enzyme, except †, recombinant truncated enzyme (containing residues 25 to 383 of human SQS; Ward WHJ *et al.*, unpublished data, cited with permission), which was assayed at 0.3 μM FPP. The first two columns relate to different product fractions from the same assays. Best fit values are shown with 95% confidence limits in parentheses. The units for v_0 are fmol product/sec/μg protein. The standard assay was performed in a volume of 200 μL.

TABLE 2. Kinetic parameters for the time course of inhibition of squalene synthase

BPQ (μM)	Rate equation	v_{ss} (fmol SQ/sec/μg protein)	v_1 (fmol SQ/sec/μg protein)	v_2 (fmol SQ/sec/μg protein)	k (sec^{-1})
Without preincubation:					
0	6	15.3 ± 0.2			
0.01	6	15.0 ± 0.2			
0.25	5		16.7 ± 0.6	−(8 ± 5)	0.0006 ± 0.0002
2.5	5		16 ± 2	0.5 ± 0.5	0.006 ± 0.001
100	5		6 ± 8	0.29 ± 0.06	0.05 ± 0.07
With preincubation:					
0	6	14.5 ± 0.1			
0.01	6	11.7 ± 0.2			
0.25	6	2.46 ± 0.07			
2.5	5		1.8 ± 0.1	−(10 ± 10)	0.0001 ± 0.0002
100	6	0.263 ± 0.009			

Experimental details, procedures for data analysis, and symbol definitions are given in Methods and Results. Rat liver microsomes were the source of enzyme. Best fit values are shown ± SE.

concentration (Fig. 4B–E). The effects of preincubation are similar for BPQ-OH.

Initial inspection of dose-response data suggests a single phase when there is no preincubation (Fig. 5). However, the systematic distribution of residual differences between the best fit line and the experimental observations indicates that conventional kinetics are not obeyed [30, 32]. When there is a preincubation, the residuals tend to be satisfactory for the sensitive phase and more systematic for the insensitive phase (Fig. 5).

Characteristics of Inhibition by BPQ

In some experiments, the inhibitor-sensitive phase follows tight binding kinetics (see below). That is, the concentration of inhibitor added to the assay is similar to the concentration of binding sites, so that there is significant depletion of free BPQ. Conventional rate equations assume that the free inhibitor concentration is approximately the same as the total concentration. This assumption does not hold in tight binding kinetics, which requires special rate equations [31, 33, 36]. Consideration of this factor when deriving rate equations allows estimation of the concentration of inhibitor binding sites (see Methods).

There is close agreement between fraction 1 and fraction 2 results for several species. This is exemplified by the data for rat microsomes (Table 1). The experimental variation in fraction 1 is greater than in fraction 2, so that there is a higher degree of uncertainty in the parameter values. The rat fraction 2 data are best described by the rate equation for biphasic kinetics where one phase is tight binding, Eqn (3). When compared to the nontight binding relationship, Eqn (1), the value of P = 1.9% (see Methods).

The apparent inhibition constants for the sensitive and insensitive phases respectively are K'_{is} = 4.5 nM and K'_{ii} = 1300 nM (Table 1). Thus, the two phases differ in sensitivity by a factor of approximately 300. In both fractions, the sensitive phase accounts for 77% of the total activity. Biphasic kinetics also are seen for BPQ against SQS in human and marmoset microsomes, and recombinant truncated human SQS (Fig. 3C–E). The compound displays a lower affinity for SQS from these sources (Table 1). The K'_i values are around 5- to 12-fold higher than against rat microsomes. The data were not sufficient to prove tight binding in these species (for marmoset P = 25%; for human microsomes P = 47%; for recombinant human, P = 77%). The results, therefore, were analysed assuming conventional kinetics, Eqn (1).

Biphasic kinetics for inhibition of microsomal SQS are detected for a number of quinuclidines that are related in structure to BPQ (see [26]). This behaviour is seen using microsomes from a range of mammals, including hamsters and dogs. Different structural types of SQS inhibitors also exhibit biphasic kinetics, as illustrated by the data on AA (Figs. 2 and 3F).

Inhibition of the Sensitive Phase Follows Tight Binding Kinetics

Conventional enzyme kinetics give a Hill coefficient (or slope factor) of 1 when each ligand molecule binds independently to each enzyme molecule in a 1:1 stoichiometry (see Ref. 31). The sensitive phase for the dose-response curve for BPQ against rat microsomes sometimes exhibits a slope of greater than one (Fig. 3A and B). This could be due to tight binding, or cooperative kinetics. Cooperative kinetics are reasonable for this system because SQS has at least two binding sites, as shown by its ability to bind two molecules of FPP. Both tight binding and cooperative rate equations give a similar high quality of fit. However, the following considerations indicate that tight binding occurs in this system.

1. When BPQ is characterised at higher concentrations of rat microsomal protein, analysis assuming tight binding kinetics gives the expected results; that is, a linear in-

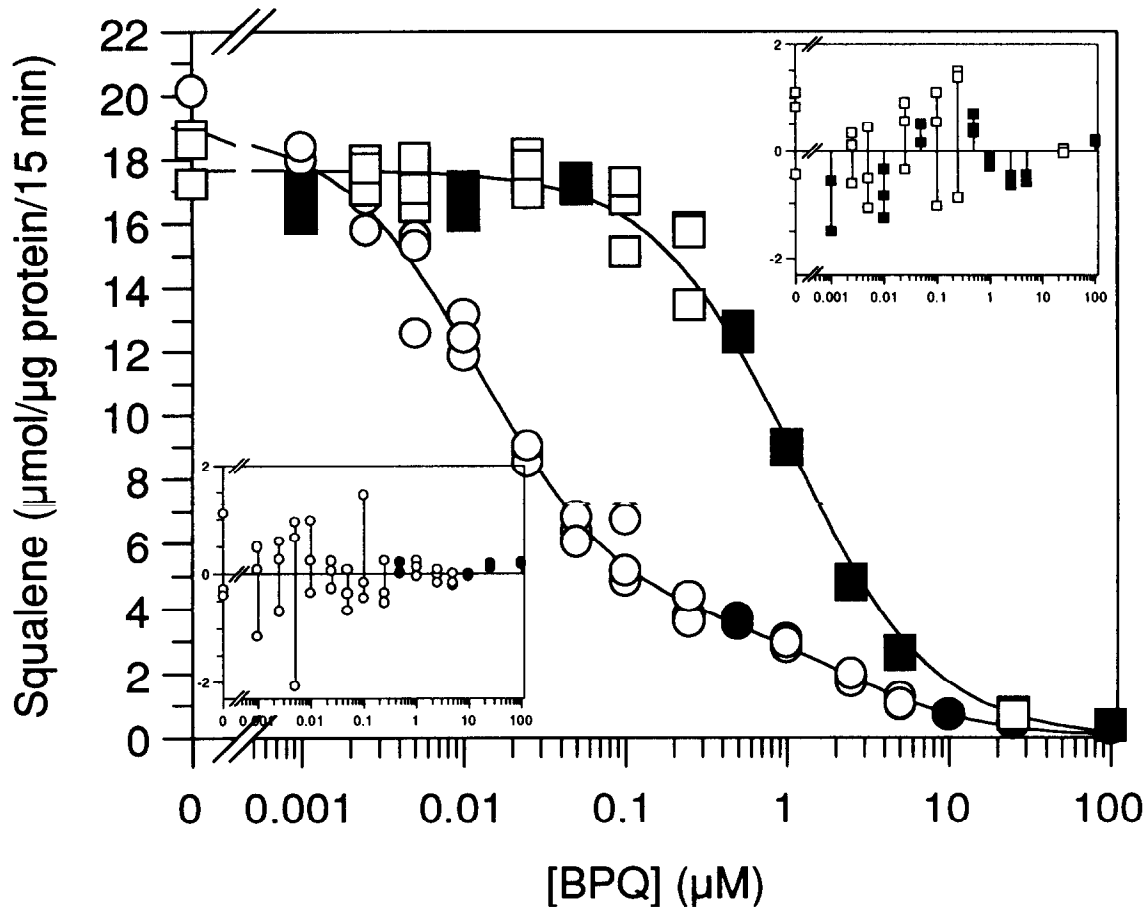

FIG. 5. Dose-response curves with and without preincubation of rat microsomes and BPQ. Assays were performed as described in Methods with (●, ○), or without (■, □) 15-min preincubation. The best fit line with preincubation is described by Eqn (1) with v_{0s} = 15.3 µmol squalene/µg protein/15 min, v_{0i} = 3.8 µmol squalene/µg protein/15 min, K'_{is} = 12 nM, K'_{ii} = 2.3 µM. That without preincubation is given by Eqn (2) and v_{0s} = 17.7 µmol squalene/µg protein/15 min, v_{0i} fixed at zero, K'_{is} = 1.1 µM. The insets show the differences between observed and calculated rates. Filled symbols are shown where all replicates lie on the same side of the best fit line.

crease in the estimated concentration of sites and no change in K'_{is} (Fig. 6). The rate equation for cooperative kinetics gives estimates of affinity and slope factor that change with protein concentration.

2. The slope becomes 1 when BPQ is characterised at lower concentrations of rat microsomal protein. Kinetics are no longer expected to be tight binding when the enzyme concentration is reduced well below that of the inhibitor.
3. Less potent quinuclidines give a slope of 1. This is consistent with tight binding kinetics for BPQ, but not with cooperative kinetics because a similar cooperative effect would be expected for inhibitors that are analogous in structure.
4. More potent compounds (e.g. AA) follow tight binding kinetics.

The Phase with a Higher Affinity for BPQ is Associated with Low FPP Concentrations

The potency of BPQ against rat microsomal SQS has been measured at a range of FPP concentrations between 1.6 and 19.5 µM (Table 3). Inhibition follows biphasic non-tight binding kinetics (Eqn 1). To avoid depletion of FPP at reduced concentrations of the substrate, these experiments were performed at lower protein concentrations (down to 5 µg/assay) than those in Table 1 (20 µg/assay), explaining why the behaviour was no longer tight binding. The uninhibited rate associated with the sensitive phase (v_{0s}) remains almost constant at around 15 fmol squalene/sec/µg protein, indicating saturation even at the lowest FPP concentration. The value of K'_{is} (around 5 nM) also does not vary in this range of FPP concentrations, suggesting that inhibition follows either uncompetitive or noncompetitive kinetics (see Ref. 31). The inhibited rate linked with the insensitive phase (v_{0i}) increases from 3.0 to 8.6 fmol squalene/sec/µg protein as the FPP concentration is elevated.

Mechanism of Inhibition by BPQ

To determine the kinetic mechanism for inhibition in the sensitive phase, BPQ was varied at a range of FPP concentrations including those where the substrate was not satu-

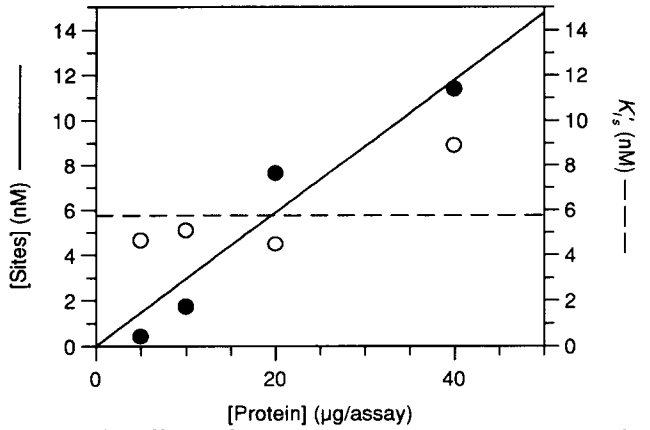

FIG. 6. The effects of varying protein concentrations on the kinetic parameter values for tight binding inhibition by BPQ. These values were calculated by fitting Eqn (3) to measured rates of squalene production by rat liver microsomes under the standard assay procedure (see Materials and Methods). The solid line (concentration of binding sites, ●) has an intercept fixed at zero, and the broken line (K'_{is} values, ○) has a slope fixed at zero. An F-test (see Methods) was used to decide which parameters vary. For the concentration of sites, P = 65% when the intercept varied. For the K'_{is} values, P = 29% when the slope is varied.

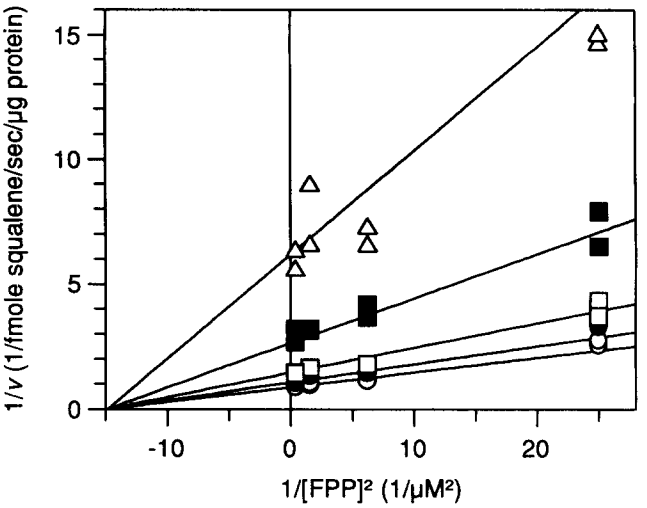

FIG. 7. Mechanism of inhibition of rat microsomal SQS by BPQ. Details are given in Materials and Methods. The concentrations of BPQ were as follows: ○, 0 nM; ●, 1 nM; □, 3 nM; ■, 9 nM; Δ, 27 nM. The best fit lines are shown, and were calculated using Eqn (10) and the parameter values given in the text.

rating (Fig. 7). First, data at each substrate concentration were analysed to estimate the contribution from each phase. Values for the insensitive phase were subtracted prior to multivariate nonlinear regression to fit a number of rate equations to the sensitive phase rates. The concentrations of both inhibitor and substrate were varied in the same fit to allow comparison of rate equations by considering the residual sum of squares. As in Table 3, the estimated contribution from the insensitive phase is decreased at lower FPP concentrations, being 12% of the total at 1.6 μM FPP and 0% at 0.2 μM substrate.

A recent detailed study [20] demonstrates that the FPP-dependence of a recombinant yeast SQS obeys a rate equation that can be rearranged to the form:

$$v = V[FPP]^2/(K_a + K_b[FPP] + [FPP]^2) \quad (7)$$

where V, K_a and K_b are apparent constants that may vary according to the concentration of NADPH. V is the maximal velocity, K_b is the apparent dissociation constant for the second molecule of FPP, and K_a is the product of the apparent dissociation constants for the first and second molecules of FPP. BPQ could bind to free enzyme (apparent dissociation constant K_{i1}), or after the first molecule of FPP (apparent dissociation constant K_{i2}), or after the second molecule of FPP (apparent dissociation constant K_{i3}). Such behaviour would follow the equation:

$$v = V[FPP]^2/\{K_a(1 + [I]/K_{i1}) + K_b[FPP](1 + [I]/K_{i2}) + [FPP]^2(1 + [I]/K_{i3})\} \quad (8)$$

When this relationship is fitted to the measured velocities, the value of K_b is very low and there is considerable uncertainty (high SE) in K_{i2}. This result suggests that the levels of E.FPP are very low. If they are insignificant, then K_b tends to zero and the following equation describes the rate:

$$v = V[FPP]^2/\{K_a(1 + [I]/K_{i1}) + [FPP]^2(1 + [I]/K_{i3})\} \quad (9)$$

This relationship implies that the K_b and K_{i2} terms in Eqn (8) are redundant, a conclusion that is proved by comparing the fits using the F-test (P = 81%). Equation (9) describes mixed noncompetitive inhibition. It contains an extra parameter relative to three other relationships [31, 34], allow-

TABLE 3. The effects of varying FPP concentration

FPP (μM)	v_{Os} (fmol SQ/sec/μg protein)	v_{Oi} (fmol SQ/sec/μg protein)	K'_{is} (nM)	$(v_{Oi}/v_{Os} + v_{Oi})$ (%)
1.6	14 (12–16)	3.0 (1.0–4.9)	4.6 (2.8–7.6)	18
3.68	15 (14–17)	4.4 (2.8–6.0)	3.2 (2.2–4.9)	22
8.64	15 (14–17)	6.4 (4.6–8.4)	5.1 (3.5–7.3)	30
19.5	17 (14–19)	8.6 (6.1–11)	5.8 (3.5–9.6)	34

Experimental details and procedures for data analysis are given in Methods. The assays were performed with rat microsomes and parameter values were calculated by fitting Eqn (2) to the measured rate of squalene formation. Best fit values are shown with 95% confidence limits in parentheses.

ing comparison using the *F*-test. For competitive inhibition, K_{i3} is absent because it tends to infinity. In uncompetitive inhibition, K_{i3} is retained and K_{i1} tends to infinity. Pure noncompetitive inhibition loses a parameter because $K_{i1} = K_{i3}$. These data justify the additional parameter when mixed inhibition is compared with competitive and uncompetitive inhibition ($P < 0.001\%$ and $P = 0.17\%$, respectively), but not when it is compared with pure noncompetitive inhibition ($P = 32\%$), so that the measured velocity is best described by the relationship:

$$v = V[FPP]^2/\{K_a(1 + [I]/K_i) + [FPP]^2(1 + [I]/K_i)\} \quad (10)$$

Data also were analysed by using a Hill coefficient for inhibitor binding, but this did not improve the quality of fit ($P = 87\%$, $h = 0.99 \pm 0.05$). After comparison with several other models, Eqn (10) continues to give the best description of the measured rates. This relationship gives an adequate description of the kinetics by the criteria given in the Methods section. It was rearranged into the following form to estimate the confidence limits of K_i and K_a:

$$v = V[FPP]^2/\{10^{LK_a}(1 + [I]/10^{LK_i}) + [FPP]^2(1 + [I]/10^{LK_i})\} \quad (11)$$

where LK_a and LK_i are the logs of K_i and K_a. The best fit values (95% confidence limits in parentheses) are $V = 1.16$ (1.11–1.20) fmol squalene/sec/μg protein, $K_a = 0.068$ (0.058–0.079) μM^2, and $K_i = 4.4$ (3.9–5.0) nM. The double reciprocal plot of $1/v$ against $1/[S]^2$ gives lines that intersect on the abscissa (Fig. 7), as expected for pure noncompetitive inhibition [31, 34]. V is expected to be similar to v_{0s}, which is estimated at higher values in the range 8.5–17 fmol/sec/μg at 19.6 μM FPP (Tables 1 and 3), reflecting variation between experiments. The variation is lower (v_{0s} = 14–17 fmol/sec/μg) when the uninhibited rate at 1.6–19.5 μM FPP is estimated in a single experiment (Table 3).

DISCUSSION

Characteristics of Rat Liver Microsomal SQS

In the absence of inhibitor, the total rate at 19.6 μM FPP is 11–26 fmol squalene/sec/μg protein, of which 8.5–17 fmol/sec/μg protein are associated with the BPQ-sensitive phase (Tables 1 and 3). These values are in good agreement with previous measurements for rat liver of 5 and 30 fmol/sec/μg protein [17, 37]. The specific activities are lower for the primate microsomes than for the rat (Table 1). This may reflect differences in the abundance of SQS, or differences in the turnover rate of each enzyme molecule.

From the data in Table 1, there is 63–83% coupling between the two steps catalysed by microsomal SQS. A high degree of coupling also has been seen for yeast microsomal SQS in the absence of detergents [38]. Conversely, Sasiak and Rilling [39] report only one mole squalene formed for each 6 moles of presqualene pyrophosphate (see Fig. 1) in the presence of detergent. These conditions appear to perturb the function of SQS because an enzyme should be unlikely to release a significant proportion of a bound intermediate.

Assuming that the concentration of inhibitor binding sites for the sensitive phase equals the concentration of active sites, then each active site turns over at a rate of 0.0056 sec^{-1} ($=k_{cat}$). This is lower than published k_{cat} values of 0.1 to 7.4 s^{-1}, which were measured in the presence of detergents [17–20, 39, 40]. Detergents accelerate SQS, possibly by increasing a rate-limiting release of product [17, 19, 20], especially because squalene is very hydrophobic and is unlikely to partition from the active site and into solution.

Our results for the sensitive phase agree with previously published work in terms of the concentration of FPP required to give half-maximal rate. We find that the substrate concentration giving half-maximal rate, $S_{0.5} \approx 0.26$ μM (the square root of $K_a = 0.068$ μM^2), compared well to values of 0.5 to 1 μM for rat liver [17, 25, 37] and 0.3 μM for hog liver [41]. The value of k_{cat}/K_m is a measure of the catalytic efficiency, and we find $k_{cat}/S_{0.5} = 2.2 \times 10^4$ s^{-1} M^{-1}. This is similar to that of 8.3×10^4 s^{-1} M^{-1} for a truncated yeast SQS [19] and within the range of 10^4–10^8 s^{-1} M^{-1} that is typical for enzyme-catalysed reactions [42].

The affinities of rat, human, and marmoset SQS for BPQ fall within a 16-fold range (Table 1). This observation is consistent with biphosphonate SQS inhibitors having similar potencies against the human and rat enzymes [9], and with the observation of 85% sequence identity between rat and human SQS [13–16].

The concentration of BPQ binding sites associated with the sensitive phase is 7.6 nM when there is 100 μg microsomal protein per cm^3 in the assay (Table 1). This value is in close agreement with published results. Bergstrom *et al.* [25] report that the zaragozic acid SQS inhibitors are very tight binding, giving an IC_{50} of ≈ 5 nM at 110 μg/cm^3 rat microsomal protein. The measured IC_{50} under these conditions is equal to 50% of the concentration of binding sites, leading to an estimate of 9.1 nM SQS at 100 μg/cm^3 protein. The M_r of rat SQS is 48,000 [13], so that 7.6 nM enzyme in 100 μg protein/cm^3 corresponds to SQS being 0.4% of the protein in microsomes. This figure agrees closely with the report of a 160-fold purification from rat liver microsomes to give homogeneous SQS [17]. Here, the enzyme accounts for 0.6% of the microsomal protein. When the data for recombinant SQS (Fig. 3D) are analysed assuming tight binding, Eqn (3), the concentration of sites is estimated as 10 nM (95% confidence intervals 0.04–3000 nM), which is consistent with the value of 34 nM calculated by dividing the protein concentration by M_r.

Inhibition of SQS by BPQ Follows Biphasic Kinetics

Preincubation of the inhibitor and enzyme could load SQS with BPQ, which dissociates slowly during the assay, invalidating the steady-state approximation [see 31, 34, 35]. The time-course of product accumulation was investigated both with and without preincubation (Fig. 4; Table 2).

There is no gradual increase in rate after preincubation with BPQ (Fig. 4B–E), showing that dissociation is either too fast, or too slow, to detect. (This protocol allows reasonably accurate estimation of affinity even if dissociation is too slow to detect, because addition of FPP to start the assay only leads to a small dilution from 180 to 200 μL.) Conversely, slow binding is seen when there is no preincubation (Fig. 4C–E). The steady-state approximation is valid during the sensitive phase when there is a preincubation (BPQ at 0.01 and 0.25 μM, Fig. 4B, C). At 2.5 μM BPQ, binding is slow even after preincubation (Fig. 4D), correlating with a change between sensitive and insensitive phases. The steady-state approximation holds at 100 μM BPQ after preincubation because the high inhibitor concentration allows faster equilibration (Fig. 4E). With no preincubation, the value of v_1 at each concentration BPQ is comparable with the uninhibited rate, and the value of k increases with inhibitor concentration, as expected for a process that is dependent upon association of BPQ and enzyme (Table 2). At 100 μM BPQ, the value of v_2 without preincubation is very similar to that of v_{ss} after preincubation. The rate after preincubation with 100 μM BPQ, therefore, probably reflects the steady state, whereas the time-course without preincubation reflects the approach to steady state.

The systematic residuals in dose-response studies performed without preincubation (Fig. 5) indicate that conventional kinetics are not obeyed [30, 32]. This is most marked in the sloping region of the curve that contains the data governing the estimate of potency. At several BPQ concentrations, all replicates fall on the same side of the best fit line, rather than being distributed both above and below it as expected for an adequate rate equation [30, 32]. Some uneven distribution of residuals is expected by chance with this small number of replicates. However, the dose-response data do reflect the failure of the steady state seen in the time courses (Fig. 4). Detection of systematic residuals requires precise measurements and careful examination of rates. It is far easier to see the artefactual nature of the kinetics without preincubation in the time-course studies (Fig. 4) than in the dose-response analysis (Fig. 5). These factors, and use of protocols without preincubation, may explain why there are no previous reports of biphasic dose-response behaviour. Alternatively, slow binding may occur only for certain inhibitors under specific conditions. With preincubation, the residuals tend to be satisfactory for the sensitive phase and more systematic for the insensitive one (Fig. 5), again reflecting the validity of the steady-state approximation. Similar behaviour is seen in the other dose-response data where there is preincubation (Fig. 3). Thus, both time-course and dose-response studies show that data from the sensitive phase after preincubation are the easiest to interpret.

Preincubation in the absence of FPP allows detection of biphasic kinetics, with BPQ binding at high affinity during the sensitive phase. The insensitive phase may be due to SQS that is not inhibited during the sensitive-phase binding BPQ at lower apparent affinity due to competition by FPP, explaining why it is not detected at low FPP (Table 3). The cause of biphasic kinetics is not clear. For example, SQS may exist in phosphorylated and unphosphorylated forms that bind BPQ differently. Additional possibilities include partial proteolysis, oxidation, conformation changes, or isoforms. Alternatively, biphasic kinetics may result from inhibitor binding along two different branches of a reaction pathway. An unbranched mechanism involving binding of a second molecule of inhibitor to an enzyme-inhibitor complex could not explain the detection of two v_0 values. Biphasic time-courses have recently been reported for inhibition of SQS by zaragozic acid A [43]. The cause of this behaviour is not clear, but it may be the same as the origin of the biphasic dose-response curves seen in the present work.

We observe that inclusion of high concentrations of thiol reagents or protease inhibitors during microsome preparation and enzyme assay has no effect on the number of kinetic phases. It could be that different enzymes from various subcellular locations generate the biphasic kinetics, perhaps because of posttranslational modifications. There are reports of SQS in microsomes [11, 44] and peroxisomes [45]. Different complementary DNA sequences have been reported for human SQS, raising the possibility that various forms of the enzyme originate from different individuals [40]. However, biphasic kinetics are seen for homogeneous SQS, suggesting that this behaviour is an intrinsic property of the enzyme. A purified recombinant yeast SQS exhibits biphasic FPP-dependence in the formation of presqualene pyrophosphate in the absence of NADPH and inhibitors [20], and a homogeneous recombinant human SQS follows biphasic kinetics with respect to BPQ (Fig. 3D).* Furthermore, although there are two SQS mRNAs in a number of rat and human tissues [14, 45], the two sequences differ only in their 3′-untranslated regions [14].

Addition of pyrophosphate increases inhibition by ammonium analogues of putative carbocation intermediates in the SQS reaction, probably by mimicking an ion pair that is formed during catalysis [47]. Up to 10% of the FPP is converted into pyrophosphate in our assays, suggesting that the kinetics of the cationic quinuclidines may be affected by accumulation of the product. The highest levels of pyrophosphate occur when the rate is fastest. However, the accumulation of squalene at high rates after preincubation with BPQ is linear with time (Fig. 4B, C), implying that the pyrophosphate formed is insufficient to affect the kinetics. Addition of 775 μM pyrophosphate markedly increases the potency of a quinuclidine related to BPQ (K'_{is} decreases 75-fold). There is no significant change in K'_{is} at 1.6 μM pyrophosphate. These observations, coupled with increased contribution from the inhibitor sensitive phase with no

* Ward WHJ, Holdgate GA, Davidson RG, Pioli D, Abbott M and Charles AD, Unpublished data.

change in K'_{is} as FPP (and pyrophosphate produced) is lowered (Table 3), show that accumulation of up to 2 μM pyrophosphate is unlikely to cause the 290-fold difference between K'_{is} and K'_{ii} for BPQ (Table 1).

The FPP concentration in rat liver is around 0.5 μM [48]. Thus, the BPQ-sensitive phase is responsible for most of the SQS activity in the absence of inhibitors. However, there appears to be at least a 6-fold increase in radiolabelled FPP following inhibition of SQS in Hep G2 cells [25] or rat liver [26]. The radiolabel derives from biosynthetic precursors and may reflect similar increases in total FPP levels. If so, the activity of the BPQ-resistant phase may become significant, especially in human and marmoset liver where the insensitive phase contributes a greater proportion of the total activity (Table 1). Thus, occurrence of the BPQ-insensitive phase may have consequences for the transfer of activity of SQS inhibitors across species *in vivo*.

A Kinetic Mechanism for Inhibition of SQS by BPQ

Variation of FPP and BPQ in the same experiment has allowed characterisation of the kinetic phase that is most active at low FPP concentrations (Fig. 7). This phase approximates the total rate at physiological substrate levels. The insensitive phase is difficult to characterise because it has low activity and does not approximate to steady state. The inhibitor sensitive phase is accurately described by Eqn (10), where second order kinetics are followed with respect to FPP. Previous publications have described various types of kinetics with respect to this substrate. Several apparently demonstrate first-order kinetics [38, 41, 49], and others seem to assume first-order kinetics during characterisation of inhibitors [21, 22, 40, 50]. Conversely, Shechter and Bloch [51] and Mookhtiar *et al.* [20] propose complex kinetics including a term that is second-order with respect to FPP. These differences may be due to changes in mechanism according to the assay conditions, or may reflect the range of substrate concentrations, or methods of data analysis.

In the absence of BPQ, Eqn (10) reduces to:

$$v = V[\mathrm{FPP}]^2/(K_a + [\mathrm{FPP}]^2) \qquad (12)$$

which describes the FPP-dependence in Fig. 7. In a thorough study of a truncated soluble recombinant yeast SQS, it is reported that FPP-dependence follows Eqn (7) [20], which contains an extra term, K_b[FPP], to reflect binding of a single molecule of FPP. Failure to detect this term in the current work is probably because the second FPP molecule binds at high affinity (K_b is low), so that the levels of complexes with a single molecule of FPP bound are low compared to those with containing no FPP, or two molecules of the substrate. Thus, there is only a subtle difference between the behaviour in Fig. 7 and that described by Mookhtiar *et al.* [20]. It is not clear whether or not there is a second phase of FPP-dependence at high substrate concentrations in the absence of BPQ. The second phase in the presence of BPQ may be due to competition between inhibitor and FPP. Inhibition of SQS by BPQ follows first-order kinetics, whereas the FPP-dependence follows second-order kinetics, Eqn (10), possibly because it binds strongly at only one of the two FPP-sites. The two FPP-sites appear to have different binding specificities (see Ref. 5), and our failure to detect a complex containing a single molecule of FPP suggests that the second molecule binds with a higher affinity than the first.

The noncompetitive kinetics for the cationic quinuclidine SQS inhibitor contrast with the FPP-competitive kinetics seen for anionic inhibitors such as the FPP-analogues, squalestatins, and bisphosphonates [9, 21–23, 25, 40, 43, 50]. This does not prove that FPP and BPQ use different sites. BPQ binds slowly and at a high affinity in the sensitive phase, suggesting that dissociation may be slow enough to prevent competition by FPP during the assay. Alternatively, noncompetitive kinetics may occur because BPQ associates with SQS after FPP. It may bind to E.NADP$^+$, or at one of the two farnesyl sites to E.presqualene pyrophosphate or E.squalene complex (when presqualene pyrophosphate or squalene is bound at the other farnesyl site). Thus, noncompetitive kinetics may involve slow dissociation of an enzyme.product complex that is bound by the inhibitor. Similar behaviour has been reported for aldose reductase inhibitors, which utilise the binding site for glucose but follow noncompetitive, or uncompetitive, kinetics [52, 53]. We speculate that BPQ functions as an analogue of proposed carbocationic intermediates in the SQS reaction mechanism (see Ref. 5), with the hydrophobic side-chain occupying a farnesyl binding site and the charged nitrogen mimicking a carbocation. This hypothesis is supported by the increased potency of quinuclidines in the presence of pyrophosphate, and observation of kinetic competition between an FPP analogue and BPQ.*

Analysis of SQS Inhibition Data

Slow, tight binding and the occurrence of two kinetic phases mean that care is required for accurate analysis of SQS inhibition. The importance of using a rate equation for biphasic kinetics is illustrated using the data for inhibition of human microsomal SQS by BPQ. An apparently reasonable fit (Fig. 3C) is obtained using the Hill equation:

$$v = v_0/(1 + [\mathrm{I}]^{sl}/\mathrm{IC}_{50}{}^{sl}) \qquad (13)$$

where v_0 is the rate in the absence of inhibitor and *sl* is the slope factor. This equation is often used to analyse biological data (see Refs. 29 and 32), and estimates IC_{50} = 1700 nM and *sl* = 0.45. The relationship for biphasic kinetics, Eq (1), leads to K'_i values of 70 and 12,000 nM (Table 1). Analysis assuming single-phase kinetics generates misleading results because it averages potency across the two

* Ward WHJ, Holdgate GA, Davidson RG, Pioli D, Abbott M and Charles AD, unpublished data.

phases, which vary in magnitude (especially between species and according to FPP concentration), and the ratio of K'_{ii}/K'_{is} varies according to the structure of the inhibitor (Table 1). We find that numerous SQS inhibitors exhibit biphasic kinetics (see Table 1 and [26]. The ability to detect multiple phases is dependent upon several factors, especially having a sufficient difference between the two K'_i values and both phases giving a significant contribution to the overall rate. There is also variation between assays that may be associated with different preparations of microsomes or purity of the FPP substrate. These factors, lack of preincubation, or use of different inhibitors, may explain why previous studies have not reported biphasic kinetics.

The importance of tight binding is illustrated using the data for inhibition of rat microsomal SQS by AA. An apparently reasonable fit (Fig. 3F) is obtained using the relationship for biphasic non-tight binding kinetics, Eqn (1). This analysis gives K'_{is} = 1.2 nM. The relationship for tight binding kinetics, Eqn (3), gives K'_{is} = 0.3 nM. Failure to allow for the effects of tight binding leads to underestimation of potency and an apparent affinity that varies according to the concentration of binding sites. These problems have been encountered when characterising other potent SQS inhibitors [25].

In conclusion, careful assay design and data analysis are required to avoid the misleading effects of slow, tight binding and multiple phases during inhibition of SQS by BPQ.

We thank the following for their contributions, advice, and support: John Preston, Larry W. Furlong, Peter J. Harrison (in memoriam), Jonathon O'Connell, Reg K. Goodwin, David Griffiths, David S. Clarke, Alan J. Foubister, David P. Tuffin, and Leslie R. Hughes.

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