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RETENTION OF THE LABEL DURING THE CONVERSION OF [3-^{3}H] SQUALENE INTO (3*S*)-2,3-OXIDOSQUALENE CATALYZED BY MAMMALIAN SQUALENE OXIDASE

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Squalene epoxidase is the only known flavoprotein that catalyzes the epoxidation of an olefin. In order to test the possibility of a catalytic non-heme metal-based mechanism, the conversion of chemically synthesized [3-^{3}H]squalene into [^{3}H]2,3-oxidosqualene, by partially purified pig liver squalene epoxidase, was studied. No exchange of the labeled hydrogen could be observed, ruling out a mechanism involving,e.g.,an iron carbene type species at C-3. © 1995 Academic Press, Inc.

Squalene epoxidase (EC 1.14.99.7), a microsomal enzyme, catalyzes the conversion of squalene to (3*S*)-2,3-oxidosqualene. The properties of the enzyme from rat liver were extensively studied by Bloch and co-workers in the seventies [1-3] and the enzyme, after solubilization with detergent, was purified to apparent homogeneity by Ono et al [4]. It was found that the enzymatic epoxidation reaction required molecular oxygen, FAD, NADPH and NADPH-cytochrome P450-reductase. Interest in this enzyme, which was found to be a control site in the cholesterol biosynthetic pathway [5], was recaptured during the recent years with the development of new hypocholesterolemic drugs. Thus, NB-598 was found to be a remarkably potent inhibitor of the mammalian enzyme [6]. In addition, it was shown that squalene epoxidase is able to catalyze the transformation of (3*S*)-2,3-oxidosqualene into (3*S*,22*S*)-2,3:22,23-dioxidosqualene, a metabolite that accumulates in cells treated with 2,3-oxidosqualene-lanosterol cyclase inhibitors [7], and which is a precursor of (24*S*)-24,25-epoxycholesterol, a known repressor of HMG-CoA reductase [8], the major regulatory enzyme of cholesterol synthesis.

The molecular mechanism of squalene epoxidase, which would be of interest for example in the rational design of inhibitors [9,10], remains virtually unknown. Basically, one is left to

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extrapolate from the mechanism of other flavoprotein monooxygenases catalyzed reactions and hypothesise that e.g. a 4a-flavin hydroperoxide type of intermediate is the active oxygen-transfer species [11]. Although chemical models of 4a-hydroperoxyflavins have been shown enzymic epoxidations remains to be validated, especially in the case of 2,3-oxidosqualene epoxidase which represents the only known example of a flavoprotein catalyzing such a reaction. In most cases, epoxidation reactions are catalyzed by hemoproteins such as cytochrome P-450 monooxygenases [14] and peroxygenases [15] or by non-heme iron enzymes found e.g. in micro-organisms, such as *Pseudomonas oleovorans* monooxygenase [16] or methane monooxygenase, a complex that also comprises a flavoprotein [17]. Although squalene epoxidase is not a hemo-protein, in the present context largely dominated by the difficulty to obtain pure enzyme in large enough quantities, the intervention of a metal such as iron in the catalytic activity of this enzyme cannot be totally ruled out.

It was previously shown that epoxidations of alkenes carried out by metalloenzymes could involve the exchange of a vinylic protons with the medium [18]. In order to test the occurrence of such an exchange, which might be the signature of a metal-containing system and of a specific reaction intermediate such as an oxametallacyclic species, we have followed the fate of H-3 of squalene during its epoxidation by partially purified pig liver squalene epoxidase.

MATERIALS AND METHODS

Chemicals- [4,8,12,13,17,21-^{3}H]Squalene was purchased from NEN-Dupont (France) and [3-^{3}H]squalene was synthesized as described [19].

Enzymes - NADPH-cytochrome P-450 reductase was solubilized from pig liver microsomes with emulphogene BC-720, an non-ionic detergent, and purified by affinity chromatography on 2',5'-ADP-Sepharose 4B (Pharmacia), essentially as described in literature [20].

Squalene epoxidase was partially purified from pig liver microsomes according to a procedure that was adapted from Bai and Prestwich [21]. Briefly, microsomes (17.5 mg protein/ml) resuspended in 20 mM Tris,HCl buffer, pH 7.4, containing 0.1 mM EDTA and 1mM DTT (Buffer A) were solubilized with emulphogene (detergent/protein ratio = 2). After centrifugation for 60 min at 10^5g, the supernatant (170 ml) was applied to a 4.3x5.8 cm DEAE-cellulose DE53 (Whatman) column. After washing with buffer A containing 0.75% emulphogene and 20 mM NaCl (500 ml), the enzyme was eluted (0.5 ml/min) by increasing NaCl concentration to 75 mM. The effluent was immediately applied to a 1.8x12.5 cm AffiGel blue column (Bio Rad) that had been pre-treated with buffer A containing 0.5% emulphogene (buffer B) and BSA (10 mg/ml), washed with buffer B containing 2 M NaCl and equilibrated with buffer B. The column was then washed (100 ml) with buffer B containing 0.2 M NaCl, followed by 150 ml buffer B containing 1 M NaCl in order to elute the enzyme.

2,3-Oxidosqualene-lanosterol cyclase was solubilized from pig liver microsomes with emulphogene BC-720 and partially purified (175-fold) as described previously [22].

Analytical procedures- Compounds were separated by TLC on silica gel plates (Merck 60 F_{254}) and the radiochromatograms were obtained with a Berthold TLC analyzer LB 283.

Squalene epoxidase assay- The activity was determined in buffer B (final volume 100 µl) containing 30 µM FAD, 1 mM NADPH and 80 mU NADPH-cytochrome P-450 reductase.

Squalene epoxidase assay- The activity was determined in buffer B (final volume 100 µl) containing 30 µM FAD, 1 mM NADPH and 80 mU NADPH-cytochrome P-450 reductase. The reaction was initiated with the addition of [^{3}H]squalene (10 µM; 3x10^4 dpm) in 2-butanol. After an incubation of 30 min at 37°C, the medium was extracted with 2x500 µl dichloromethane and the reaction products analyzed by TLC with hexane/ethyl acetate (95:5, v/v) as solvent. R_f values were 0.95 for squalene and 0.55 for 2,3-oxidosqualene.

Fate of the tritium atom during the epoxidation of [3-^{3}H]squalene - To a reaction mixture consisting of 116 µg partially purified squalene epoxidase, 0.16 U NADPH-cytochrome P-450 reductase in buffer B (final volume 400 µl) containing 30 µM FAD and 1 mM NADPH, [3-^{3}H] squalene (10 µM; 180 000 dpm) was added. After 30 min at 37°C, the reaction was stopped at - 78°C and the aqueous phase was recovered by lyophilization. The radioactivity of the aqueous phase and of the residue, redissolved in water, was determined with a LKB 1209 liquid scintillation counter.

Transformation of [3-^{3}H]squalene into [^{3}H]lanosterol - A reaction was run under identical experimental conditions as above. After 30 min, the medium was extracted with 2x500 µl dichloromethane to recover unreacted [3-^{3}H]squalene and formed [^{3}H]2,3-oxidosqualene. A 1% solution of Tween 80 in acetone (10 µl) was then added to the extracts and the solvents were evaporated. The residue was redissolved in the presence of partially purified 2,3-oxidosqualene-lanosterol cyclase (128 µg protein) in 10 mM potassium phosphate buffer, pH 7.4, containing 0.5% emulphogene (100 µl final volume). After 30 min at 37°C, the reaction mixture was worked-up as above and the products separated by TLC using hexane/ethyl acetate (85: 15, v/v). The band corresponding to lanosterol (R_f 0.42) was scrapped-off the plate and extracted from silica with dichloromethane.

Oxidation of [^{3}H]lanosterol into [^{3}H]lanosten-3-one and fate of the H-3 - Swern reagent was obtained by adding to 2.5 ml dichloromethane (at -10°C), oxalyl chloride (100 µl) followed by DMSO (170 µl) [23]. A solution (1 ml) in anhydrous dichloromethane of the [^{3}H]lanosterol obtained above, 200 mg lanosterol (carrier) and [^{14}C]cholesterol (10^4 dpm; internal standard) was then added slowly under magnetic stirring. After 25 min the reaction was slowly treated with 700 µl triethylamine, followed after return to room temperature, by 5 ml water. Reaction products were extracted with 5ml dichloromethane. The aqueous medium was lyophilized to recover the water phase, whose radioactivity content was determined. The organic phase was dried over anhydrous sodium sulphate, was counted using a double ^{3}H/^{14}C label counting technique or evaporated and the residue analyzed by TLC with using hexane/ethyl acetate (60:40, v/v). Under these experimental conditions all lanosterol (R_f 0.5) was transformed into lanosten-3-one (R_f 0.64); the band corresponding to the 3-ketosterols (which was positive with a 2,4-dinitrophenylhydrazine test) was scrapped-off the plate and counted.

Results and Discussion

Squalene epoxidase was solubilized from pig liver with emulphogene, a non-ionic detergent, and partially purified by an adaptation of the tandem DEAE-cellulose/AffiGel blue procedure of Bai and Prestwich [7]. The preparation, obtained with a 35-fold purification factor and

The reconstituted system, in the presence of NADPH cytochrome P-450 reductase, molecular oxygen, FAD and NADPH transformed chemically synthesized [18][3-^{3}H]squalene (**1**) into 2,3-oxidosqualene (**2**) (Figure 1). In a representative experiment monitored by a radio-TLC assay, 14 % of substrate **1** (180 000 dpm) was epoxidized and essentially no radioactivity was found in the water phase (45 dpm) which was obtained quantitatively from the reaction

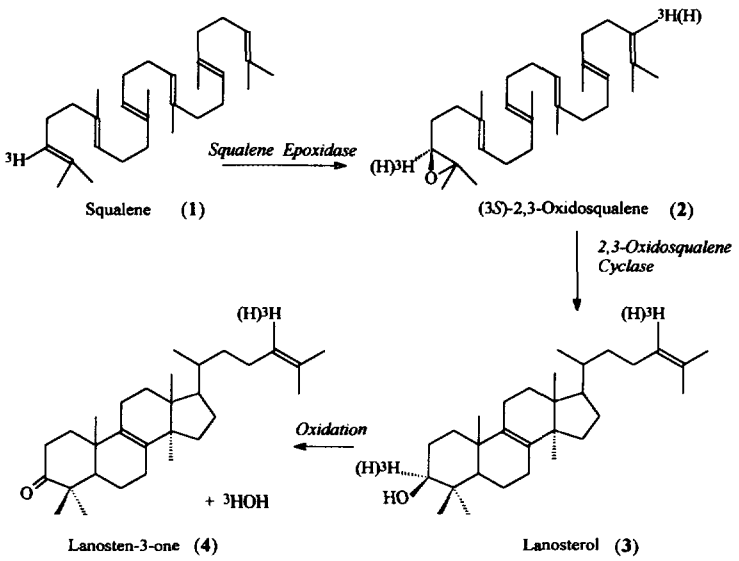

Fig. 1. Fate of the label of [3-^{3}H]squalene after epoxidation into 2,3-oxidosqualene, catalyzed by pig liver squalene epoxidase. Sequence of reactions allowing the transformation of 2,3-oxidosqualene to lanosterol lanosten-3-one.

medium by lyophilization. All the radioactivity (179 800 dpm) was recovered in the reaction residue. This result indicates that the enzyme catalyzed epoxidation (step **1** → **2**) does not involve the loss of the vinylic proton at C-3. Since we do not anticipate, during the monoepoxidation process, a discrimination by the enzyme between the two terminal double bonds of squalene, a complete exchange of the vinylic hydrogen at the epoxidized double bond should have resulted in a measurable transfer of 7% (about 12 500 dpm) of the initial label to the water.

To ascertain this result, 2,3-oxidosqualene (**2**) was further transformed into lanosterol (**3**) by pig liver 2,3-oxidosqualene-lanosterol cyclase. The aim was to determine, by oxidation of the 3β-ol of **3**, the proportions of the label at positions 3 and 24 in lanosterol (**3**), and consequently at positions 3 and 22 in 2,3-oxidosqualene (**2**) obtained by enzymatic epoxidation of **1** (Fig. 1). Under our experimental conditions an average of 50% of cyclization of **2** was achieved and the formed [^{3}H]lanosterol (**3**) was isolated by TLC. In a representative experiment, **3** (1 310 dpm) was mixed with tracer [^{14}C]cholesterol, and quantitatively oxidized under mild conditions into **4**, using a Swern reagent. [^{3}H]Lanosten-3-one (**4**) recovered from the reaction medium by extraction with dichloromethane, contained about 51% (675 dpm) of the original label. According to a dual ^{3}H/^{14}C label counting technique, the ^{14}C-counts found in the organic phase represented over 95% of the initial counts, indicating that the extraction was

nearly quantitative. The water phase of the reaction **3** → **4**, which was recovered quantitatively by lyophilization, contained 47% (620 dpm) of the tritium originally associated to lanosterol (**3**). This result confirms that about half of the ^3H in 2,3-oxidosqualene (**2**) was bound to C3, and therefore the observed lack of vinylic proton exchange during the epoxidation **1** → **2** was not due to some improbable isotope effect which would have directed the epoxidation predominantly to the 22,23-double bond of **1**.

Loss of a vinylic hydrogen in the epoxidation of an olefin has been interpreted, e.g. in certain cytochrome P-450 catalyzed reactions, as evidence of an oxametallacyclic species along the reaction pathway that can rearrange to an iron carbene with accompanying exchange of the hydrogen at the ligated position [18, 24]. The finding that epoxidation of squalene proceeds the reaction pathway that can rearrange to an iron carbene with accompanying exchange of the hydrogen at the ligated position [18, 24]. The finding that epoxidation of squalene proceeds with no measurable loss of hydrogen from carbon-3, seems to rule out the occurrence of such a mechanism for the pig liver squalene epoxidase catalyzed reaction. It should be noted, however, that epoxidation of olefins by *Pseudomonas oleovorans*, a non-heme iron monooxygenase, also proceeds without hydrogen exchange [25] and that a sequestering, in the active site, of the exchangeable proton cannot be totally ruled out.

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