PROPERTIES OF A 2,3-OXIDOSQUALENE-CYCLOARTENOL CYCLASE FROM *OCHROMONAS MALHAMENSIS*

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1. Introduction

The intermediate role of 2,3-oxidosqualene in the conversion of squalene into lanosterol is well established [1–3]. Similarly in plants, the formation of 2,3-oxidosqualene has been demonstrated [4, 5], together with its cyclization to cycloartenol [6–8], which probably replaces lanosterol as the first stable tetracyclic triterpene formed from squalene during phytosterol biosynthesis [9–11]. We previously reported an active 2,3-oxidosqualene-cycloartenol cyclase preparation from *Ochromonas malhamensis* [12] which cyclizes radioactive 2,3-oxidosqualene exclusively to cycloartenol. We now report the isolation, solubilisation and partial purification of this cyclase, together with a comparison of some of its physical properties with those of the 2,3-oxidosqualene-lanosterol cyclases from pig liver [13] and yeast [14].

2. Materials and methods

2.1. *Preparation of microsomal fraction*

Ochromonas malhamensis (933/1A, Cambridge Culture Collection) was grown for 7 days at 27° in 10 l batches on a standard medium [15], with continuous illumination and aeration. The cells were collected by centrifugation, washed with ice-cold 0.1 M potassium phosphate buffer (pH 7.4) and resuspended in sufficient of the same buffer to produce a thick paste. This was passed through a French Pressure Cell (8–10,000 lbs/sq. inch), and the ruptured cells were centrifuged at 5000 *g* for 15 min, the supernatant re-centrifuged at 20,000 *g* for 20 min and this supernatant centrifuged at 105,000 *g* for 90 min. Both the pellet (microsomal sediment) and supernatant were quick frozen in acetone/solid CO_2 and stored at −20° without appreciable loss of activity over several months.

2.2. *Preparation and purification of enzyme*

The method of enzyme preparation was essentially similar to that of Dean et al. [16]. The redissolved 0–30% ammonium sulphate precipitate was dialysed, centrifuged at 105,000 *g* for 2 hr, the supernatant quick frozen in small batches and stored at −20° prior to use (fraction A). Enzymic activity was assayed at each stage (table 1).

Further purification of the cyclase was obtained by Sephadex gel filtration. Fraction A (34 mg) was concentrated by vacuum dialysis into 3 ml of 0.001 M potassium phosphate buffer pH 7.4 + 0.05% sodium deoxycholate + 0.35 M KCl, and applied to a Sephadex G-200 column (1.5 × 30 cm), equilibrated and subsequently eluted with the above solution. A single peak of cyclase activity (assayed under standard conditions) was observed shortly after the void volume of the column. The cyclase specific activity of the peak compared to that of fraction A was increased 5-fold, so that the total purification of the cyclase at this stage was 120-fold (cf. table 1). The molecular weight of the cyclase estimated by the method of Andrews [17] was 190,000. It is just conceivable that a cyclase subunit could possibly be removed on Sephadex gel filtration, so that the remaining protein catalysed formation of a product other than cycloartenol. This possibility was checked by incubation of the combined remaining fractions that contained cyclase activity after gel filtration with radioactive

Table 1
Activity of 2,3-oxidosqualene-cycloartenol cyclase during isolation from *Ochromonas malhamensis.*

Enzyme preparation	Total protein (gm)	Total activity (mμmole/hr)	Specific activity (mμmole/hr/mg)	Purification	% Recovery
Broken cells	53.1	24.7×10^3	0.47	1.0	100
105,000 *g* Supernatant	13.7	0.4×10^3	0.03	–	1
105,000 *g* precipitate (microsomes)	2.42	3.9×10^3	1.62	3.4	16
Solubilised microsomes	1.84	8.2×10^3	4.39	7.2	33
0–30% $(NH_4)_2SO_4$ Dialysed, centrifuged 105,000 *g* supernatant	0.16	1.9×10^3	11.40	24.2	8

substrate and identification of the only product as cycloartenol by gas-radiochromatography and recrystallization with carrier material to constant specific radioactivity (8570, 8882, 8621, 8813 dpm/mg).

2.3. *Enzyme assay*

Fraction A was used for studying the properties of the cyclase. The enzyme buffer was changed from 0.1 M (most suitable for storage) to 0.01 M (optimal for assay) and excess salt and free deoxycholate removed by passage of each batch through a Sephadex G-25 column (0.9 X 30 cm) equilibrated with 0.01 M potassium phosphate buffer pH 7.4 and subsequently eluting with the same buffer. The enzyme was eluted in the void volume.

The standard assay mixture (in a total volume of 1 ml) contained potassium phosphate buffer pH 7.4 (10 μmole) potassium chloride (350 μmoles), sodium deoxycholate (1 mg), 2,3-oxido[^{3}H] squalene* (43 mμmole of active isomer; 75,000 dpm) and enzyme (1 mg of protein).

The enzyme, salts and deoxycholate (total volume 0.9 ml) were preincubated anaerobically for 30 min at 37°** and the reaction started by the addition of the substrate emulsified in 0.1 ml of 0.3% Tween 80 in 0.01 M potassium phosphate buffer pH 7.4. Incubations were carried out anaerobically at 37° for 30 min and the reactions terminated by addition of 1 ml of 10% methanolic potassium hydroxide. Following the addition of 1 mg each of carrier 2,3-oxidosqualene and cycloartenol, the mixture was saponified either for 1 hr at 90° or overnight at 5°. The nonsaponifiable fraction was extracted with ethyl acetate (3 X 2 ml), evaporated to dryness and subjected to TLC on rhodamine 6G-impregnated silica gel developed with benzene:ethyl acetate (9:1). Cycloartenol and 2,3-oxidosqualene had R_f values of 0.3 and 0.6, respectively. The radioactivity associated with cycloartenol was eluted with ether and expressed as a percentage of the total recovered radioactivity from the whole plate.

* Prior to determination of temperature optimum, assays were carried out at 30°.

** Synthesised as previously described from [2-$^3H_2$] mevalonic acid [6].

3. Results

3.1. *Properties of the cyclase enzyme*

Cyclase activity of fraction A assayed under standard conditions using varying concentrations of sodium deoxycholate was stimualted at low levels, optimal at 0.1% (15 mμmole substrate/hr) and inhibited at levels greater than 0.5%.

Assay of cyclase activity (fraction A) at different concentrations of KCl showed that in the absence of KCl, the enzyme was inactive. KCl stimulated cyclisation at concentrations up to 0.35 M (15 mμmole/hr) whereas at higher levels enzyme activity fell off slightly.

The pH optimum of the cyclase determined under conditions of constant ionic strength was in the region

Table 2
Effect of sulphydryl reagents and iminosqualene on 2,3-oxisqualene cyclase activity. Preincubations were carried out at 37° for 30 min: assays 1 and 3 contained enzyme and buffer (containing deoxycholate and KCl), whereas 2 and 4 contained in addition dithiothreitol and iminosqualene (emulsified in Tween 80), respectively. After 30 min, substrate was added to assays 1, 2 and 4 and pHMB was added immediately before substrate to 3. Incubation was continued for 30 min, when all were stopped.

Assay No.	Incubation contents	Relative cyclase activity
1	Control	1.00
2	+5 mM Dithiothreitol	1.91
3	+0.1 mM pHMB	0.32
4	+0.02 mM 2,3-Iminosqualene	0.58

7.0–7.3. Cyclase had an apparent K_m of 100 μM and a temperature optimum for a 30 min incubation of 37°. Cyclase activity showed a linear relationship when determined at different levels of excess substrate. Under these conditions, the specific activity of the crude enzyme (fraction A) was 17 mμmole/hr/mg protein.

Dithiothreitol markedly stimulated cyclisation while *p*-hydroxymercuribenzoate (PHMB) caused a 70% inhibition at the level used (table 2). However, there was only very little reactivation of the PHMB inhibited enzyme. Whereas 2,3-iminosqualene is a powerful inhibitor of the pig liver cyclase causing complete inhibition of the enzyme [18], it only caused 42% inhibition of the *Ochromonas* enzyme when the same ratio of substrate/inhibitor was used.

4. Discussion

In agreement with the results on pig liver cyclase, the *Ochromonas* enzyme is mainly particulate, being sedimented at 105,000 *g*, but probably strongly membrane bound (table 1). The total enzymic activity is also doubled on treatment of the microsomal fraction with deoxycholate in agreement with similar findings for the liver cyclase [16]. The dependence of the *Ochromonas* enzyme on deoxycholate and a high salt concentration for optimal activity is a further similarity between the two systems. The effect of KCl is presumably one of ionic strength rather than specific activation, and it is not unreasonable to expect that, as in the pig liver enzyme, other ionic salts would have the same effect. In contrast to the pig liver and *Ochromonas* enzymes, the yeast cyclase appeared to be soluble, was optimally active in solutions of low ionic strength, stimulated by Triton X-100 and inhibited by deoxycholate [14]. The pig liver and *O. malhamensis* cyclases differed in their apparent K_m values (25 μM and 100 μM, respectively), molecular weights (90,000 and 190,000, respectively) and extent of iminosqualene inhibition. However, the apparent K_m value should be treated with caution, for the following reasons:

(a) The level of endogeneous substrate is unknown.

(b) It is assumed that all the added substrate is sufficiently emulsified to reach the enzyme active site.

(c) The rate of reaction over 30 min might not be the true initial rate. The possible involvement of a sulphydryl group at the active site of the *Ochromonas* enzyme warrants further investigation.

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