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Squalene epoxide cyclase and microemulsion

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Squalene epoxide cyclase was extracted from microsomal preparations of rat liver using anionic, cationic and non-ionic microemulsions. The anionic microemulsion was the best with respect to protein solubilisation, extracted cyclase activity and stability of this activity over time. The activity assay showed cyclase activity to be higher in anionic microemulsion than in buffer in the presence of surfactant. Calcium chloride in the anionic microemulsion had a stabilising effect and less total protein seemed to be extracted.

Squalene epoxide cyclase; Microemulsion; Micelle; Detogert; (Rat liver)

1. INTRODUCTION

The use of microemulsions and related media for enzymic reactions and for the studies of hydrophobic proteins has recently been illustrated [1–3]. Our work on alcohol dehydrogenases from horse liver [4,5] and from *Thermoanaerobium brockii* [6] has led to an optical resolution of a compound of low-water solubility by enzymic conversion in microemulsion [7]. These studies were extended to cholesterol oxidase and, through an examination of the enzyme kinetics in microemulsions, we discovered that this enzyme was inactivated by hydrogen peroxide [8]. Addition of catalase avoided this inactivation and thus afforded the preparative oxidation of cholesterol and related sterols in microemulsions and other heterogeneous media [9,10]. We now present an extension of our study of enzymes in microemulsion to a membrane-bound enzyme which acts on a substrate of low-water solubility: squalene epoxide cyclase (EC 5.4.99.7) [11,12].

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2. MATERIALS AND METHODS

SDS (Fluka) and tetradecyl trimethylammonium bromide (CTAB) (Fluka) were recrystallized from ethanol, washed with pentane and dried under vacuum for 3 days. Triton X-100 (Sigma) was used as obtained. Cyclohexane for UV spectroscopy (Fluka) was used without further purification. *n*-Butanol was purified by fractional distillation.

Rat liver microsomes were prepared according to published procedures [13] and were resuspended in 0.1 M potassium phosphate buffer at pH 7.5 to afford a protein concentration of 5 mg/ml, as determined by a modified Lowry method [14]. All the following experiments were carried out at 20°C, unless otherwise noted.

2.1. *Protein and squalene epoxide cyclase solubilisation in microemulsions*

The microsomal suspension was mixed at 20°C with the surfactant, 1-butanol as cosurfactant, and cyclohexane (weight ratio as indicated in table 1) and was stirred for 10 min. After a specified incubation time, the protein content of the microemulsion was determined after centrifugation at 10000 × *g* for 3 min by the absorption intensity at 280 nm of the supernatant. The squalene epoxide cyclase activity of the supernatant was determined by the extent of ^{3}H-labelled squalene epoxide conversion to lanosterol. The published method for this activity determination in buffer [13] was modified so that it could be used for microemulsions. A solution of squalene epoxide (racemic in all experiments: 120 nmol) and of ^{3}H-labelled squalene epoxide (100000 cpm) in toluene (30 μl) was added at specified times to the microemulsion supernatant (1 ml) from centrifugation as above. After 1 h incubation at 20°C, a 60%

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Table 1

Composition of the microemulsions in weight ratio

Microemulsion	Surfactant	Cosurfactant 1-butanol	Cyclohexane	Microsomal suspension in buffer (ml)	Buffer
	SDS				
Ab-1	1.2	2.0	6.3	0.5	0
Ab-2	1.2	2.0	5.8	0.5	0.5
Ab-3	1.2	2.0	5.3	0.5	1.0
Ab-4	1.2	2.0	5.3	1 (0.1 M $CaCl_2$)	0.5
Ab-5	1.2	2.0	5.3	1 (1 M $CaCl_2$)	0.5
	CTAB				
Cb-1	1.5	2.0	6.0	0.5	
Cb-2	1.5	2.0	5.5	0.5	0.5
Cb-3	1.5	2.0	5.0	0.5	1.0
	Triton X-100				
Nb-1	2.7	2.0	4.3	0.5	0.5

A, C and N: anionic, cationic and non-ionic surfactant; b: 1-butanol

solution of potassium hydroxide (0.2 ml) was added and the medium was dried under vacuum. The oily residue was then extracted twice with ether (5 ml). After concentration, the compounds were separated by thin-layer chromatography on silica plates, according to the published procedure [13]. Since the ^{3}H-labelled squalene epoxide is racemic, its maximal conversion to lanosterol should be 50% and was found with our sample to be 40%. The cyclase activity is therefore calculated from % of the radioactivity which was incorporated and counted in the isolated lanosterol (maximum 40000 cpm) and expressed in nmol of squalene epoxide converted per h to lanosterol.

2.2. *Cyclase activity in buffer and calcium chloride effect*

To a solution of squalene epoxide (20 nmol) and ^{3}H-labelled squalene epoxide (100000 cpm) in toluene (15 μl) was added a solution of Tween 80 (100 μl:50 mg/ml in ethanol). The solvents were then removed under vacuum. The microsomal suspension (0.5 ml) was then added to the residues with stirring. After 30 min at 37°C a 6% potassium hydroxide solution (1 ml) was added and the separation of the products was performed as described [13]. Calcium chloride was added as solid or as 1 M solution in buffer to the microsomal preparations so that the calcium chloride content of the microsomal preparations was 0, 1 mM, 100 mM and 1 M, and the cyclase activity determined.

3. RESULTS

When rat liver microsomes were suspended in microemulsions the protein content of the microemulsion, after centrifugation, was determined from the absorption at 280 nm and was found to be constant between 0.5 and 8 h (table 2). The protein concentration of the microemulsion Nb-1 could not be determined because of the detergent absorption at 280 nm. Anionic microemulsions seemed to extract more total proteins from microsomes than did cationic microemulsions. The water content of anionic microemulsions seemed to have no effect on the amount of the total protein extracted; whereas, for cationic microemulsions, the amount of the total protein extracted increased with the water content. The calcium chloride and pH effects were studied in the anionic microemulsions: anionic microemulsions containing calcium chloride extracted less total protein; variation of the pH from 6 to 10 did not influence the quantity of total protein solubilised in the anionic microemulsions.

Concerning the cyclase activity solubilised in the microemulsions, several effects have clearly been demonstrated. The total solubilised cyclase activity was higher in anionic microemulsions than in cationic and non-ionic microemulsions. In the anionic microemulsions, the solubilised cyclase activity was independent of the water content, the activity was more stable. Calcium chloride had three effects: less total protein was solubilised, the cyclase seemed to be more selectively solubilised and to be more protected from inactivation.

In the activity test in buffer at 37°C, using Tween 80 as surfactant, the microsomal preparation showed an activity of 60 nmol/h per ml of microsomal preparation, compared with the activi-

Table 2

Amount of protein and of squalene epoxide cyclase activity solubilised from microsomes by microemulsions

Microemulsion	Absorption at 280 nm (protein quantity)	Cyclase activity in % of cpm counted to 40000 cpm[a] after the following periods (h) incubation in microemulsion (0.05 ml of microsomal suspension)			Activity[b] in nmol/h after 0.5 h incubation	Activity in nmol/h for an absorption of 1.0 at 280 nm
		0.5	2	8		
Ab-1	0.8	18	9	6	11	14
Ab-2	0.84	20	11	8.5	12	14.3
Ab-3	0.86	21	15	13	12.5	14.5
Ab-4[c]	0.33	20	17.5	16	12	36.4
Ab-5[c]	0.25	19	17	16	11.2	45
Cb-1	0.06	5	4.3	4.0	3	50
Cb-2	0.13	9	7.5	6	5.5	41
Cb-3	0.20	13	12	10.5	7.8	40
Nb-1	–	0.4	0.4			

[a] See text
[b] For 0.05 ml of microsomal suspension in microemulsion
[c] Corrected

ty of 240 nmol/h per ml of microsomal preparation in anionic microemulsion at 20°C.

In buffer, the calcium chloride had a stimulating effect on the cyclase activity at 1 mM calcium chloride concentration: 90 nmol/h per ml of microsomal preparation. At higher calcium chloride concentration, the cyclase activity decreased: 25 for 10 mM; 15 for 100 mM and 14 for 1 M.

4. DISCUSSION

Squalene epoxide cyclase, a microsomal enzyme acting on a substrate of low-water solubility, was of interest for demonstrating the usefulness of microemulsions for solubilisation of a microsomal enzyme in an active form and as enzymic assay medium more compatible with its substrate. We found that the anionic microemulsion was the most efficient of the microemulsions tested here for the amount of total protein and for cyclase activity released from the microsomes. As previously found for other enzymes, cyclase activity was more stable at higher water content. In contrast, the cationic microemulsion solubilised less total protein, so that the cyclase seemed to be more selectively extracted.

The effect of calcium chloride on the partitioning of enzymes within micellar phases had been previously studied [15–18] and it was also found in this study to have an effect on the solubilisation of the cyclase. The solubilised cyclase activity in buffer increased at low calcium chloride concentration, but decreased at higher concentration. In the microemulsion, the cyclase was more selectively extracted from microsomes and protected from inactivation by the presence of calcium chloride.

Non-ionic microemulsion extracted only weak cyclase activity. This result is in sharp contrast to the reported solubilisation of the cyclase from hog liver microsomes using buffer containing a non-ionic surfactant, Emulphogene BC 720 [19]. At this time it is not possible to say whether this difference is attributable to species differences in the microsomes used or to essential differences in solubilisations using microemulsions or solutions of non-ionic surfactants. In this study, solubilised cyclase activity was higher with anionic microemulsion than with buffer in the presence of Tween 80. Again, it is not possible at present to say whether cyclase is more active in microemulsions or whether it is not fully available when it is at least partly bound to the microsomes or whether the substrate diffusion is faster in microemulsion than

in buffer. Further work on purified cyclase should give the answer to several of these questions and to others, such as the calcium chloride effects. We note that cholesterol oxidase showed maximum velocity in the range 3–8 $mmol \cdot min^{-1}$ Enz compared to 1–3 $mmol \cdot min^{-1} \cdot mg^{-1}$ Enz in microemulsions [8]. It should prove interesting to continue such comparisons after more extensive studies have been completed on both enzyme systems. For now, we can certainly conclude that microemulsions will play an important role in further studies of solubilisation of membrane-bound proteins and their hydrophobic substrates.

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