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SOLUBILIZATION OF CAROTENOGENIC ENZYMES OF *APHANOCAPSA*

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Abstract—Twelve non-ionic and eight ionic detergents, over a 0.3–3.0% (w/v) concentration range, were tested for their effect on the conversion of [3-^{14}C]geranylgeranyl pyrophosphate into phytoene, lycopene and β-carotene by a thylakoid preparation of *Aphanocapsa* in order to establish which compounds were the most suitable for enzyme solubilization studies. With the exception of Zwittergent 3-08, the ionic detergents were very inhibitory. In contrast, the non-ionic surfactants were generally less severe, with Brij 78- and Tween 40-treated preparations retaining 69 and 62% of their carotenogenic activities, respectively, at a 3% concentration. The single most effective method for solubilizing the carotenogenic enzymes was by treatment with 1% Tween 40 for 1 hr at 4°. The amounts of acyl lipids removed from the membranes were different with each detergent tested.

INTRODUCTION

A photosynthetic membrane preparation of the unicellular cyanobacterium *Aphanocapsa* is one of the most efficient systems for studies on the *in vitro* biosynthesis of carotenoids, since it converts [3-^{14}C]GGPP into β-carotene in high yields [1], as well as forming xanthophylls from carotene precursors [2, 3]. It has also been used extensively in studies on the mode of action of bleaching herbicides [4–6].

Recent studies with this cell-free system have shown that the enzymes responsible for carotenoid formation are membrane bound and therefore require solubilization before protein purification procedures can be attempted. The complexity of the interactions between lipids and proteins in membranes necessitates an empirical approach to this problem, in order to determine the most suitable solubilizing agent which also retains the enzymic activities of the proteins [7, 8]. Consequently, we have screened twenty detergents for their effects on the carotenogenic enzymes of *Aphanocapsa*. The surfactants displaying minimal inhibitory effects have been used to solubilize the proteins. In addition, the types and quantities of acyl lipids removed from the thylakoids by some detergents have been determined.

Abbreviations: Phytoene, 7,8,11,12,7',8',11',12'-octahydro-ψ,ψ-carotene; lycopene, ψ,ψ-carotene; β-carotene, β,β-carotene; GGPP, geranylgeranyl pyrophosphate; MVA, mevalonic acid; Chaps, 4-(3-cholamidopropyl)-dimethylammonio-1-propane sulphonate; Poly 10, polyoxyethylene-10-tridecyl ether; Z3-08–Z3-16, Zwittergent series (*N*-alkyl-*N*-*N*-dimethyl-3-ammoniopropane sulphonates, designated 3*n*, where *n* is the number of carbon atoms in the alkyl group); sulphoquinovosyl diglyceride (SQDG), 6-sulpho-α-DL-quinovosyl-1(1-1')-2',3'-diacyl-D-glycerol; MGDG, monogalactosyl diacylglycerol; DGDG, digalactosyl diacylglycerol; PG, phosphatidylglycerol; S_{105}, supernatant fraction after centrifugation at 105 000 *g* for 90 min at 4°.

RESULTS

The effects of detergents on in vitro *carotene biosynthesis by* Aphanocapsa *membranes*

Amongst the twenty detergents used in this study, seven ionic (Chaps, cholate, deoxycholate, Z3-10, Z3-12, Z3-14 and Z3-16) and eight non-ionic (Tween 20, Tween 85, Poly 10, Tergitol TMV, Brij 96, Triton X-100, octylglucoside, Lubrol PX) inhibited the incorporation of [3-^{14}C]GGPP into β-carotene by more than 80% at a 1% (w/v) concentration. In general, the non-ionic detergents, with the exception of octylglucoside, were less inhibitory than the ionic surfactants. At a 3% concentration, Brij 78 and Tween 40 retained 69 and 62% respectively, of the control activities of β-carotene formation (Fig. 1). Even at 10% (w/v), Tween 40 treated preparations retained 36% of this activity (data not shown).

The effects of the detergents on phytoene and lycopene formation were variable, but in several cases stimulation of incorporation of [3-^{14}C]GGPP occurred, e.g. Tween 85 (Fig. 1). Within the Zwittergent series, Z3-08 was the least inhibitory, and caused a stimulation of lycopene formation (Fig. 1).

Estimations of the protein concentrations of the S_{105} fractions of detergent-treated and control preparations (Fig. 1) indicated that the surfactants had released some proteins from the membranes, but no obvious correlation was apparent between the effectiveness of protein solubilization and the severity of inhibition of carotene formation.

Solubilization of carotenogenic enzymes

Attempts to solubilize the carotenogenic enzymes of *Aphanocapsa* were made using the three detergents found to be least inhibitory with crude membrane preparations, i.e. Tween 40, Brij 78 and Z3-08 (Fig. 1). Membranes were treated with each detergent in three different ways: for 30 and 60 min at 4° and for 15 min at 20°. The cytosolic (S_{105}) fractions from untreated and detergent-treated prepar-

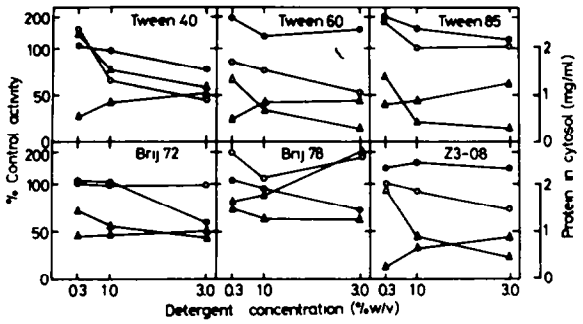

Fig. 1. Carotenogenic enzyme activities and protein release of *Aphanocapsa* membranes after preincubation with non-ionic and ionic detergents for 1 hr at 4°. Enzyme activities were assayed by the incorporation of [3-^{14}C]GGPP (0.25 μCi) into phytoene (○), lycopene (●), and β-carotene (△), and are expressed as percentage control values (643, 2061 and 1944 dpm in phytoene, lycopene and β-carotene, respectively). Protein concentrations (▲) were estimated in cytosolic (S_{105}) fractions. The protein content of the control was 0.30 mg/ml.

ations were then assayed for carotenogenic activities using [^{14}C]phytoene and [^{14}C]lycopene as substrates for 'phytoene dehydrogenase' (phytoene to lycopene) and 'lycopene cyclase' (lycopene to β-carotene), respectively.

Maximum solubilization under the conditions used was found with 1% Tween 40, when preincubated for 1 hr at 4° (Table 1). Under the same conditions, 1% Z3-08 was the second most effective solubilizing agent. Brij 78-treated thylakoids exhibited negligible solubilization under any of these situations. An increase in the concentration of Tween 40 from 1 to 3% greatly reduced the enzymic activities in the cytosol, as did preincubation at 20°.

An alternative protocol was also carried out with 1% Tween 40. The membrane preparation was treated with the detergent for 1 hr at 4°, the S_{105} fraction collected and assayed for carotenogenic activities. The pellet from the centrifugation was reincubated with 1% Tween 40 on two further occasions, thus producing three cytosolic fractions [$S_{105}(1)$, $S_{105}(2)$, $S_{105}(3)$]. The incorporation patterns showed that further enzyme was released from the membranes by repeat extractions, with a total of *ca* 38% of the phytoene to lycopene and lycopene to β-carotene enzyme activities being solubilized (Table 2).

Removal of lipids from Aphanocapsa *membranes by detergents*

It was apparent from the initial screening of 15 surfactants that certain detergents were potent inhibitors of carotenoid biosynthesis, whilst some "mild" surfactants were barely inhibitory, yet achieved negligible solubilization of the carotenogenic enzymes (Table 1). In order to establish whether these phenomena were related to the ability of the detergents to extract certain lipids from the thylakoid membranes, two experiments were carried out. Firstly, the amount of [^{35}S]SQDG released from thylakoids was determined with 5 detergents, at concentrations of 0.1, 1.0 and 3.0% (Table 3). The three 'mild' detergents, i.e. Tweens 40 and 60 and Z3-08, released very little of the available sulpholipid (maximum 2.2%), but both inhibitory detergents, Triton X-100 and octylglucoside, removed large quantities of SQDG, e.g. 73% by 3% octylglucoside.

The second experimental approach involved the analysis of the fatty acid moieties of acyl lipids released from the thylakoids by five detergents (Table 4). Octylglucoside (1% w/v), the most inhibitory of the five detergents selected, extracted over 92% of the acyl lipids, whereas

Table 1. Solubilization of carotenogenic enzymes from *Aphanocapsa* membranes with Tween 40, Brij 78 and Z3-08

		Incorporation pattern in S_{105} fractions, as % control activities* of untreated membranes		
Detergent	Treatment	[^{14}C]Phytoene† into lycopene	β-carotene	[^{14}C]Lycopene‡ into β-carotene
None	—	0.5	0.7	0.6
1% Tween 40	30 min, 4°	5.5	9.0	17.0
	1 hr, 4°	27.3	34.3	27.9
	15 min, 20°	1.8	0.7	0.6
3% Tween 40	30 min, 4°	10.2	0.2	0.8
	1 hr, 4°	0.4	0.2	0.9
	15 min, 20°	2.6	0.8	0.9
1% Brij 78	30 min, 4°	1.5	0.1	0.1
	1 hr, 4°	0.2	0.1	0.0
	15 min, 20°	0.1	0.0	0.0
1% Z3-08	30 min, 4°	3.8	5.8	2.6
	1 hr, 4°	12.1	13.1	17.2
	15 min, 20°	4.9	0.9	0.8

All membrane preparations contained 80 μg chlorophyll.

*Control activities were 14 021 and 32 757 dpm into lycopene and β-carotene from [^{14}C]phytoene and 19 726 dpm from [^{14}C]lycopene into β-carotene.

†111 808 dpm.

‡145 862 dpm.

Table 2. Repeat extractions of *Aphanocapsa* membranes with 1% Tween 40

Fraction	Incorporation pattern in S_{105} fractions, as % of control activities* of untreated membranes		
	[^{14}C]Phytoene into lycopene	β-carotene	[^{14}C]Lycopene into β-carotene
S_{105} (1)	26.3	22.8	27.1
S_{105} (2)	7.1	3.0	6.0
S_{105} (3)	4.7	1.3	4.6

Membrane preparations contained 120 μg chlorophyll.
*Control activities were 3571 and 8275 dpm into lycopene and β-carotene from 29 888 dpm [^{14}C]phytoene and 6960 dpm into β-carotene from 13 074 dpm [^{14}C]lycopene.

Table 3. Solubilization of [^{35}S]SQDG from *Aphanocapsa* membranes by detergents

Detergent (%w/v)	[^{35}S]Sulphoquinovosyl diglyceride released into the S_{105} fraction (μmol)
Tween 40	
0.1	0.016
1.0	0.013
3.0	0.047
Tween 60	
0.1	0.008
1.0	0.024
3.0	0.045
Z3-08	
0.1	0.003
1.0	0.016
3.0	0.023
Triton X-100	
0.1	0.355
1.0	0.513
3.0	1.319
Octylglucoside	
0.1	0.015
1.0	0.792
3.0	1.522

Membrane preparations contained 300 μg chlorophyll and were prelabelled with [^{35}S]SQDG (2.09 μmol). They were treated with detergents for 1 hr at 4°.

Tween 40 and 60 released about 20% of these lipids from the thylakoids. A comparison of the ratio of C_{16}:C_{18} fatty acids revealed that the Tweens had preferentially removed acyl lipids containing C_{18} fatty acid residues, but the other three detergents did not show such selectivity (Table 4).

DISCUSSION

The marked sensitivity of the carotenogenic enzymes of *Aphanocapsa* to surfactants was also found in an earlier investigation with *Phycomyces* [9]. Since GGPP is an immediate precursor of the carotenes, the effects of the detergents must be on the carotenogenic enzymes *per se*, rather than earlier enzymes such as prenyl transferase, which has been shown to be stimulated by Tween 80 in tomato fruits [10]. The results also verify the importance of an empirical approach to the problem of solubilizing carotenogenic enzymes; although Tween 40 is the most suitable with *Aphanocapsa* thylakoids, Tween 60 was found to be the best for *Phycomyces* [9], whilst Chaps, a potent inhibitor of carotene formation in both *Aphanocapsa* and *Phycomyces* [9], has recently been shown to be the detergent of choice for daffodil chromoplasts [11]. The stimulation of incorporation of [3-^{14}C]GGPP into phytoene and lycopene by some non-ionic detergents e.g. Tween 85 and by Z3-08 (Fig. 1) has been shown previously for 'phytoene synthetase' of *Phycomyces* [9], prenyl transferase [11] and also enzymes associated with non-terpenoid pathways [12]. The precise reason for this latency of enzymic activity is not known, but the presumably peripheral location of 'phytoene synthetase' and 'dehydrogenase' on the *Aphanocapsa* thylakoid may enable mild detergents to alter the active sites of the enzymes and make them more accessible to their respective substrates. Certainly the apparent stimulation of enzymic activity cannot be accounted for by the concomitant inhibition of β-carotene formation (Fig. 1).

The lack of a correlation between protein release from the thylakoids and the inhibition/solubilization of carotenogenic enzymes (Fig. 1, Table 1) indicates that no detergent is able to selectively solubilize these proteins. A recent, more detailed, study on protein solubilisation of the enzymes from daffodil using SDS gel electrophoresis has established that Chaps extracts proteins in a non-selective manner [11].

The most favourable protocol for the solubilization of the carotenogenic enzymes from thylakoids was repeated, hour-long treatments with 1% Tween 40 at 4° (Table 2). Increases in temperature to 20° or detergent concentration to 3% were not effective in this respect (Table 1), presumably because the detergent was inhibitory under these conditions.

The major lipids of cyanobacteria are MGDG, DGDG, SQDG and, to a minor degree (less than 10%), PG [13]. The fatty acid patterns of the acyl lipids are known to show great diversity [13] and in *Aphanocapsa* consist of C_{16} and C_{18} fatty acids, with palmitic (16:0) and linolenic (18:3) acids being predominant (Table 4). This composition is similar to that reported for *Anabaena variabilis* [14].

The removal of acyl lipids by detergents, as measured either directly (SQDG, Table 3) or by their constituent fatty acid residues (Table 4), shows several trends which suggest that MGDG and DGDG are present in the native environment of carotenogenic enzymes. The almost total extraction of all the lipids by 1% octylglucoside confirms that it has a harsh, non-selective action on *Aphanocapsa* thylakoids which results in almost complete inhibition of carotenogenesis by this detergent. Triton X-100 solubilizes less lipids than octylglucoside, but is also non-selective and a potent inhibitor of carotenogenesis. In contrast, Z3-08 is selective in its extraction of acyl lipids, since it removes negligible amounts of SQDG (Table 3), but some 45% of the other lipids, i.e. MGDG and DGDG, if the small amount of thylakoid PG is ignored. The ratio of C_{16}:C_{18} fatty acids of the Z3-08 extracted lipids is closely similar to the native membranes, indicating no preferential extraction in this respect. Tweens 40 and 60 are very

Table 4. The fatty acid composition of acyl lipids released from *Aphanocapsa* thylakoids by detergents

Treatment	Fatty acids (% of untreated thylakoid fatty acids)						Ratio C_{16}:C_{18} fatty acids released
	16:0	16:1	18:0	18:1	18:2	18:3	
Tween 40							
0.1%	15.8	20.3	48.8	41.8	2.5	23.5	0.82
1.0%	13.5	18.8	83.7	30.4	12.7	19.6	0.63
Tween 60							
0.1%	5.0	3.1	32.6	13.9	4.2	3.9	0.61
1.0%	16.5	3.1	67.4	68.4	11.0	13.7	0.58
Z3-08							
0.1%	20.5	29.7	18.6	38.0	8.5	18.3	1.33
1.0%	40.0	56.3	105	69.6	18.6	43.1	1.04
Triton X-100							
0.1%	50.0	34.4	109	50.6	48.3	27.5	1.19
1.0%	50.0	43.8	46.5	64.6	114	15.7	0.99
Octylglucoside							
0.1%	57.5	51.6	58.1	65.8	66.9	63.4	1.04
1.0%	100	102	55.8	82.3	78.8	92.8	1.44

Aphanocapsa thylakoids, containing 600 μg chlorophyll, were treated with detergents for 1 hr at 4°. The acyl lipids released into S_{105} fractions were isolated and their constituent fatty acids estimated. The fatty acid composition of untreated thylakoids is: 16:0, 4.0; 16:1, 0.64; 18:0, 0.43; 18:1, 0.79; 18:2, 1.18; 18:3, 1.55 μmol, and the C_{16}:C_{18} fatty acid ratio 1.18.

similar to each other with respect to their abilities to remove acyl lipids from thylakoids (Tables 3 and 4), since they remove only small amounts of SQDG (Table 3) and extract MGDG and DGDG with C_{18} fatty acid moieties in preference to C_{16} residues (Table 4). This phenomenon does not occur with Z3-08, Triton X-100 or octylglucoside. Tween 60, however, is far more inhibitory (63% at 1% w/v) than Tween 40 (13% at 1% w/v). Possibly Tween 40, which contains palmitic acid (16:0) as its fatty acid residue, is able to mimic the C_{16} fatty acids present in the native environment of the enzymes, whereas Tween 60, with a stearic (18:0) acid residue, cannot fulfil this function and therefore cannot retain the activities of the enzymes. Further experiments are needed, however, in order to prove this suggestion.

In conclusion therefore, we have obtained a solubilized, stable preparation of carotenogenic enzymes of *Aphanocapsa* which will be used in future work on the purification of the individual enzymes.

EXPERIMENTAL

Organisms and culture conditions. Aphanocapsa 6714 (Pasteur Culture Collection, Paris) was grown in a liquid minimal medium at 35° as described previously [1]. The C5 *car B*10 (−) and C9 *car R*21 (−) strains of *Phycomyces blakesleeanus* were obtained from the culture collection of the Departamento de Genetica, Universidad de Sevilla, Sevilla, Spain. Their growth and maintenance conditions have been described in an earlier publication [15].

Radiochemicals. DL-[2-^{14}C]Mevalonic acid lactone (53 mCi/mmol) and sodium [^{35}S]sulphate (34 mCi/mmol) were purchased from Amersham Buchler, Braunschweig, F.R.G. The former was converted to the Na salt prior to use [16]. [3-^{14}C]GGPP (10 mCi/mmol) was synthesized by Prof. G. Schultz, Hannover, F.R.G. according to the method of Soll and Schultz [17]. [^{14}C]-Labelled phytoene and lycopene were formed *in situ* from [2-^{14}C]MVA using cell extracts of the C5 and C9 strains of *Phycomyces*, respectively, as described in the section of enzyme assays.

Preparation of cell extracts. Cell-free systems of *Phycomyces* were prepared in 0.4 M Tris–HCl buffer pH 8.0, containing 5 mM dithiothreitol, essentially as described previously [16]. Thylakoid preparations of *Aphanocapsa* were obtained by osmotic lysis of spheroplasts as detailed in an earlier publication [1]. Cytosolic samples of *Aphanocapsa* extracts were obtained as S_{105} fractions.

Treatment with detergents. All detergents were prepared as 10 or 20% (w/v) stock solns in distilled water. Appropriate aliquots of these solns were added to *Aphanocapsa* membrane preparations to give final concentrations of 0.3, 1.0 and 3.0% (w/v). The mixtures were left over ice for 60 min prior to analysis, unless otherwise stated.

Enzyme assays. Carotenogenic enzyme activities were assayed by either determining the incorporation of [3-^{14}C]GGPP (0.25 μCi) into phytoene, lycopene and β-carotene, using the incubation conditions described previously [1], or by using a coupled assay system of DL-[2-^{14}C]MVA (0.5 μCi) with cell extracts of the C5 (phytoene-producing) or C9 (lycopene-producing) strains of *Phycomyces* added to the *Aphanocapsa* preparations. The practical details of the coupled assay have been given elsewhere [3]. All incubations were carried out for 2 hr at 35°, under aerobic conditions in fluorescent light (15 w/m^2). The [^{14}C]-labelled carotenoids were exhaustively purified by TLC [1, 2, 17, 18] and finally radioassayed by liquid scintillation counting [19].

Determination of SQDG. Aphanocapsa was grown under normal conditions [1], in the presence of sodium [^{35}S]sulphate (25 μCi; specific activity in the medium 125 μCi/mmol). Following isolation of the membranes and treatment with detergents, the [^{35}S]SQDG was isolated from the S_{105} fraction by partition with $CHCl_3$–MeOH (2:1) against satd NaCl. The hypophase was collected, washed with dilute NaCl soln and dried overy dry Na_2SO_4. The extract was then subjected to TLC on silica gel G, developed with $CHCl_3$–MeOH–H_2O (65:25:4) [2]. The band corresponding to SQDG (typical R_f 0.31) was visualized with I_2 vapour, scraped off and subjected to liquid scintillation

counting [19]. The percentage removal of this lipid from *Aphanocapsa* thylakoids was calculated by comparison with the total amount of [^{35}S]SQDG in control preparations.

Determination of fatty acids. Acyl lipids were extracted from *Aphanocapsa* membranes (control preparations) and from the S_{105} fractions of detergent-treated thylakoids with $CHCl_3$–MeOH, as described previously [20]. Prior to hydrolysis and estimation of the constituent fatty acid moieties, the acyl lipids of detergent-treated extracts were chromatographed on activated silica gel G thin layers in order to remove traces of contaminating detergents. The solvents systems were as follows: for octylglucoside-treated preparations, Me_2CO–toluene –H_2O–HOA (60:20:4:1); for Triton X-100-, Tween 40- and Tween 60-treated preparations, $CHCl_3$–MeOH–25% ammonia–H_2O (65:30:15:2.5) and Z3-08-treated thylakoids, $CHCl_3$–MeOH–H_2O (65:35:4). Bands corresponding to PG, MGDG, DGDG and SQDG were visualized with Rhodamine 6G, scraped off and eluted with MeOH at 65°. The subsequent hydrolysis of these lipids, the formation of methyl esters of the fatty acids and their quantitation by GC has been described in detail in a previous publication [21].

Other estimations. Protein concentrations of cell extracts were estimated with the Bio-Rad protein assay reagent using BSA as a standard. Where necessary, detergents were included in the reference solutions. Determinations of membrane-bound chlorophyll were made by measurement of its absorbance in 80% Me_2CO [22].

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