Taurine conjugates in the lipid fraction of *Euglena* cells and their mitochondria

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Dark-grown resting (non-dividing) cells of *Euglena gracilis* var. *bacillaris* and mutants W₃BUL (with a proplastid remnant) and W₁₀BsML (lacking plastids) incubated with ³⁵SO₄²⁻ form a series of labelled lipids which are low or absent in dividing cells. These lipids all release labelled taurine on mild acid-hydrolysis. Treatment of the labelled lipids with 2,4-dinitrofluorobenzene (DNFB) followed by acid hydrolysis does not yield labelled dinitrophenyltaurine (DNP-taurine), but treatment with DNFB after hydrolysis readily forms labelled DNP-taurine, indicating that taurine is linked to the lipids by at least the amino group. Illumination increases the labelling of these taurolipids in plastid-containing cells (wild-type and W₃BUL) but has little effect in cells lacking plastids (W₁₀BsML); labelling is highest in W₁₀ cells irrespective of illumination. This indicates that the presence of a plastid may exert a negative control on taurolipid formation which is relieved by light. The same series of labelled lipids is found in isolated purified mitochondria from mutant W₁₀, indicating that this organelle is a site for taurolipid deposition. The formation of taurolipids under non-dividing conditions may be a response to nutritional stress and these negatively charged constituents (as well as the thylakoid sulpholipid) may serve to protect membranes by repelling deleterious negatively charged oxygen species.

Introduction

Lipid extracts of cells of *Euglena gracilis* var. *bacillaris* grown in darkness or light on a complete medium with ³⁵SO₄²⁻ show a single major labelled spot on thin-layer silica gel chromatography corresponding to the thylakoid sulpholipid (diacylsulphoquinovosylglycerol); much higher labelling is observed in the light than in the dark (Saidha & Schiff, 1989). Mutant W₃BUL (W₃) retains only a proplastid remnant but also shows light-stimulated accumulation of the sulpholipid under dividing conditions. W₃ yields a chromatographic pattern much like the wild-type with a single major spot corresponding to the sulpholipid (Saidha & Schiff, 1989). Dividing cells of mutant W₁₀BsML (W₁₀) lack plastids completely (Osafune & Schiff, 1983) and show no major radioactive spot under the same conditions in either light or

darkness. W₁₀, therefore, does not make the sulpholipid (Saidha & Schiff, 1989). Although traces of other radioactive spots could occasionally be seen on these chromatograms, their nature only became apparent in a study of resting (non-dividing) cells of *Euglena* incubated with ³⁵SO₄²⁻ on a medium containing mannitol (not utilized as a carbon source for growth by *Euglena*), MgCl₂ and KH₂PO₄. Under non-dividing conditions, cells of wild-type *Euglena* and W₃ incubated with ³⁵SO₄²⁻ still form radioactive sulpholipid. In addition, wild-type, W₃ and W₁₀ accumulate substantial radioactivity in several spots that were only labelled slightly in dividing cells. As we will show in this paper, these spots contain taurine conjugates of lipids ('taurolipids'). The properties and labelling of these compounds in resting cells of *Euglena* wild-type, W₃ and W₁₀ will be presented and their possible physiological function will be discussed. A brief abstract of this work has appeared (Saidha *et al.*, 1990).

Methods

Cell culture and labelling. *Euglena gracilis* Klebs var. *bacillaris* Cori and mutants W₃ and W₁₀ were grown in darkness with shaking at 26 °C on modified Hutner's pH 3.5 medium (MHM) containing low vitamin

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Abbreviations: DNFB, 2,4-dinitrofluorobenzene; DNP-taurine, dinitrophenyltaurine.

B₁₂ (50 ng l⁻¹) and just enough sulphate to allow normal growth as described previously (Saidha *et al.*, 1988; Cogburn & Schiff, 1984). After at least three transfers in darkness, the cells were grown in darkness for 4 d. Either these cells were used directly, or they were diluted with resting medium (Stern *et al.*, 1964) containing 0.045 M-mannitol (an osmoticum not used as a carbon source), 0.01 M-MgCl₂ and 0.01 M-KH₂PO₄ at pH 5.5, incubated with shaking in darkness for 72 h (during which time cell division ceases) and then used for experiments in which resting (non-dividing) cells were required. Cells were counted in a Coulter model A counter as previously described (Zeldin & Schiff, 1967). All manipulations of dark-grown cells were done under dim green safelights (Schiff, 1972).

For labelling experiments, carrier-free ³⁵SO₄²⁻ [0.5 mCi (18.5 MBq) in 250 ml] was added to growing or resting cultures. The cultures were then shaken in darkness or in light [90 ft-c (3.08 W m⁻²) from white and red fluorescent lamps] and 50 ml samples were removed at the indicated times. The culture sample was passed through four layers of cheesecloth and was then centrifuged at 370 g for 5 min. The cell pellet received 5 ml of chloroform/methanol (2:1, v/v) and was then incubated at 37 °C for 20 min. A volume of 0.45% (w/v) NaCl solution equal to 0.25 of the volume of the solvent/cell mixture was then added and mixed thoroughly by vortexing; the mixture was then centrifuged at 370 g for 5 min. The lower organic phase was removed and taken to dryness in a stream of N₂ (Haas *et al.*, 1980).

Preparation of mitochondria from ³⁵S-labelled *W*₁₀BsM cells. In previous work we have described methods for preparation of clean, coupled mitochondria from *Euglena* using a Yeda press to break the cells. Since then, others have used sonication to break the cells which yields equally good coupled mitochondria (Moreno-Sanchez & Raya, 1987). The sonication method proved to be most convenient for breaking radioactive cells in the present work and this was combined with our previous methods for isolating and purifying the mitochondria (Gomez-Silva *et al.*, 1985; Saidha *et al.*, 1985). The total procedure is as follows. A culture of *Euglena* (1.44–1.58 × 10⁸ cells in 350 ml representing 1.75 g wet wt of cells) received carrier-free ³⁵SO₄²⁻ and was incubated for 72 h in darkness under resting conditions as described above. The culture was then centrifuged at 370 g for 5 min and the pellet was washed twice with 100 ml of Buffer A (containing 25 mM-HEPES/KOH buffer, pH 7.4, 0.25 M-sucrose and 0.005 M-EDTA) by centrifugation. The pellet obtained after the second wash was resuspended in 25 ml of Buffer A and sonicated continuously at 70% of maximum power for two intervals of 15 s each in an ice-bath using a Branson model 200 sonifier with a microtip 0.4 cm in diameter. After sonication, the suspension was centrifuged at 370 g for 5 min and the pellet was discarded. The supernatant fraction was then centrifuged at 10000 g for 10 min to obtain the crude mitochondrial pellet. This pellet was resuspended in 1–2 ml of Buffer A and 0.5–1.0 ml was layered on top of a discontinuous Percoll density gradient (consisting of steps of 60, 30, 20, 10 and 5%, w/v, Percoll) described in detail previously (Saidha *et al.*, 1985). Band A, appearing at the interface of 60% and 30% Percoll after centrifugation and containing highly purified and intact mitochondria (Gomez-Silva *et al.*, 1985) was collected by suction, diluted with 20 ml of Buffer A and the mixture was centrifuged at 10000 g for 10 min to remove Percoll. The pellet of the purified mitochondria thus obtained was extracted for lipids as described above for cells. Separation of the lipids was achieved by TLC as described below.

Separation and purification of ³⁵S-labelled lipids. The dried organic phase was resuspended in 200 µl of chloroform/methanol (2:1, v/v) and either spotted (for quantitative work) or strip-loaded (for preparative work) onto a glass silica gel thin-layer chromatographic (TLC) plate, 20 × 20 cm, previously activated by heating at 100 °C for 1 h. The plate was developed in chloroform/methanol/acetic acid/water (65:35:8:4, by vol.). After development, the dried TLC plate was exposed to X-ray film and radioactive areas corresponding to the lipids

were scraped from the plate and counted in a scintillation counter (Saidha *et al.*, 1985) for quantitative analysis; for preparative work, the strip was scraped and re-extracted with chloroform/methanol and repartitioned with NaCl as described above.

Acid hydrolysis of the unknown ³⁵S-labelled lipids. Measured samples of TLC-purified lipids in chloroform/methanol were taken to dryness. HCl and Triton X-100 were added to each sample to yield final concentrations of 1 M and 0.1% respectively (Benson *et al.*, 1959). The mixtures were heated at 100 °C for 30 min, dried under N₂ and the residual HCl removed under vacuum overnight in a desiccator over NaOH. The compounds in the hydrolysate were separated by paper electrophoresis in sodium borate (pH 8.0), sodium citrate (pH 5.8) and formic/acetic acid (pH 2.0) buffer systems (Saidha *et al.*, 1988). Known compounds including taurine and dinitrophenyl taurine (DNP-aurine) were examined for co-electrophoresis with the ³⁵S-labelled hydrolysed products by autoradiography. Co-chromatography of the products with taurine was also investigated on cellulose thin-layers in butanol/acetic acid/water (80:20:20, by vol.), propan-2-ol/ammonia/water (6:3:1, by vol.), and two-dimensionally in methanol/n-butanol/benzene/water (120:60:60:60, by vol.) followed by n-butanol/acetic acid/water (100:22:50, by vol.) (Schiff, 1959). Co-chromatography of DNP-aurine was investigated in the two-dimensional system and in the butanol/acetic acid/water and propan-2-ol/ammonia/water systems described above, and on cellulose thin-layers in chloroform/methanol/acetic acid/water (65:35:8:4, by vol.).

Other methods. DNP-aurine was synthesized from taurine using 2,4-dinitrofluorobenzene (DNFB) by the method of Pentz *et al.* (1957) scaled down 10-fold. DNP-derivatization of the ³⁵S-labelled acid-hydrolysed products of the unknown lipids was achieved by substituting the neutralized acid-hydrolysed products for taurine in the above procedure and using the reaction mixture without further purification for electrophoresis or chromatography. Taurine was visualized by ninhydrin spray (Saidha *et al.*, 1988). DNP-aurine was located by its yellow colour.

Results and Discussion

Resting cells of *Euglena gracilis* var. *bacillaris* and mutants W₃ and W₁₀ incubated in light or darkness with ³⁵SO₄²⁻ accumulate at least three novel chloroform/methanol-extractable ³⁵S-labelled compounds, which separate during chromatography on silica gel thin-layer plates. These are designated A, B and C in Fig. 1. Labelled sulpholipid is also found in all cells except those of W₁₀ (Saidha & Schiff, 1989) which lack plastids completely (Osafune & Schiff, 1983).

In general, compounds A, B and C are highly labelled in light or darkness under these conditions (Table 1). In wild-type and W₃ light stimulates the labelling of A, B and C, but in W₁₀ light has a comparatively small effect and is stimulatory to labelling of compound A, has no effect on labelling of compound B and is somewhat inhibitory to labelling of compound C. However, W₁₀ generally shows the highest labelling of the compounds compared with wild-type and W₃. The data suggest that the presence of a plastid (a proplastid or chloroplast in dark-grown or light-grown wild-type cells or a proplastid remnant in dark-grown or light-grown W₃) brings the system under negative control, since W₁₀, which lacks

Fig. 1. Autoradiograms of thin-layer silica gel plates developed in chloroform/methanol/acetic acid/water (65:35:8:4, by vol.) showing separation of ^{35}S -labelled lipids from dark-grown resting wild-type (lanes 2 and 3) cells of *Euglena gracilis* var. *bacillaris* and mutants W_3 (lanes 5 and 6) and W_{10} (lanes 8 and 9) incubated with carrier-free $^{35}\text{SO}_4^{2-}$ in the light for the indicated times. Lanes 1, 4 and 7 received [^{35}S]sulpholipid standard prepared from light-grown wild-type *Euglena* as described previously (Saidha & Schiff, 1989). Spots A, B and C represent the unknown lipids and SL indicates sulpholipid. Spot C' was not included in the various analyses but may well be another tauro lipid.

plastids, shows the greatest labelling in either light or darkness. Thus the presence of a proplastid in darkness lowers the labelling of compounds A, B and C and renders the cells responsive to stimulation of labelling of these compounds by light as observed in wild-type and W_3 . In contrast, cells of W_{10} , which lack plastids, show high and fairly uniform labelling of A, B and C in darkness or in light. Perhaps the plastid produces an inhibitor or repressor that blocks labelling of A, B and C in darkness, and light causes a lowering of this control molecule. As found previously (Saidha & Schiff, 1989), labelling of the plastid thylakoid sulpholipid is stimulated

by light in wild-type and in W_3 (Table 1), reflecting the induction of development of the proplastid into a chloroplast in wild-type cells (Saidha & Schiff, 1989) or limited development of the proplastid remnant in W_3 (Osafune & Schiff, 1980); W_{10} , of course, contains no plastids (Osafune & Schiff, 1983) and makes no sulpholipid (Table 1).

Mild acid hydrolysis releases [^{35}S]taurine from purified compounds A, B and C (Fig. 2). When purified (see Methods) ^{35}S -labelled A, B and C were exposed to 2,4-dinitrofluorobenzene (DNFB) (which substitutes free amino groups to yield DNP derivatives) and were then

Table 1. Radioactivity in taurolipids (spots A, B and C) and in sulpholipid (SL) in dark-grown wild-type resting cells of *Euglena gracilis* var. *bacillaris* and mutants W_3 and W_{10}

Radioactivity was measured 48 h after addition of carrier-free $^{35}\text{SO}_4^{2-}$ and incubation in light or darkness. ND, Not detected.

Lipid type	Radioactivity (d.p.m. per 10^4 cells)					
	Wild-type		W_3 BUL		W_{10} BSmL	
	Light	Dark	Light	Dark	Light	Dark
A	7939	1520	5587	1742	12624	8636
B	13825	3801	34317	10862	32998	32959
C	32652	9658	22219	10303	41979	73885
SL	942179	23379	103007	15150	ND	ND

acid-hydrolysed; again, free [^{35}S]taurine was found on a subsequent paper electrophoresis (Fig. 3). However, if compounds A, B and C were treated with DNFB after undergoing acid hydrolysis, a labelled compound moving to the position of authentic DNP-taurine was found, indicating that the taurine is bound to the lipid in each case at least through the amino group. Unhydrolysed compound A, B or C treated with DNFB (Fig. 3) or each of the untreated compounds (not shown) stays at the origin and does not move at all. The ^{35}S -labelled acid-hydrolysis products of compounds A, B or C show exact co-electrophoresis with authentic taurine on electrophoresis in citrate (pH 5.8), borate (pH 8.0) and formic/acetic acid (pH 2.0) buffer systems (data not shown); co-chromatography in various one-dimensional or two-dimensional solvent systems (see Methods) on silica gel and cellulose thin-layers was also demonstrated. Thus compounds A, B and C contain taurine that is linked, at least through the amino group, to a lipid.

Our previous work has indicated that a sulphate-metabolizing centre is present on the outside of the inner mitochondrial membrane of *Euglena* (Saidha *et al.*, 1985, 1988). For this reason, it was of interest to determine whether the taurolipids of *Euglena* are present in the mitochondria. Fig. 4 shows that purified mitochondria from cells of *Euglena* W_{10} BSmL contain the same collection of taurolipids as the whole cells. Thus, the mitochondria represent a significant depot for taurolipids in the *Euglena* cell. Since it is known that the loss of plastids leads to an increase in mitochondrial material in *Euglena* (Lefort, 1964), the increased taurolipid labelling seen in aplastidic mutant W_{10} compared with plastid-containing wild-type and W_3 cells (Table 1) is consistent with a mitochondrial site for taurolipids in *Euglena*.

Studies by others have demonstrated the presence of a 'taurolipid A' [identified as 2-(3-acyloxy-7,13-dihydroxy-octadecanoyl)aminoethanesulphonic acid] in *Tetra-*

Fig. 2. Acid hydrolysis of compounds A, B and C shown in Fig. 1. ^{35}S -Labelled compounds A, B and C were isolated and purified from *Euglena gracilis* var. *bacillaris* mutant W_{10} BSmL and were subjected to hydrolysis in 1.0 M-HCl as described in Methods. The hydrolysate was then subjected to paper electrophoresis in citrate buffer, pH 5.8. An autoradiogram of the electrophoretogram is shown; the positions of authentic sulphate and taurine are indicated for comparison.

hymena pyriformis (Kaya *et al.*, 1985), and a 'taurolipid B' [identified as 2-(3-acyloxy-2,7,13-trihydroxyoctadecanoyl)aminoethanesulphonic acid] in *Tetrahymena*

Fig. 3. Lipids A, B and C from light-grown wild-type resting cells of *Euglena gracilis* var. *bacillaris* exposed to light and $^{35}\text{SO}_4^{2-}$ for 72 h, subjected to various treatments followed by paper electrophoresis (diagrammatic representation) in citrate buffer (pH 5.8). Upper section, key to content of electrophoresis spots; middle section, paper electrophoretogram; bottom section, protocol for treatments of lipids A, B and C preceding electrophoresis. The positions of taurine and DNP-taurine standards on the electrophoretogram are also shown.

thermophila (Kaya *et al.*, 1986), as well as other related compounds in cells of *Tetrahymena thermophila* (Kaya & Sano, 1991); the tauro lipids 'A' and 'B' were shown to be present in the mitochondrial-lysosomal fraction of *T. pyriformis* and *T. thermophila* (Kaya & Nohara-Uchida, 1986). The *Euglena* tauro lipids may be quite similar to the *Tetrahymena* tauro lipids, but this remains to be proven. (The letter designations for the various tauro lipids in the two organisms were chosen independently and arbitrarily by the investigators.) Since *Tetrahymena* is a ciliated protozoan and *Euglena* is closely related to the flagellated protozoa, it is possible that similar tauro lipids might be found in other protozoan groups and species. Interestingly, three other lipids are known that contain a taurine configuration [the capnoids (capnine and *N*-acylcapnine) found in bacteria of the genus *Capnocytophaga* (Godchaux & Leadbetter, 1980) and 1-deoxyceramide-1-sulphonic acid found in the diatom *Nitzschia alba* (Anderson *et al.*, 1978, 1979)], but in these taurine is linked by carbon-carbon bonds to a long carbon skeleton.

Taurine has been implicated indirectly in the preservation of membrane integrity (e.g. Hayes *et al.*, 1975; see Wright *et al.*, 1986, for a review). The sulphonic acids in general (including the sulpholipid of the thylakoid membrane and the tauro lipids of the mitochondria) might be very appropriate for such a function. The sulphonic acid group has a low *pK* and, therefore, is ionized at physiological pH, is very unreactive and is very resistant to release by hydrolysis from the carbon to which it is attached. Thus as a membrane constituent, it would provide stable negative charges under a variety of conditions that might repel negatively charged deleterious chemical species such as some toxic derivatives of oxygen. The increased tauro lipid formation in resting cells may represent a stress response to nutritional deprivation connected with the protection of mitochondrial membranes.

Two different forms of ATP sulphurylase are found in *Euglena* (Li *et al.*, 1991); one is soluble and the other is mitochondrial. The mitochondrial enzyme is found together with the enzymes of adenosine 3'-phosphate 5'-

Fig. 4. Resting cells of *Euglena gracilis* var. *bacillaris* mutant $W_{10}BSmL$ were labelled with $^{35}SO_4^{2-}$ and mitochondria were prepared as described in Methods. Both cells and purified mitochondria were extracted with chloroform/methanol and the resulting lipid extracts were chromatographed on a silica gel thin-layer plate. An autoradiogram of the dried thin-layer plate after chromatography is shown. Note the correspondence of the major lipid spots obtained from whole cells and mitochondria.

phosphosulphate (PAPS) formation and sulphate reduction to sulphite and cysteine on the outside of the inner mitochondrial membrane (Saidha *et al.*, 1985, 1988). The plastid receives the reduced sulphur for sulpholipid formation from the mitochondrion as sulphite or cysteine (Saidha & Schiff, 1989); sulphite might also serve as the source of sulphur for the formation of mitochondrial taurolipids.

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