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A sulphate metabolizing centre in *Euglena* mitochondria

Tekchand SAIDHA, Song-Qing NA, Jiayang LI and Jerome A. SCHIFF*
Photobiology Group, Biology Department, Brandeis University, Waltham, MA 02254, U.S.A.

We have previously shown that a sulphate activating system is present on the outside of the inner mitochondrial membrane of *Euglena gracilis* Klebs. var. *bacillaris* Cori, but efforts to couple this system to ATP produced from oxidative phosphorylation were unsuccessful. In the present work we show that the concentration of P_i ordinarily used to support oxidative phosphorylation in these mitochondria (10 mM) inhibits sulphate activation completely; by reducing the concentration of P_i 10-fold, both processes proceded normally. Sulphate activation under these conditions is inhibited nearly completely by the uncouplers of oxidative phosphorylation dinitrophenol (0.1 mM) and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (0.2 μM). Sulphate reduction to form free cysteine, most of which appears outside the organelle, and in the cysteine of mitochondrial protein can be demonstrated in the same preparations, is membrane-bound and is inhibited by chloramphenicol (100 μg/ml), NaN_3 (5 mM), KCN (100 μM), dinitrophenol (0.1 mM) or CCCP (0.2 μM). Digitonin fractionation of the mitochondria into mitoplasts, outer membranes and an intermembrane fraction show that reduction of $^{35}SO_4^{2-}$ to form free cysteine and cysteine of protein is located on the mitoplasts; adenosine 5′-phosphosulphate sulphotransferase, the first enzyme of sulphate reduction, is found in the same location. Sulphate activation is highly enriched in the mitochondrial fraction of *Euglena*; the small amount found in the chloroplast fraction can be attributed to mitochondrial contamination. Thus, in *Euglena*, sulphate activation and reduction are contained in a sulphate metabolizing centre on the outside of the mitochondrial inner membrane; this centre appears to supply the mitochondrion and the rest of the cell with the products of sulphate activation as well as with reduced sulphur in the form of cysteine. Mitochondria from wild-type *Euglena* cells and from W_{10}BSmL, a mutant lacking plastids completely, appear to be similar in the properties studied.

INTRODUCTION

Our previous studies have shown that *Euglena* mitochondria contain the enzymes capable of sulphate activation and reduction (Saidha *et al.*, 1985; Brunold & Schiff, 1976). The enzymes involved in sulphate activation (ATP sulphurylase, EC 2.7.7.4; adenylylsulphate kinase, EC 2.7.1.25, and inorganic pyrophosphatase, EC 3.6.1.1) are thought to be located on the outside of the mitochondrial inner membrane (Saidha *et al.*, 1985). In these experiments the coupled energy metabolism of the mitochondrion was not employed; ATP (or ADP utilized via the mitochondrial adenylate kinase) was supplied in the incubation mixture.

In the work to be described, it has become possible to study sulphate metabolism linked to the energy metabolism of the mitochondrion. We have found that inorganic phosphate (P_i) is an inhibitor of sulphate activation, but conditions can be found where P_i is supplied at concentrations that allow oxidative phosphorylation to take place but do not inhibit sulphate activation. Using these more physiological conditions we are able to show that, like sulphate activation, sulphate reduction to free cysteine appears to be located on the outside of the inner membrane of *Euglena* mitochondria; subsequently, this amino acid is incorporated into mitochondrial proteins. A brief abstract of this work has appeared previously (Saidha & Schiff, 1986).

EXPERIMENTAL

Growth of the organism

Wild-type *Euglena gracilis* Klebs var. *bacillaris* Cori was used for isolation of both chloroplasts and mitochondria when both were required. W_{10}BSmL, a mutant lacking chloroplasts and proplastids (Osafune & Schiff, 1983), was used to prepare mitochondria for most of the other experiments. We have not detected any differences between the mitochondrial preparations from the two types of cells.

Cells of W_{10}BSmL were grown in darkness for 5 days on a medium containing reduced levels of vitamin B-12 and highly purified intact (fraction A) mitochondria were prepared as described previously (Saidha *et al.*, 1985). Dark-grown wild-type cells were used to innoculate cultures on the same low vitamin B-12 medium except that the excess sulphate in the medium was decreased by lowering the $MgSO_4$ concentration 10-fold and adding $MgCl_2$ to keep the Mg^{2+} concentration the same as in the normal medium. This reduced level of sulphate is fully adequate for normal cell growth and does not affect the final cell density achieved (Cogburn & Schiff, 1985). After growth in darkness for 96 h the cultures were moved into light (90 foot candles, 3.08 W m^{-2}, from equal numbers of cool white and red fluorescent lamps) for an additional 48 h to allow chloroplast development

Abbreviations used: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; APS, adenosine 5′-phosphosulphate.
* To whom correspondence and reprint requests should be addressed.

to take place. Highly purified intact chloroplasts were isolated from these cells as described previously (Gomez-Silva *et al.*, 1985*b*). The supernatant fraction obtained after pelleting the chloroplasts was then used for isolation of mitochondria using the method described previously (Saidha *et al.*, 1985) for isolation of fraction A mitochondria from the supernatant fraction obtained from centrifuging the crude homogenate of W_{10}BSmL cells. All cultures were incubated on rotary shakers (120 rev./min, at 26 °C). All media for cell growth and the glassware, plasticware, etc. used for isolation of organelles were autoclaved at 103.5 kPa (15 lbf/in^2) at 120 °C for 20 min and all transfers of cultures were made aseptically under green safelights (Schiff, 1972). All solutions used in experiments were sterilized either by autoclaving or by Millipore (0.45 μm pore size) filtration as appropriate.

In some experiments fraction A mitochondria from W_{10}BSmL were further fractionated into mitoplasts, outer membrane vesicles and intermembrane components by digitonin treatment (1 mg of digitonin/9 mg of protein) as described previously (Saidha *et al.*, 1985).

Sulphate activation

Adenosine 3′-phosphate 5′-phospho[^{35}S]sulphate formed from $^{35}SO_4^{2-}$ and ATP in the reaction mixture described in Table 1 was assayed as follows. After incubation of the mitochondria (300–750 μg of protein) or mitochondrial fraction (5–20 μg of protein) or chloroplasts (50–85 μg of chlorophyll representing 20–35 μg of protein) in individual experiments, the suspension was chilled on ice for 10 min and then centrifuged for 10 min in a Microfuge at 4 °C (about 10000 *g*). The supernatant fluid obtained after pelleting the mitochondria was treated with charcoal and the eluted nucleotides were assayed for radioactivity in adenosine 3′-phosphate 5′-phospho[^{35}S]sulphate after paper electrophoresis in borate buffer; the details of these methods have been presented previously (Saidha *et al.*, 1985).

ATP-sulphurylase activity was determined by measuring the enzymic liberation of P_i from ATP in the presence of molybdate and inorganic pyrophosphatase (Wilson & Bandurski, 1958). Conditions for the assay of APS kinase were as described previously (Burnell & Whatley, 1975); the APS-dependent formation of ADP is coupled to the oxidation of NADH which is measured spectrophotometrically.

Sulphate reduction

The formation of free [^{35}S]cysteine from $^{35}SO_4^{2-}$ by mitochondria or mitochondrial fractions was measured after incubation in the reaction mixture given in Table 2. The reaction mixture was centrifuged in a Microfuge as above, and a known amount of the clear supernatant fluid was passed through a Dowex 50 × 8 (100–200 mesh) column (4.0 cm × 1.1 cm) in the H^+ form as described previously (Hodson *et al.*, 1968). Distilled water (15 ml) was passed through the column followed by 3 ml of 3M-NH_4OH. The pooled NH_4OH fractions were lyophilized and the sulphur amino acids present were oxidized with performic acid as described by Hodson *et al.* (1968). The oxidized sulphur-containing amino acids (after addition of 10 μg each of non-radioactive cysteic acid and methionine sulphone) were separated by paper electrophoresis (E-C Apparatus Co.) using a formic/acetic buffer at pH 2.0 (Efron, 1960; Hodson *et al.*, 1968). The amino acid spots were visualized by spraying with ninhydrin [0.1% in butanol/acetic acid (100:3, v/v)] and heating at 100 °C for 5 min. The spots were excised, cut into small pieces and after addition of scintillation fluid were counted in a Beckman model LS-150 liquid-scintillation counter (Saidha *et al.*, 1985). The colour from the ninhydrin reaction did not produce significant quenching and did not interfere with the counting of the samples.

Incorporation of $^{35}SO_4^{2-}$ into amino acids of mitochondrial protein was measured in the Microfuge pellet. The proteins of the pellet were oxidized with performic acid and hydrolysed by the method of Moore (1963) as modified by Hodson *et al.* (1968). Acid was removed at reduced pressure over solid NaOH. The samples were then subjected to electrophoresis to separate the oxidized sulphur-containing amino acids and counted as above.

Incorporation of the radioactivity from $^{35}SO_4^{2-}$ into constituents of the mitochondrial pellet was also measured by the filter disc method of Manns & Novelli (1961) except that after spotting, the filter disc, was immersed in 10% (w/v) trichloroacetic acid containing 100 mM-Na_2SO_4 and 100 mM-cysteine, rather than leucine, to exchange with free $^{35}SO_4^{2-}$ and [^{35}S]cysteine. Since this technique includes further treatment of the filters with hot trichloroacetic acid and with hot ethanol/ether solutions, ester and lipid sulphate should be removed and only incorporation into sulphur of cysteine and methionine of protein should remain. To check this, discs prepared as described were further treated with 15 mg of Pronase (Calbiochem) in 5 ml of 50 mM-NH_4HCO_3 (pH 7.8) at 37 °C for 24 h; fresh Pronase (15 mg) was added and the incubation was continued for another 24 h. After centrifugation at 10000 *g*, an aliquot of the supernatant fluid was subjected to electrophoresis on Whatman 3MM filter paper in formic/acetic buffer (pH 2.0) as described above. Only one spot was found after autoradiography, and this was at the position expected for cysteine. Thus the filter paper assay is a reliable assay for incorporation of radioactivity into cysteine of protein; as will be noted subsequently, the system studied does not form labelled methionine from $^{35}SO_4^{2-}$.

Other assays

Succinate dehydrogenase (EC 1.3.99.1) was assayed at 30 °C by the method of Davis & Merrett (1974). Protein was measured by the method of Bradford (1976), with bovine serum albumin as the standard. Total chlorophyll (a + b) was determined according to Bruinsma (1961) as modified by Cunningham & Schiff (1986).

Assay of APS sulphotransferase

Highly purified mitochondria (fractions A and B combined), isolated as before (Saidha *et al.*, 1985), were resuspended in a solution containing 25 mM Hepes/KOH (pH 7.4), 0.25 M-sucrose and 5 mM-EDTA. The suspension was then subjected to three cycles of freezing in liquid N_2 and thawing and was then centrifuged at 170000 *g* for 2 h. The supernatant fraction was applied to a Sephadex G-150 column equilibrated with a solution containing 10 mM-Tris/HCl (pH 7.8), 1.0 mM-2-mercaptoethanol and 10% (v/v) glycerol. Elution was carried out with the same buffer. Fractions containing ATP sulphurylase activity as determined by the molybdolysis

assay (Wilson & Bandurski, 1958) were pooled. This ATP-sulphurylase preparation, lacking APS sulphotransferase activity, was used to generate adenosine 5′-phospho[^{35}S]sulphate from $^{35}SO_4^{2-}$ and ATP.

ATP sulphurylase (5 units; 1 unit = 1 μmol of product formed/min) together with 3 units of inorganic pyrophosphatase (1 unit = 2 μmol of product formed/min), 1.0 mM-$MgCl_2$, 1.0 mM-sodium ATP and 1.0 mCi of carrier-free $^{35}SO_4^{2-}$ in a total volume of 10 ml was incubated at 30 °C for 8 h. The reaction mixture was then heated at 70 °C for 3 min. The adenosine 5′-phospho[^{35}S]sulphate content was measured by paper electrophoresis of an aliquot of the heated reaction mixture together with non-radioactive APS on Whatman 3MM paper in 20 mM-sodium citrate buffer (pH 5.8) at 1800 V for 50 min followed by localization of spots under 253.7 nm u.v. light, excision and determination of radioactivity by scintillation counting. The remainder of the heated reaction mixture was stored at −20 °C for use in the APS sulphotransferase assay.

A portion (100 μl) of the heated reaction mixture containing 10^6 c.p.m. of adenosine 5′-phospho[^{35}S]sulphate together with 100 mM-Tris/HCl (pH 8.6), 30 mM-$MgCl_2$, 30 mM-dithiothreitol and 0.2–2.0 mg of mitochondrial protein to be assayed for APS sulphotransferase activity were contained in a total volume of 1.0 ml; incubation was for 30 min at 30 °C. An aliquot of the incubated reaction mixture was transfered to a Conway diffusion dish, the acid-volatile radioactivity was released and was trapped in base over a period of 8 h as described previously (Schiff & Levinthal, 1968; Tsang & Schiff, 1976). A portion (50 μl) of the base was spotted on Whatman 3 MM filter paper, 2.3 cm in diameter, dried and the radioactivity determined by scintillation counting as above.

RESULTS AND DISCUSSION

In previous experiments (Saidha *et al.*, 1985) ATP for sulphate activation was supplied to the organelles in the incubation medium; alternatively, ADP was supplied which was converted to ATP for sulphate activation via adenylate kinase, also shown to be present on the outside of the mitoplast. For reasons that were unclear at the time it was not possible to obtain sulphate activation under conditions known to favour internal formation of ATP from ADP via oxidative phosphorylation, although the optimal conditions for oxidative phosphorylation in *Euglena* mitochondria had been established in our studies of coupled phosphorylation (Gomez-Silva *et al.*, 1985*a*) and protein synthesis linked to oxidative phosphorylation (Delorme *et al.*, 1986).

It soon became obvious that the major problem was the presence of P_i in the reaction mixtures used to support coupled phosphorylation. P_i is usually added at a concentration of 10 mM (Tokunaga *et al.*, 1976; Gomez-Silva *et al.*, 1985*a*), but lower concentrations can be used as well (Delorme *et al.*, 1986). As can be seen in Fig. 1, P_i is inhibitory to sulphate activation and the formation of adenosine 3′-phosphate 5′-phosphosulphate from sulphate in intact *Euglena* mitochondria is completely inhibited by 10 mM-KH_2PO_4. Fortunately, concentrations of inorganic phosphate can be found that are still adequate for oxidative phosphorylation (Delorme *et al.*, 1986) but do not inhibit sulphate activation; 1.0 mM appears to be optimal under our conditions (Fig. 1).

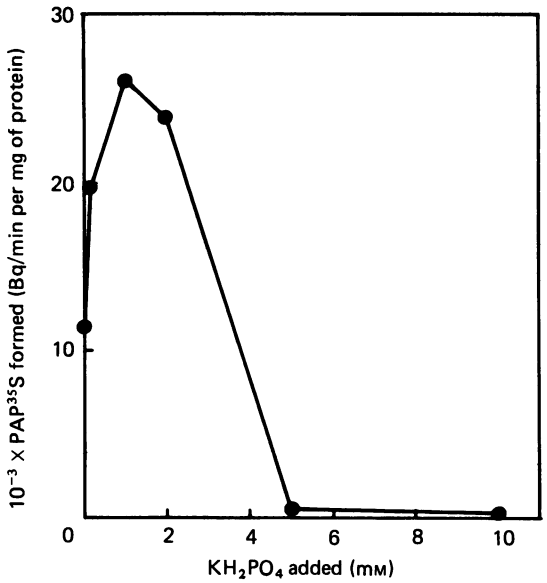

Fig. 1. Inhibition of formation of adenosine 3′-phosphate 5′-phosphosulphate (PAP^{35}S) from $^{35}SO_4^{2-}$ in *Euglena* mitochondria by P_i

The complete system (with ADP) given in Table 1 was used, except that the concentration of inorganic phosphate was varied.

Robbins & Lipmann (1958), working with ATP sulphurylase and inorganic pyrophosphatase of yeast, showed that the reactions catalysed by ATP sulphurylase and inorganic pyrophosphatase could be reversed to form sulphate and ATP if very high P_i concentrations (100 mM) were employed; 10 mM P_i did not cause measurable reversal. We have repeated these experiments with *Euglena* mitochondria and have obtained the same results (not shown). Therefore it is unlikely that this type of reversal is responsible for the inhibition of sulphate activation by P_i that we have observed (Fig. 1). We have also found (results not shown) that APS kinase is not inhibited by 10 mM-P_i. Therefore it seems likely that P_i inhibits at the forward end of the ATP sulphurylase reaction; competition of anions with sulphate has been described previously for this enzyme from *Penicillium* (Farley *et al.*, 1978). This inhibition by P_i may serve a regulatory role in mitochondrial sulphate activation.

Having established compatible conditions for oxidative phosphorylation and sulphate activation, we studied the properties of the system to determine whether the two processes were coupled. As can be seen in Table 1, exp. 1 shows that ADP and Mg^{2+} are indispensible; ADP would be necessary as a phosphate acceptor in oxidative phosphorylation and Mg^{2+} is required for the ATP sulphurylase reaction (Saidha *et al.*, 1985) among others. Deletion of phosphate or the substrate for oxidation (malate, in this case) lowers the activity by about half; the remaining activity can be attributed to endogenous reserves of substrate and phosphate in the intact organelles. Deletion of the sucrose osmoticum lowers the activity by about 60%; heating abolishes the activity completely. Exp. 2 shows that sulphate activation is inhibited 80–90% by the uncouplers dinitrophenol and CCCP at concentrations previously shown to block oxidative phosphorylation in these organelle prepara-

Table 1. Properties of the sulphate activating system coupled to oxidative phosphorylation in *Euglena* mitochondria

The complete reaction mixture for intact mitochondria under oxidative phosphorylating conditions contained, in a total volume of 2.65 ml: Tricine/KOH, pH 7.6, 17 mM; $MgCl_2$, 18 mM; KH_2PO_4, 1.0 mM; DL-malic acid, 7.0 mM; sucrose, 250 mM; sodium ADP, 1.5 mM or sodium ATP, 1.0 mM; mitochondrial protein, 0.75–1.00 mg; K_2SO_4, 1.0 mM and $^{35}SO_4^{2-}$ (carrier-free), 32 μCi. The incubation mixture was shaken for 1 h at 30 °C.

Expt. no.	Additions	10^{-3} × Adenosine 3′-phospho 5′-phospho[^{35}S]sulphate formed (Bq/h per mg of protein)	Activity (%)
1.	Complete (with ADP)	45.90	100
	−ADP	1.86	4
	$-MgCl_2$	1.79	5
	$-P_i$	22.00	48
	−malate	22.60	49
	−sucrose	18.20	40
	Complete (with ADP), enzyme heated at 100°C, 5 min	0	0
2.	Complete (with ADP)	21.40	100
	+0.1 mM-Dinitrophenol	3.29	15
	+0.2 μM-CCCP	2.30	11
3.	Complete (with ATP)	32.50	100
	+0.1 mM-Dinitrophenol	29.00	89
	+0.2 μM-CCCP	29.60	91

tions (Gomez-Silva *et al.*, 1985*a*). The uncouplers have no direct effect on the enzymes of sulphate activation since replacement of ADP by ATP (Table 1, exp. 3) eliminates the inhibitory effects of the uncouplers. Thus, under the conditions presented in Table 1, sulphate activation utilizes the ATP produced from oxidative phosphorylation.

We next turned to a study of the location and function of the sulphate reducing system under these physiological conditions. Although a number of the enzymic activities connected with sulphate reduction have been detected in *Euglena* mitochondria (Brunold & Schiff, 1976), the location of the system within the mitochondria, its activity in the organelle and its relation to oxidative phosphorylation have not been established.

Highly purified intact mitochondria form free cysteine (measured as cysteic acid) from sulphate when incubated with the complete system (see Table 2 for experimental details). As previously found for the formation of adenosine 3′-phosphate 5′-phosphosulphate (Saidha *et al.*, 1985) most (93%) of the free cysteine formed by intact mitochondria accumulates in the medium surrounding the mitochondria; only about 7% is found within the organelle. Measurement of cysteine uptake (results not shown) indicates that about 5% of cysteine added to the mitochondria is found within the mitoplasts after 15 min of incubation. As shown in Table 2, sulphate reduction is inhibited by concentrations of azide and cyanide that block electron transport in *Euglena* mitochondria (Buetow & Buchanan, 1965; Gomez-Silva *et al.*, 1985*a*); uncouplers also inhibit at concentrations known to block oxidative phosphorylation (Gomez-Silva *et al.*, 1985*a*). These results are consistent with the fact that APS is the activated sulphate compound used in the sulphate reducing pathway of *Euglena* (Brunold & Schiff, 1976); any compound which blocks electron transport or coupled phosphorylation should block ATP formation and, therefore, sulphate activation to form APS. Cycloheximide, a specific inhibitor of translation on cytoplasmic ribosomes of *Euglena*, does not cause much inhibition of cysteine formation, but the substantial inhibition by chloramphenicol is surprising. Chloramphenicol inhibits translation on mitochondrial ribosomes in *Euglena* (Avadhani & Buetow, 1972; Bovarnick *et al.*, 1974; Delorme *et al.*, 1986); either chloramphenicol has an effect on the enzymes of sulphate reduction, or blocking translation on mitochondrial ribosomes results in a signal that inhibits the reduction of sulphate to form cysteine. We have already shown (Table 1) that the uncouplers do not substantially inhibit the sulphate activating system itself; the same result was found for the other inhibitors as well since sulphate activation (measured as formation of adenosine 3′-phosphate 5′-phosphosulphate) was inhibited only 21% by chloramphenicol, 9% by cycloheximide, 7% by KCN and 23% by sodium azide at the same concentrations as in Table 2.

Oxidation of the free amino acids formed and separation by paper electrophoresis showed that cysteic acid was labelled but there was no label in methionine sulphone. Apparently *Euglena* mitochondria do not convert cysteine to methionine under these conditions.

Table 2 also shows data for incorporation of label from $^{35}SO_4^{2-}$ into protein using the filter disc method. Oxidation of the labelled protein followed by acid hydrolysis yielded only radioactive cysteic acid; no label was found in methionine sulphone, consistent with the lack of conversion of cysteine to methionine already noted. The labelling of the cysteine of protein (Table 2) showed the same pattern of inhibition as formation of free cysteine from sulphate. This would be expected if the free cysteine formed from sulphate is incorporated into protein. The inhibition by chloramphenicol would be expected, in this instance, since cysteine incorporation into protein would require the participation of the mitochondrial ribosomes.

Table 2. Influence of various compounds on the incorporation of label from $^{35}SO_4^{2-}$ into free cysteine and into proteins by highly purified mitochondria from *Euglena*

Experiments were performed using the complete system shown in Table 1 containing ADP, except that 250 μCi (5×10^8 d.p.m.) of $^{35}SO_4^{2-}$ was used and non-radioactive sulphate was omitted. Incorporation of radioactivity into free cysteine was measured as cysteic acid and into protein (incorporation into cysteine of protein) by the filter disc method (see the Experimental section). In expt. 1, 100% value for free cysteine = 4.87×10^5 Bq/min per mg of protein; 100% for protein synthesis = 4.82×10^5 Bq/min per mg of protein (average of two experiments). In expt. 2: 100% value for free cysteine = 2.52×10^5 Bq/min per mg of protein; 100% for protein synthesis = 3.36×10^5 Bq/min per mg of protein. In expt. 2 the control contained 0.4% ethanol, the same final amount added along with dinitrophenol and CCCP.

		Incorporation into:	
Expt. no.	Compound added	Free cysteine (%)	Protein (%)
1.	None	100	100
	Chloramphenicol (100 μg/ml)	19	24
	Cycloheximide (20 μg/ml)	81	62
	NaN_3 (5 mM)	14	3
	KCN (100 μM)	3	2
2.	None	100	100
	Dinitrophenol (0.1 mM)	16	6
	CCCP (0.2 μM)	20	27

Data concerning the localization of the sulphate reducing system are shown in Table 3. The mitochondria were fractionated into mitoplasts, mitochondrial outer membranes and intermembrane components as before (Saidha *et al.*, 1985) using digitonin. Nearly all of the sulphate reducing activity forming free cysteine, as well as the APS sulphotransferase activity (the first enzyme of sulphate reduction), is found to be associated with the mitoplast, as previously found for the sulphate activating system (Saidha *et al.*, 1985). The labelling of the cysteine of protein starting with $^{35}SO_4^{2-}$ is also found in the mitoplast fraction, as would be expected since sulphate activation and sulphate reduction are associated with the mitoplasts and the ribosomal protein synthesizing system is found inside the mitoplasts. As may be seen in Table 3, the recoveries of activity in the various fractions are quite good.

The ready access of the reducing system to substrates, such as APS formed from sulphate activation, and the appearance of most of the free cysteine formed in the surrounding medium, argues for a location outside the permeability barrier of the mitochondrion, the mitochondrial inner membrane.(The outer membrane is not thought to be a significant permeability barrier to small molecules.) Thus a sulphate metabolizing centre appears to be located on the outside of the mitochondrial inner membrane of *Euglena*. Perhaps further studies will reveal other enzymes of sulphur metabolism in this location. The enzymes located here are strategically placed to make use of ATP from oxidative phosphorylation exported from the mitoplast, or ADP (via adenylate kinase) or ATP from outside the organelle, e.g. from glycolysis. Having both processes in one location would facilitate the handing along of intermediates from the activating system to the reducing system.

Table 3. Localization of sulphate metabolizing activities within *Euglena* mitochondria as determined by digitonin fractionation

The complete system for sulphate reduction (labelling of free cysteine and cysteine of protein) is given in Table 2. Details of the assay for APS-sulphotransferase are described in the Experimental section. Note: ATP-sulphurylase and inorganic pyrophosphatase are present on the outside of the mitochondrial inner membrane. To ensure that all the fractions of the mitochondria have enough of these enzymes to catalyse the formation of APS, the sulpho donor for reduction, purified ATP-sulphurylase (1 unit) and inorganic pyrophosphatase (5 units) (Sigma, yeast) was added to incubations of all fractions of the mitochondria in experiments assaying incorporation of $^{35}SO_4^{2-}$ into cysteine and protein. Values in parentheses denote percentages.

	Amount recovered from 100 μl of mitochondria				
Activity or constituent	Unfractionated mitochondria 1	Mitoplast 2	Inner membrane 3	Outer membrane 4	Total recovered (2)+(3)+(4)
$10^{-4} \times$ APS sulphotransferase (Bq/min)	65.50 (100)	46.76 (71)	1.76 (3)	0.08 (0.1)	(74)
$10^{-5} \times$ Free cysteine from $^{35}SO_4^{2-}$ (Bq/min)	4.23 (100)	3.28 (77)	0.32 (7)	0.01 (0.2)	(85)
$10^{-5} \times$ Protein from $^{35}SO_4^{2-}$ (Bq/min)*	0.53 (100)	0.45 (85)	0.02 (4)	0.03 (6)	(94)
$10^{-5} \times$ Protein from $^{35}SO_4^{2-}$ (Bq/min)†	0.38 (100)	0.34 (89)	0.04 (11)	0.03 (8)	(107)
Protein content (mg)	1.00 (100)	0.72 (72)	0.20 (20)	0.02 (2)	(94)

* Incorporation into protein was measured by the filter disc method.

† Incorporation into cysteine of protein was measured as cysteic acid after oxidation and acid hydrolysis (see the Experimental section for details).

Table 4. Sulphate activation in the chloroplast and mitochondrial fractions from wild-type *Euglena* cells

Assay methods are described in the Experimental section. Abbreviation: PAP^{35}S, adenosine 3′-phosphate 5′-phospho[^{35}S]-sulphate.

Activity	Mitochondrial fraction	Chloroplast fraction	Chloroplast fraction (%)
ATP-sulphurylase (μmol/h per mg of protein)	3.92	0.23	5.5
APS kinase (μmol/h per mg of protein)	0.47	0.00	0
$10^{-3} \times$ PAP^{35}S formed from $^{35}SO_4^{2-}$ (Bq/h per mg of protein)	372.00	45.50	10.9
Succinate dehydrogenase (μmol/h per mg of protein)	384.00	40.30	9.5

Since sulphate activation and reduction can be localized within *Euglena* mitochondria, and since these processes are found in the chloroplasts of higher plants such as spinach (Schwenn & Trebst, 1976) it was of interest to determine whether these processes are also present in *Euglena* chloroplasts. As far as we have been able to determine, *Euglena* chloroplasts do not take up sulphate readily and do not convert what is taken up to other sulphur compounds (results not shown). Table 4 shows various activities related to sulphate activation in highly purified mitochondria and chloroplasts from light-grown wild-type *Euglena* cells. Since contamination of the chloroplast fraction by small amounts of adherent mitochondria is unavoidable, succinate dehydrogenase activity was also measured as an estimate of the amount of mitochondrial material present. The data of Table 4 show that although ATP sulphurylase, APS kinase and the formation of adenosine 3′-phosphate 5′-phospho-[^{35}S]sulphate from $^{35}SO_4^{2-}$ are high in freeze–thawed mitochondria, only about 5–10% of these values are obtained for the freeze–thawed chloroplasts. Since about 10% of the succinate dehydrogenase activity is found in the chloroplasts as well, the small amount of sulphate activating activity found in the chloroplast fraction can be attributed to mitochondrial contamination.

CONCLUSIONS

The results presented in this paper clearly show that highly purified *Euglena* mitochondria operating under physiological conditions can provide ATP for sulphate activation through coupled phosphorylation. These mitochondria are also able to form all of the necessary intermediates for sulphate reduction, since they form the sulphur of free cysteine from sulphate and ATP. The cysteine formed can be used for protein synthesis by the mitochondria. As we have previously shown (Delorme *et al.*, 1986), ATP for protein synthesis can be supplied by oxidative phosphorylation under these conditions.

We have previously presented evidence which suggests that the sulphate activating system is located on the outside of the mitochondrial inner membrane (Saidha *et al.*, 1985). Similar evidence in the present paper suggests that the sulphate reducing system is present in the same location. The sulphate reducing system is not soluble within the mitochondrion but appears to be firmly bound to the outside of the mitoplast.

At present we suppose the system to operate as follows. ATP from oxidative phosphorylation crosses the mitoplast membrane in exchange for ADP (Hanson & Day, 1980), or ADP or ATP are supplied from outside; in either case, ATP for sulphate activation is supplied to the ATP sulphurylase on the outside of the inner mitochondrial membrane. The APS formed is either converted to adenosine 3′-phosphate 5′-phosphosulphate or reacts with APS sulphotransferase, also on the outside of the inner membrane where reduction occurs to form free cysteine. The source of electrons for sulphate reduction in the intact mitochondrial system has not yet been identified. Since reduced nicotinamide nucleotides support activity of organic thiosulphate reductase in broken preparations of *Euglena* cells or mitochondria (Brunold & Schiff, 1976) these were tried in intact mitochondria but their addition did not increase the reduction of sulphate to cysteine (results not shown). Either the sulphate reducing step is already saturated with endogenous reduced nicotinamide nucleotide, is inaccessible to the added nicotinamide nucleotide or the intact mitochondrial sulphate reducing system receives its reducing power directly from some other carrier in the electron transport chain, perhaps through transmembrane electron transport across the mitochondrial inner membrane. Once free cysteine is formed, most of it is found outside the mitochondria.

The location and operation of the sulphate metabolizing centre on the outside of the mitochondrial inner membrane ensures the efficient formation of adenosine 3′-phosphate 5′-phosphosulphate for esterification reactions inside and outside the mitochondrion, APS for sulphate reduction on the mitochondrial inner membrane and cysteine for protein synthesis within the mitochondrion and for the formation of reduced sulphur compounds elsewhere in the cell.

Although *Euglena* mitochondria contain the enzymes of sulphate activation and reduction, *Euglena* chloroplasts appear to lack the enzymic machinery for sulphate utilization. *Euglena* chloroplasts, however, contain the usual thylakoid sulpholipid (Saidha & Schiff, 1987; Bingham & Schiff, 1979*a*,*b*). One would expect, therefore, that *Euglena* mitochondria will be required to convert $^{35}SO_4^{2-}$ to intermediates that can be utilized by the chloroplast for sulpholipid formation. Indeed, as we have recently found, incubation of the two organelles together is necessary to obtain labelling of the *Euglena* chloroplast sulpholipid using [^{35}S]sulphate (Saidha & Schiff, 1987).

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