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Oxidative Phosphorylation during Glycollate Metabolism in Mitochondria from Phototrophic *Euglena gracilis*

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Mitochondria were isolated by gradient centrifugation on linear sucrose gradients from broken cell suspensions of phototrophically grown *Euglena gracilis*. An antimycin A-sensitive but rotenone-insensitive glycollate-dependent oxygen uptake was demonstrated in isolated mitochondria. The partial reactions of glycollate–cytochrome *c* oxidoreductase and cytochrome *c* oxidase were demonstrated by using *Euglena* cytochrome *c* as exogenous electron acceptor/donor. Isolated mitochondria contain glycollate dehydrogenase and glyoxylate–glutamate aminotransferase and oxidize exogenous glycine. A P:O ratio of 1.7 was obtained for glycollate oxidation, consistent with glycollate electrons entering the *Euglena* respiratory chain at the flavoprotein level. The significance of these results is discussed in relation to photorespiration in algae.

Photorespiration, in higher plants, is a light-dependent oxygen uptake and CO_2 release occurring during photosynthesis, the CO_2 loss greatly decreasing net photosynthetic CO_2 fixation (Jackson & Volk, 1970). The oxygen uptake in photosynthesis results from glycollate biosynthesis in the chloroplast and glycollate oxidation mediated by the enzyme glycollate oxidase (glycollate–oxygen oxidoreductase, EC 1.1.3.1). Detailed investigations by Tolbert (1963) established a sequence of reactions: 2 glycollate $\rightarrow$ 2 glyoxylate $\rightarrow$ 2 glycine $\rightarrow$ 1 serine+CO_2 $\rightarrow$ glycerate $\rightarrow$ phosphoglycerate, resulting in the conversion of glycollate into phosphoglycerate and ultimately sucrose by an energetically wasteful process. Only the glycine $\rightarrow$ serine conversion has been shown to yield ATP, and although the probable yield for this conversion is 2mol of ATP for each mol of serine formed (Bird *et al.*, 1972*a*), 1mol of ATP is required for the conversion of glycerate into phosphoglycerate.

In higher plants, the energy released in the oxidation of glycollate to glyoxylate is dissipated by the combined action of a flavoprotein-linked glycollate oxidase and catalase (H_2O_2–H_2O_2 oxidoreductase, EC 1.11.1.6) (Tolbert *et al.*, 1968). In *Euglena*, however, glycollate is oxidized by glycollate dehydrogenases, and catalase is absent (Lord & Merrett, 1971*a*; Graves *et al.*, 1971; Collins & Merrett, 1975). In particular, we have demonstrated the presence of a glycollate dehydrogenase in a mitochondrial fraction from *Euglena* which links to oxygen via the respiratory chain (Collins & Merrett, 1975). The possibility arises that *Euglena* may derive energy by phosphorylation linked to glycollate oxidation over and above that derived from the conversion of glycine into serine (Bird *et al.*, 1972*a*,*b*).

Materials and Methods

Growth of alga

Euglena gracilis Klebs strain z was grown phototrophically at 25°C and 6000lx light-intensity in the medium of Cramer & Myers (1952). Cultures, after 48h of growth on 5% (v/v) CO_2 in air followed by 24h growth in air, were harvested in the early exponential phase of growth.

Isolation of mitochondria

Mitochondria were prepared in bulk by differential centrifugation of phototrophically grown *Euglena* as described previously (Collins & Merrett, 1975). Purified mitochondria were prepared by density-gradient centrifugation of *Euglena* extracts (Collins & Merrett, 1975). Pumpkin (*Cucurbita pepo*) mitochondria were prepared from cotyledons of 8-day-old dark-grown seedlings as described previously (Brown *et al.*, 1974).

Enzyme assays

NADH–cytochrome *c* oxidoreductase (EC 1.6.99.3) and glycollate–cytochrome *c* oxidoreductase (EC 1.1.2.–) were assayed in a Gilford series 2000 recording spectrophotometer at 30°C. In each case, the assay mixture contained the sorbitol–EDTA–Hepes [2-(*N*-2-hydroxyethylpiperazin-*N*′-yl)sulphonic acid] buffer of Sharpless & Butow (1970*a*) and 1mg of oxidized cytochrome *c*. With NADH as substrate, cytochrome oxidase (EC 1.9.3.1) activity was blocked by the addition of 1mM-KCN. With glycollate as substrate, cytochrome re-oxidation was prevented by performing the assay anaerobically after gassing with

O_2-free N_2 in Thunberg cuvettes. NADH oxidation was followed at 340nm and cytochrome reduction at 550nm (horse heart cytochrome *c*) or 558nm (*Euglena* cytochrome *c*-558). The extinction coefficient used for both cytochromes was that of horse heart cytochrome *c*, i.e. 18500 litre·mol^{-1}·cm^{-1}.

Cytochrome oxidase was assayed spectrophotometrically at 550nm (horse heart and parsnip) or 558nm (*Euglena* cytochrome) at 30°C. The assay mixture contained the sorbitol–Hepes–EDTA–bovine serum albumin buffer of Sharpless & Butow (1970*a*) and reduced cytochrome. At least three different concentrations of cytochrome were used, and the rates extrapolated to infinite cytochrome concentration (Fowler *et al.*, 1962).

Determination of oxygen uptake and phosphorylation

Oxygen uptake by mitochondrial suspensions was measured with a Clark-type oxygen electrode at 30°C in the sorbitol–Hepes–EDTA–bovine serum albumin buffer of Sharpless & Butow (1970*a*). Oxidative phosphorylation was measured in the same apparatus in an assay mixture of volume 1ml containing 5mM-P_i, 5mM-$MgCl_2$, 2mM-ATP, 33mM-glucose, 1mg of bovine serum albumin and 3 units of hexokinase (EC 2.7.1.1). Phosphorylation was measured as a decrease in P_i on addition of substrate compared with a substrate blank. P_i was measured by the method of Atkinson *et al.* (1973) after precipitation of protein by the addition of 0.1vol. of 60% (w/v) $HClO_4$. Under similar conditions P:O ratios of approx. 3.0 were obtained as a routine with NADH and purified pumpkin mitochondria. In some experiments, phosphorylation was confirmed by measuring glucose 6-phosphate in an assay containing 2mM-ADP instead of ATP (Slater, 1967). Glucose 6-phosphate was measured enzymically with $NADP^+$ and glucose 6-phosphate dehydrogenase (EC 1.1.1.49).

Protein determination

Protein was measured by the method of Lowry *et al.* (1951), by using a calibration curve prepared for crystalline bovine serum albumin.

Materials

Euglena cytochrome *c*-558 was obtained from cells of *Euglena* grown heterotrophically on the medium of Hutner *et al.* (1956) and purified by the method of Pettigrew *et al.* (1975). The cytochrome was purified within 2 days of initial cell harvesting and used for enzyme assays immediately. The product had a purity index of E_{558} (reduced)/E_{280} (oxidized) = 0.88. Parsnip (*Pastinaca sativa*) cytochrome *c* was extracted and purified as described by Brown & Boulter (1974). The cytochromes were prepared in the oxidized or reduced forms by treatment with ferricyanide or ascorbate respectively with subsequent desalting.

Horse heart cytochrome *c* (type III), ADP, ATP, nicotinamide nucleotides, glucose 6-phosphate dehydrogenase (type XV) and hexokinase (type C-130, glucose 6-phosphate dehydrogenase-free) were purchased from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K.

Results and Discussion

Partial reactions of the respiratory chain during glycollate oxidation

The redox potential of glycollate/glyoxylate (E_0 = 0.087V; Zelitch, 1955) would lead one to expect that glycollate would donate electrons to the respiratory chain at the flavoprotein–cytochrome *b* level. The oxidation of glycollate by *Euglena* mitochondria should therefore be insensitive to rotenone, but sensitive to antimycin A and cyanide. This was shown to be so by experiments with crude mitochondria (Collins & Merrett, 1975) and also with purified mitochondria. Thus, 0.5μM-rotenone inhibited oxygen uptake by 18%, 3μM-antimycin A inhibited oxygen uptake by 95% and 1mM-cyanide completely inhibited oxygen uptake in experiments using purified *Euglena* mitochondria and 40mM-glycollate. The transfer of glycollate electrons directly to 2,6-dichlorophenol-indophenol catalysed by *Euglena* mitochondrial glycollate dehydrogenase was inhibited by cyanide (Collins & Merrett, 1975), so the use of the cyanide as an inhibitor of cytochrome oxidase during glycollate oxidation is ambiguous.

Further evidence for the participation of the electron-transport chain in glycollate oxidation was obtained from an investigation of the partial reactions involving cytochrome *c*, i.e. glycollate–cytochrome *c* oxidoreductase and cytochrome oxidase. Preliminary work on the NADH–cytochrome *c* oxidoreductase indicated that very low rates were obtained when horse heart cytochrome *c* was used as exogenous electron acceptor (Table 1). However, the observed rate could be increased manyfold by the use of *Euglena* cytochrome *c*, indicating a specific requirement for *Euglena* cytochrome. The *Euglena* cytochrome *c* was also used as exogenous electron acceptor for glycollate–cytochrome *c* oxidoreductase (Table 1), the observed activity being less than for NADH–cytochrome *c* oxidoreductase. The reoxidation of reduced cytochrome *c* by cytochrome oxidase was prevented by carrying out the reaction under anaerobic conditions, since cyanide, the classic inhibitor of cytochrome oxidase, would also inhibit glycollate dehydrogenase. Previous investigations have failed to detect cytochrome oxidase in *Euglena* by using horse heart cytochrome *c* (Krawiec & Eisenstadt, 1970; Lord & Merrett, 1971*a*), but when

Table 1. *Partial reactions of NADH and glycollate oxidation*

Partial reactions involving cytochrome *c* were assayed spectrophotometrically in sorbitol–Hepes–EDTA–bovine serum albumin buffer at 30°C.

Electron donor	Electron acceptor	Mitochondrial type and purity	Specific activity (nmol/min per mg of mitochondrial protein)
NADH (10mM)	Cytochrome *c* (horse heart)	*Euglena* (crude)	18
NADH (10mM)	Cytochrome *c* (*Euglena*)	*Euglena* (crude)	250
Glycollate (40mM)	Cytochrome *c* (*Euglena*)	*Euglena* (purified)	6
None	Cytochrome *c* (*Euglena*)	*Euglena* (purified)	None detectable
Cytochrome *c* (*Euglena*)	O_2	*Euglena* (purified)	350
Cytochrome *c* (parsnip or horse heart)	O_2	*Euglena* (purified)	None detectable
Cytochrome *c* (horse heart)	O_2	Pumpkin (purified)	160
Cytochrome *c* (parsnip)	O_2	Pumpkin (purified)	410
Cytochrome *c* (*Euglena*)	O_2	Pumpkin (purified)	110

Table 2. *Oxidative phosphorylation with purified Euglena mitochondria*

Uptake of oxygen by mitochondrial suspensions (100–250μg of mitochondrial protein per assay) was measured by using an oxygen electrode at 30°C. Phosphorylation was measured (1) as an uptake of P_i, or (2) as formation of glucose 6-phosphate in the presence of glucose and hexokinase.

Substrate	Oxygen uptake (μg-atoms/assay)	Phosphorylation (μg-atoms of P/assay)		P:O ratio	
		(1)	(2)	(1)	(2)
None	None detectable	None detectable	0.02	—	—
NADH (10mM)	1.0	1.8	—	1.8	—
Glycollate (40mM)	0.1	0.19	0.15	1.9±0.5 (5)	1.5±0.3 (4)
Glycine (10mM)	0.1	0.09	—	0.9	—

Euglena cytochrome *c* was used as substrate good rates of oxidation were observed (Table 1), comparable with those obtained for purified pumpkin mitochondria with cytochrome *c* from a variety of sources (Table 1).

The unusual nature of the *Euglena* respiratory chain, in having a relatively specific requirement for the *Euglena* cytochrome *c* for the demonstration of partial reactions, particularly cytochrome oxidase, was in contrast with most other mitochondrial respiratory chains. Mammalian mitochondria apparently function equally well with cytochromes from representative vertebrates, insects and fungi (Byers *et al.*, 1971) and also with the *Euglena* cytochrome (Davis *et al.*, 1972). Similarly, pumpkin mitochondria will oxidize representative mammalian, plant and *Euglena* cytochromes at comparable rates (Table 1). These observations are probably related to the unusual nature of the *Euglena* cytochrome (Pettigrew, 1973; Lin *et al.*, 1973).

Oxidative phosphorylation in mitochondria from phototrophic Euglena

There were no indications of respiratory control in our purified mitochondria, with either glycollate or NADH as substrate. This lack of respiratory control could be the result of either mitochondrial damage, or the operation in mitochondria from phototrophic cells of substrate oxidation by non-phosphorylating pathways as occurs in mitochondria from bleached, heterotrophically grown *Euglena* cells (Buetow & Buchanan, 1965).

Phosphorylation was determined by measuring the decrease in P_i, compared with a control without substrate, in an assay system containing glucose and hexokinase to regenerate ADP (Table 2). Substrate oxidation was followed after an initial equilibration with reaction medium, omitting substrate, to avoid artifacts caused by some initial uptake of P_i into the mitochondria. Glycollate oxidation was accompanied by phosphorylation, P:O ratios of 1.9±0.5 (S.E.M., five determinations) being obtained (Table 2). NADH oxidation, measured in the presence of fluoride to facilitate entry of NADH into mitochondria (Buetow & Buchanan, 1965), gave lower P:O ratios than the expected value of 3.0 (Table 2). Even so the P:O ratio of 1.8 obtained was greater than that obtained with mitochondria isolated from heterotrophically grown cells, where P:O ratios for NADH oxidation were only about 0.5 (Buetow & Buchanan, 1965).

However, the situation in mitochondria from bleached cells is complicated by the presence of a non-phosphorylating cyanide-insensitive pathway and a phosphorylating antimycin-insensitive pathway for the transfer of electrons to oxygen (Sharpless & Butow, 1970*a*,*b*).

Since the amounts of phosphate uptake in the phosphorylation experiments were small, approx. 4% of the total P_i for the glycollate oxidation, it was considered desirable to confirm the uptake of P_i into ATP by a more sensitive method. The results obtained by measuring phosphorylation as formation of glucose 6-phosphate in the presence of glucose and hexokinase (Slater, 1967) were comparable with the phosphate-uptake results, and the two methods gave the same P:O ratio within the standard errors shown in Table 2. Experiments in which phosphorylation was measured by both methods gave results which were consistent within ±20%.

Unlike higher-plant mitochondria, those from phototrophic *Euglena* cells contain a glycollate dehydrogenase and a glyoxylate–glutamate aminotransferase (EC 2.6.1.4) (Collins & Merrett, 1975) and these two enzymes can effect the overall conversion of glycollate into glycine. The conversion of glycine into serine occurs in the mitochondria of higher plants, P:O ratios of 1 being obtained with glycine as substrate (Bird *et al.*, 1972*b*). If glycollate electrons enter the respiratory chain at the flavoprotein level, as suggested by inhibitor experiments, a theoretical P:O ratio of 2.0 would be expected. Glycine oxidation in *Euglena* mitochondria gave a P:O ratio of 0.9 (Table 2), so that any phosphorylation resulting from the subsequent oxidation of glycine will tend to decrease the observed P:O ratio for glycollate oxidation.

The production of ATP resulting from glycollate metabolism in the mitochondria may be advantageous to *Euglena* under some growth conditions, but as little is known about mitochondrial function in green algae we are unable to conclude whether this is a unique feature of *Euglena* metabolism or is of general occurrence in green algae. The yield of ATP from glycollate metabolism is not great enough to support heterotrophic growth, as *Euglena* will not grow on glycollate in the dark. However, even in the presence of available CO_2, glycollate markedly enhances the photoheterotrophic growth rate of some algae (Lord & Merrett, 1971*b*) and this could be the result of glycollate-dependent ATP formation in the mitochondria. The presence of peroxisome-type particles in phototrophic *Euglena* cells (Collins & Merrett, 1975) provides an alternative pathway for the metabolism of glycollate. These organelles possess a similar enzyme complement to leaf peroxisomes and could have a gluconeogenic function, as postulated for leaf peroxisomes (Tolbert & Yamazaki, 1969). Although photorespiration in algae has not been characterized (Merrett & Lord, 1973), oxygen uptake in the light could result from the oxidation of ribulose 1,5-diphosphate by molecular oxygen, catalysed by the oxygenase activity of ribulose diphosphate carboxylase (EC 4.1.1.39), to yield one molecule each of phosphoglycollate and phosphoglycerate (Bowes *et al.*, 1971). The subsequent metabolism of glycollate, arising from phosphoglycollate, in the mitochondria could make an additional contribution to a light-dependent oxygen uptake, but as yet the extent of these reactions in the intact cell has not been determined.

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