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QUARTERLY

Coenzyme Q releases the inhibitory effect of free fatty acids on mitochondrial glycerophosphate dehydrogenase✪

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Data presented in this paper show that the size of the endogenous coenzyme Q (CoQ) pool is not a limiting factor in the activation of mitochondrial glycerophosphate-dependent respiration by exogenous CoQ_3, since successive additions of succinate and NADH to brown adipose tissue mitochondria further increase the rate of oxygen uptake. Because the inhibition of glycerophosphate-dependent respiration by oleate was eliminated by added CoQ_3, our data indicate that the activating effect of CoQ_3 is related to the release of the inhibitory effect of endogenous free fatty acids (FFA). Both the inhibitory effect of FFA and the activating effect of CoQ_3 could be demonstrated only for glycerophosphate-dependent respiration, while succinate- or NADH-dependent respiration was not affected. The presented data suggest differences between mitochondrial glycerophosphate dehydrogenase and succinate or NADH dehydrogenases in the transfer of reducing equivalents to the CoQ pool.

Mitochondrial glycerophosphate dehydrogenase (mGPDH), together with cytosolic glycerophosphate dehydrogenase (cGPDH), form the glycerophosphate shuttle (Estabrook & Sacktor, 1958; Bucher & Klingenberg, 1958). This shuttle is involved, alongside with the malate-aspartate shuttle (Scholz *et al.,* 2000), in the reoxidation of cytosolic NADH, bypass-

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Abbreviations: BSA, bovine serum albumin; cGPDH, cytosolic glycerophosphate dehydrogenase; CoQ, coenzyme Q; CoQ_3, short-chain homolog of CoQ; mGPDH, mitochondrial glycerophospahte dehydrogenase.

ing complex I. In contrast with the malate-aspartate shuttle, the glycerophosphate shuttle is highly active only in insect flight muscle cells (Estabrook & Sactor, 1958; Bolter & Chefurka, 1990) and in brown adipose tissue of newborn mammals and in hibernating mammals (Houštěk *et al.*, 1975). However, some publications showed an important role of this shuttle also in other mammalian organs, such as placenta (Olivera & Meigs, 1975; Swierczynski *et al.*, 1976), testes (MacDonald & Brown, 1996) or pancreatic β-cells (Ishihara *et al.*, 1996) and in the regulation of various physiological and pathological processes, such as thermogenesis (Lardy *et al.*, 1995), diabetes (Senner *et al.*, 1993) or obesity (Lardy *et al.*, 1989). Brown *et al.* (2002) demonstrated a lethal effect of elimination of the two genes for mGPDH and cGPDH in mice.

In spite of this increasing interest, there are still many problems not fully clarified, related to the complex system of factors regulating mGPDH expression in various organs and its participation in the regulation of the cell energy provision system.

In a previous study we found that activity of mGPDH is highly stimulated by CoQ_3, a short-chain homolog of coenzyme Q (Rauchová *et al.*, 1992). The aim of the present study was to further clarify the mechanism of the CoQ_3 activating effect on mGPDH. Because in brown adipose tissue mitochondria, mGPDH is highly stimulated by removal of endogenous free fatty acids (FFA) (Houštěk & Drahota, 1975; Rauchová & Drahota, 1984), we tested whether activation of mGPDH is related to the limited pool size of endogenous CoQ as the acceptor of reducing equivalents from the highly active mGPDH, or whether this activation indicates that CoQ_3 can compete with the endogenous FFA and release their inhibitory effect.

MATERIALS AND METHODS

Brown adipose tissue of adult, male Syrian hamsters adapted at 4°C for 3 weeks was used. Mitochondria were isolated in 0.25 M sucrose, 10 mM Tris/HCl, 1 mM EDTA, pH 7.4 as described by Hittelman *et al.* (1969) and stored at –70°C. Enzyme activities and respiration were measured using fresh or frozen-thawed mitochondria.

Glycerophosphate and succinate cytochrome *c* reductases activities were determined by measuring the rate of cytochrome *c* reduction at 550 nm in a medium containing 50 mM KCl, 20 mM Tris/HCl, 1 mM EDTA, 2 mM KCN, 100 μM cytochrome *c* and 50–75 μg mitochondrial protein/ml, pH 7.4. The reaction was started by addition of 25 mM glycerophosphate. The enzyme activity was expressed as nmol cytochrome *c* reduced per min per mg protein using an extinction coefficient of 19.1. The activity of glycerophosphate and succinate dehydrogenase was determined using dichlorophenol indophenol (DCIP) as an artificial electron acceptor as described previously (Rauchová *et al.*, 1993).

Oxygen consumption was measured with a High Resolution Oxygraph (OROBOROS, Austria) in a medium containing 100 mM KCl, 20 mM Tris/HCl, 4 mM K-phosphate, 3 mM $MgCl_2$, 1 mM EDTA at pH 7.2. The oxygraphic curves presented are the first derivative of oxygen tension changes. For calculation and presentation of oxygraphic data OROBOROS software was used (Gnaiger *et al.*, 1995). Oxygen consumption is expressed as pmol or nmol O_2 per second per mg protein. Proteins were determined according to Lowry *et al.* (1951) using bovine serum albumin as a standard.

RESULTS

In this study we extend our previous findings (Rauchová *et al.*, 1992) that the activating effect of CoQ_3 is specific for glycerophosphate cytochrome *c* reductase and cannot be detected when succinate cytochrome *c* reductase activity is measured. Data in Table 1 demonstrate the antimycin-sensitive and insensitive portion of the glycerophosphate and succinate

Table 1. Activation of glycerophosphate and succinate cytochrome *c* reductase activity of brown adipose tissue mitochondria by CoQ_3 and menadione

	Enzyme activity (nmol/min per mg protein)		
	Control	+ CoQ_3 (20 μM)	+ Menadione (800 μM)
Glycerophosphate cytochrome *c* reductase			
Total activity	422.9 ± 31 (100%)	781.2 ± 23 (100%)	718.9 ± 5 (100%)
AA-sensitive	392.7± 31 (93%)	700.3 ± 15 (90%)	123.0 ± 64 (10%)
AA-insensitive	30.2 ± 4 (7%)	80.8± 30 (10%)	643.4 ± 68 (90%)
Succinate cytochrome *c* reductase			
Total activity	265.4 ± 33 (100%)	250.0 ± 15 (100%)	196.3 ± 39 (100%)
AA-sensitive	251.1 ± 34 (93%)	185.2 ± 11 (74%)	85.9 ± 22 (44%)
AA-insensitive	17.7 ± 2 (7%)	65.9 ± 4 (44%)	110.4 ± 18 (56%)

Experimental conditions were the same as in Fig 1. Glycerophosphate was 25 mM, succinate 25 mM, antimycin A 1 μM. Data presented are means of four experiments ± S.E.M.

cytochrome *c* reductase activities and compare the activating effect of CoQ_3 with that of menadione. We found that both reductases were inhibited by 93% by antimycin A. The inhibitory effect of antimycin A on glycerophosphate cytochrome *c* reductase was nearly completely restored by menadione, but succinate cytochrome *c* reductase activity was restored by the same menadione concentration only by 50%. Because, in contrast to menadione, CoQ_3 added in the presence of antimycin A cannot shuttle electrons from glycerophosphate dehydrogenase to cytochrome *c,* its activating effect must be connected with a modification of the mGPDH function.

In further experiments we compared the rates of cytochrome *c* reductase activity in the presence of glycerophosphate and/or succinate. As demonstrated in Fig. 1, the rate of cytochrome *c* reduction is significantly higher when both substrates are present in the medium. Similar data were also obtained by polarographic measurements. The rate of oxygen uptake in the presence of glycerophosphate was further increased by subsequent additions of succinate and NADH (Fig. 2, Table 2). All these data clearly indi-

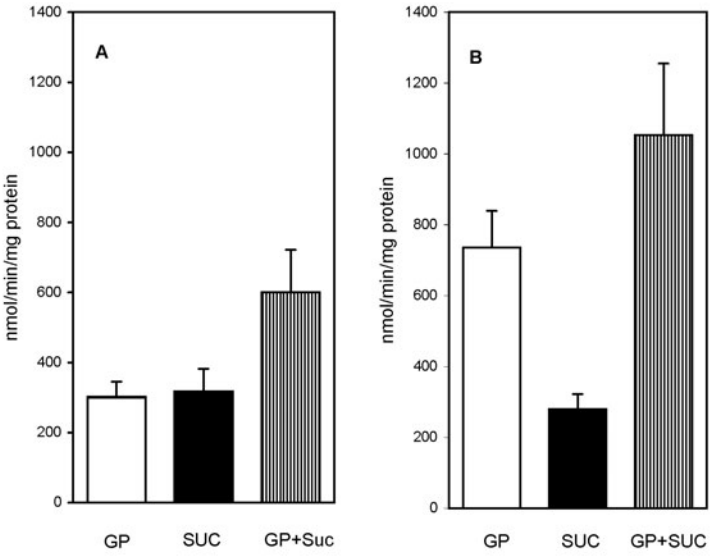

Figure 1. Glycerophosphate and succinate cytochrome *c* reductase activities (nmol/min per mg protein) of brown adipose tissue mitochondria.

Where indicated, glycerophosphate (GP) was 25 mM and succinate (SUC) 25 mM or both substrates were present. Activities were determined in the absence (A) and in the presence (B) of 0.2% fatty acid free bovine serum albumin. Frozen-thawed mitochondria were used. Data presented are means of four experiments ±S.E.M.

cate that the endogenous CoQ pool cannot be the limiting factor for the rate of mGPDH activity and that the activating effect of CoQ_3 must be due to modification of mGPDH activity.

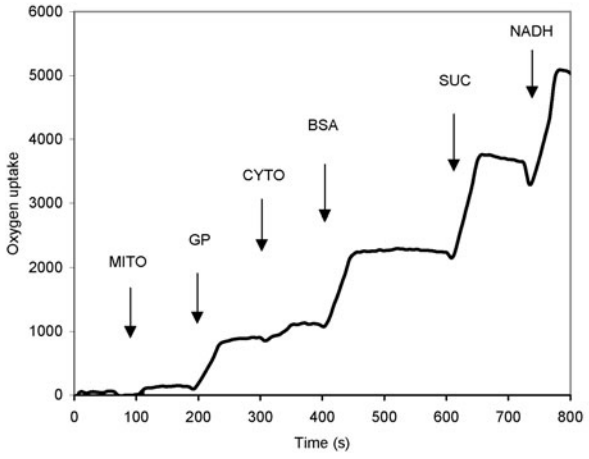

Figure 2. Oxygen consumption by brown adipose tissue mitochondria in the presence of various respiratory substrates.

To the incubation medium containing 100 mM KCl, 10 mM Tris/HCl, 4 mM K-phosphate, 3 mM $MgCl_2$, 1 mM EDTA (pH 7.2), frozen-thawed brown adipose tissue mitochondria (MITO), 0.1 mg protein/ml of medium, 10 mM glycerophosphate (GP), 25 μM cytochrome *c* (CYTO), 0.4% bovine serum albumin (BSA), 10 mM succinate (SUC) and 0.2 mM NADH were added as indicated. The oxygraphic curve is the first derivative of oxygen concentration changes. Oxygen uptake is expressed as pmol oxygen per second per mg protein. The same results were obtained using three preparations of mitochondria.

In our previous papers we found that the mGPDH activity is inhibited by endogenous FFA and that the inhibitory effect of endogenous fatty acids can be released by fatty acid oxidation (Bulychev *et al.*, 1972) or by their extraction by added bovine serum albumin (BSA) (Houštěk & Drahota, 1975; Rauchová & Drahota, 1984). Data presented in Fig. 3 demonstrate that BSA and oleate induced pronounced changes of glycerophosphate cytochrome *c* oxidoreductase activity. Both BSA and oleate had a less pronounced effect on the activity of glycerophosphate dichlorophenol indophenol oxidoreductase. These data are thus in agreement with our previous proposal that free fatty acids inhibit the transfer of reducing equivalents from glycerophosphate dehydrogenase to the CoQ pool (Rauchová & Drahota, 1984).

In further experiments we tested to what extent CoQ_3 can modify the inhibition of mGPDH by added oleate and we found that CoQ_3 can fully restore glycerophosphate-dependent respiration inhibited by oleate (Fig. 4, Table 3). However, the activating effect of CoQ_3 was less efficient than that of bovine serum albumin. Added CoQ_3 compensated only the inhibition caused by added oleate and even higher concentrations of added CoQ_3 were not able to increase the oxygen uptake to values obtained after addition of BSA. Also the activating effect of BSA on

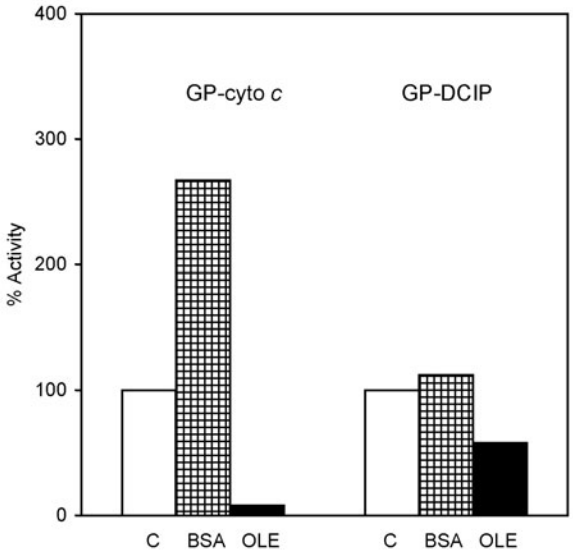

Figure 3. The effect of bovine serum albumin and Na-oleate on glycerophosphate cytochrome *c* oxidoreductase (GP-cyto *c*) and glycerophosphate dichlorophenol indophenol oxidoreductase (GP-DCIP).

Bovine serum albumin (BSA) was 0.2% and Na-oleate (OLE) was 15 μM. C indicates control samples. Frozen-thawed mitochondria were used. The same results were obtained using three preparations of mitochondria.

Table 2. Respiration of brown adipose tissue mitochondria in the presence of various substrates

Additions	nmol oxygen per second per mg protein	
Glycerophosphate (10 mM)	0.97	39%
GP + cyt *c* (25 μM)	1.22	49%
GP + cyt *c* + BSA (0.1 %)	2.48	100%
+ Succinate (10 mM)	4.08	164%
+ NADH (0.2 mM)	5.51	228%

Experimental conditions are the same as described in Fig. 2. Similar results were obtained in three experiments with mitochondria isolated from four hamsters.

Table 3. Release of the oleate-induced inhibition of mGPDH by CoQ_3.

Additions	Oxygen uptake (nmol per second per mg protein)	
	Without cytochrome *c*	With cytochrome *c* (25 μM)
10 mM glycerophosphate	1.37 (100 %)	1.36 (100 %)
+Na-oleate (15 μM)	0.51 (37 %)	0.40 (29 %)
+CoQ_3 (50 μM)	1.05 (77 %)	1.30 (96 %)
+BSA (0.1 %)	2.15 (169 %)	3.76 (276 %)

Experimental conditions were the same as described in Fig 3. Similar results were obtained in three experiments with mitochondria isolated from four hamsters.

glycerophosphate-dependent respiration was higher than that of CoQ_3 (Fig. 5) and CoQ_3 could not further activate glycerophosphate-dependent oxygen consumption in the presence of BSA (Table 4).

DISCUSSION

Activity of mGPDH is regulated by many factors, such as calcium ions (MacDonald & Brown, 1996), acyl CoA esters (Bukowiecki & Lindberg, 1974), free fatty acids (Drahota & Houštěk, 1976; Rauchová & Drahota, 1984; Rauchová *et al.* 1993) or intermediates of glycolysis (Swierczynski *et al.*, 1977). Its biogenesis is under the control of thyroid and steroid hormones (Weitzel *et al.*, 2001).

Regulation by FFA is of particular importance because the inhibitory effect of FFA is completely reversible. When fatty acids bound to isolated mitochondria are oxidized (Bulychev *et al.*, 1972) or removed by BSA treatment (Drahota & Houštěk, 1976; Rauchová & Drahota, 1984) the inhibitory effect disappears. The mechanism of this inhibitory effect has not yet been fully clarified. It seems that FFA do not interact directly with the catalytic site of the enzyme as do acyl-CoA esters (Bukowiecki & Lindberg, 1974), but modify the transfer of reducing equivalents to coenzyme Q or to artificial acceptors.

The inhibitory effect of FFA is specific for glycerophosphate oxidase or cytochrome *c* reductase activity. Succinate oxidase or cytochrome *c* reductase activity is not inhibited by FFA nor activated by BSA (Houštěk & Drahota, 1976). This supports our previous finding that the transfer of reducing equivalents from mGPDH to the coenzyme Q pool has a different mechanism than that from succinate and NADH dehydrogenases, most

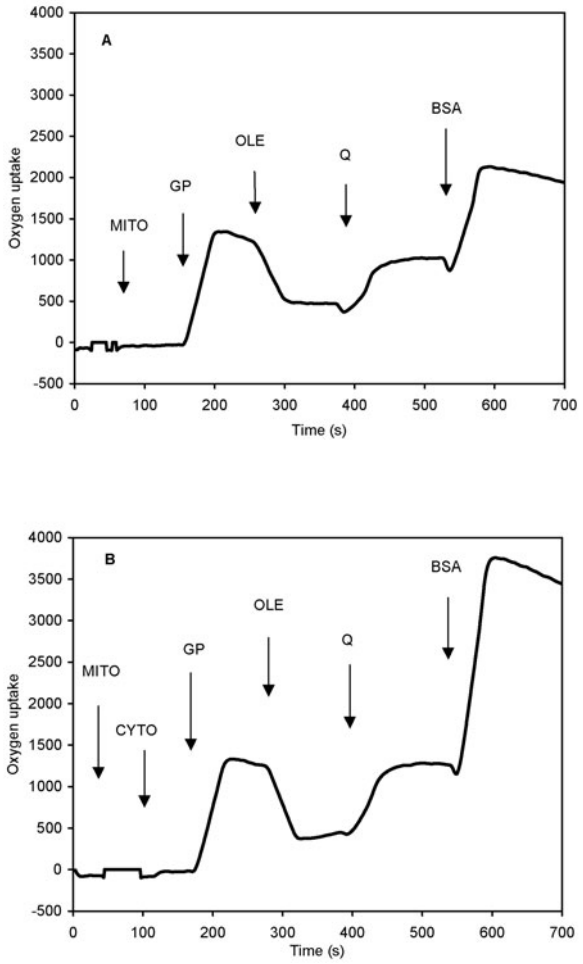

Figure 4. Inhibition by oleate of glycerophosphate-dependent oxygen consumption and the release of the inhibition by CoQ_3 in the absence (A) and in the presence (B) of cytochrome *c*.

Where indicated, freshly isolated mitochondria (MITO) 0.1 mg protein/ml, cytochrome *c* (CYTO) 25 μM, glycerophosphate (GP) 10 mM, Na-oleate (OLE) 15 μM, coenzyme Q_3 (Q) 20 μM and bovine serum albumin 0.2% (BSA) were added. The oxygraphic curves are the first derivatives of oxygen tension changes. Oxygen uptake is expressed as pmol oxygen per second per mg protein. The same results were obtained using three preparations of mitochondria.

probably due to the absence of a CoQ-binding protein in the mGHPH enzyme complex (Cottingham & Ragan 1980a; 1980b; Rauchová *et al.,*1992; 1997).

Modulation of mGPDH activity by FFA may, however, occur also through their effect on membrane fluidity. As we found in previous studies, mGPDH activity correlates with membrane fluidity changes induced by FFA, both in the intact mitochondrial membrane (Amler *et al.,* 1986) and in liposomes with incorporated mGPDH (Amler *et al.*, 1990). In insect thoracic muscle mitochondria Wojtczak & Nalecz (1979) found that the activity of mGPDH was dependent on the surface charge of the mitochondrial membrane and in liposomes it was dependent on their phospholipid composition (Nalecz *et al.,* 1980).

As demonstrated in Fig. 4, CoQ_3 can release the inhibition by added FFA. However, in these experimental conditions, CoQ_3 increased mGPDH activity only to the level obtained before the addition of oleate. This could be related to the fact that, although the activating effect of both CoQ_3 and BSA is related to fatty acid inhibition of mGPDH, evidently the mechanism of action of both substances is different. BSA is a more powerful activating agent because it can extract fatty acids from their binding sites whereas CoQ_3 activation could be explained by competition with fatty acids for the fatty acid binding sites.

Data presented in this communication describe another mechanism which participates in the regulation of mitochondrial glycerophosphate dehydrogenase, *viz*. competition of CoQ and FFA, and support the idea that CoQ, besides its role in the transport of reducing equivalents and antioxidative protection (Lenaz, 2001), has an important role also in the regulation of cell metabolic processes as, e.g., in the regulation of uncoupling proteins function (Echtay *et al.*, 2000; 2001).

The existence of a competition between FFA and CoQ_3 at the acceptor site of mGPDH also suggests that the inhibitory effect of FFA is exerted by occupying the CoQ-reducing site in the enzyme, thus preventing transfer of reducing equivalents to the CoQ pool.

Recent models of organization of the mitochondrial respiratory chain suggest the existence of specific supramolecular aggregates formed by complexes III and IV or complexes

Table 4. CoQ_3 does not activate glycerophosphate oxidation in the presence of BSA

Additions	Oxygen uptake (nmol per second per mg protein)	
10 mM glycerophosphate + 25 μM cyt *c*	1.45	(100%)
10 mM glycerophosphate + 25 μM cyt *c* + 0.1 % BSA	3.90	(269%)
10 mM glycerophosphate + 25 μM cyt *c* + 0.1 % BSA + 50 μM CoQ_3	3.32	(229%)

Experimental conditions are the same as described in Fig. 3. Similar results were obtained in three experiments with mitochondria isolated from four hamsters.

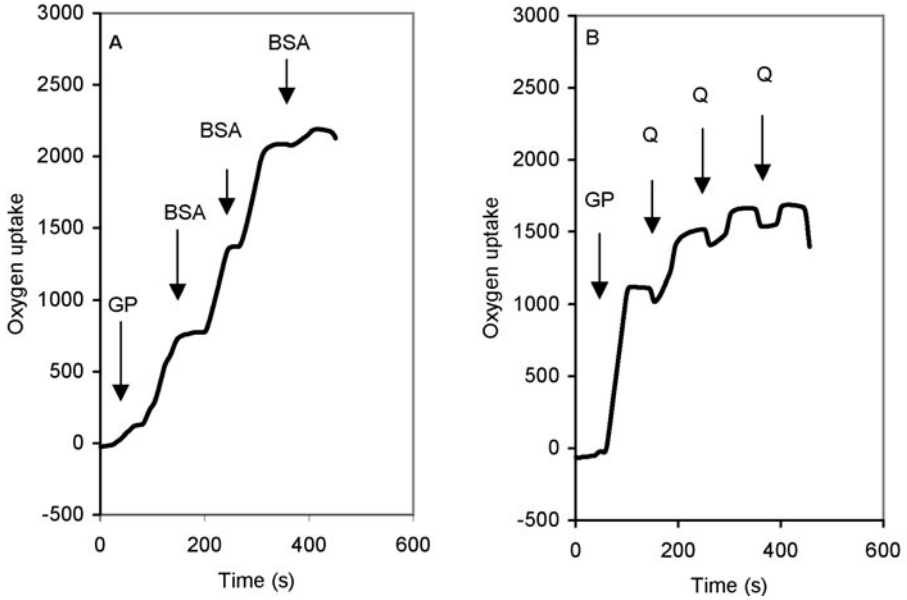

Figure 5. Activation of glycerophosphate-dependent respiration by BSA and CoQ.

Experimental conditions were the same as in Fig. 4. Where indicated glycerophosphate (GP) 10 mM, bovine serum albumin 0.2% (BSA) or coenzyme Q_3 (Q) 10 μM were added.

I, III and IV (Schagger & Pfeiffer, 2001). Succinate dehydrogenase is not involved. On the other hand, the state of mGPDH is not known although the lack of CoQ binding proteins (Cottingham & Ragan, 1980a; 1980b) is in favour of electron transfer from the enzyme to the CoQ pool. Moreover, a previous study (Rauchová *et al.,* 1997) has demonstrated a CoQ pool function for mGPDH. Thus, the transfer of reducing equivalents from succinate dehydrogenase and evidently also from glycerophosphate dehydrogenase must occur through the CoQ pool without direct interactions between individual complexes. The differences that exist between succinate and glycerophosphate dehydrogenases described in this communication and in a previous paper (Drahota *et al.*, 2002) support our hypothesis that the transfer of reducing equivalents from succinate dehydrogenase is better protected against electron leak than that from glycerophosphate dehydrogenase.

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