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THE DIVALENT CATION REQUIREMENT OF THE MITOCHONDRIAL GLYCEROL-3-PHOSPHATE DEHYDROGENASE

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SUMMARY

Coupled respiration by blowfly mitochondria has been utilized to demonstrate an absolute divalent cation requirement for glycerol 3-phosphate respiration. With ADP, phosphate and EGTA, the respiration rate (state 3) decreases as a function of the amount of oxygen reduced, to approximately 15 % of its maximum value, even at 40 mM DL-glycerol 3-phosphate; it can be increased to its maximum value by the addition of Ca^{2+}, Sr^{2+} or Mn^{2+}. The decline in state 3 rate is not due to the removal of membrane-bound calcium into the matrix by the calcium carrier, since it occurs in the presence of ruthenium red. The effect is energy-dependent since the state 3 respiration does not decrease in the presence of uncouplers. The increase in respiration upon the addition of calcium is not due to the energy-dependent calcium transport since it is sensitive to oligomycin and insensitive to ruthenium red.

The divalent cation effector site is located on the glycerol-3-phosphate dehydrogense, since state 3 (or state 4) pyruvate-proline respiration (NAD-linked) is not affected by EGTA. Yet the state 3 pyruvate-proline respiration removes calcium so effectively from the glycerol-3-phosphate dehydrogenase in the presence of EGTA, that added calcium stimulates glycerol 3-phosphate (26.4 mM) respiration about 22-fold.

Since uncouplers stimulate the inhibited glycerol 3-phosphate respiration only to a very small extent, a calcium stimulation of the rate of phenazine methosulfate reduction by glycerol 3-phosphate (26.4 mM) which bypasses all phosphorylation sites, should be detectable. Only a 3-fold stimulation was observed.

The present experiments suggest that upon complete removal of divalent cations from the dehydrogenase, glycerol 3-phosphate does not act as a homotropic effector in the coenzyme Q reductase reaction.

INTRODUCTION

Glycerol-3-phosphate dehydrogenase (EC 1.1.99.5) is located in the inner mitochondrial membrane with its glycerol 3-phosphate oxidation site at the outer surface of the inner membrane [1, 2]. Since the early studies by Estabrook and Sacktor

[3], who observed that glycerol 3-phosphate respiration is inhibited by EDTA, divalent cations have been suspected to be effectors of glycerol 3-phosphate respiration. There has been general agreement that (a) high substrate (glycerol 3-phosphate) concentrations eliminate the need for calcium at the effector site [1, 4, 5] and that (b) only a small calcium effect is observable with solubilized dehydrogenase preparations [4, 6].

The present experiments demonstrate that coupled respiration removes remaining traces of divalent cations from the dehydrogenase, resulting in very low catalytic activity even at high substrate concentrations. This condition does not permit glycerol 3-phosphate to act as homotropic effector. Additional experiments suggest indirectly that the glycerol 3-phosphate:coenzyme Q reductase is affected more dramatically by calcium than the reduction of artificial electron acceptors by glycerol-3-phosphate. Thus the coenzyme Q reductase activity of the solubilized dehydrogenase may respond more dramatically to calcium than the reduction of artificial electron acceptors.

MATERIALS AND METHODS

Mitochondria were prepared from the thoraces of 8–15-day old blowflies (*Sarcophaga bullata*) in manitol medium (0.22 M manitol/0.07 M sucrose/0.2 mM EGTA/10 mM Mops (potassium salt), pH 7.2, 4 °C) in the presence of Nagarse (6.5 μg/ml). Centrifugations were carried out as described [7].

Respiration rates were determined utilizing a Clark-type oxygen electrode manufactured by Rank Brothers, Bottisham, Cambridge, U.K. The oxygen content of the aerated medium was determined (285 μM O_2) with NADH and beef-heart submitochondrial particles. Glycerol 3-phosphate respiration was assayed in sucrose medium (0.2 M sucrose/40 mM 3-(*N*-morpholino) propanesulfonic acid, pH 7.2, 25 °C). Additions were made as described in the figures. Cytochrome *a* was determined with an Aminco-Chance spectrophotometer at 445–455 nm from the change in absorbance of the glycerol 3-phosphate-induced aerobic-anaerobic transition of carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP)-uncoupled mitochondria. This change in absorbance was not modified by the presence of Antimycin A. An extinction coefficient of 106 mM^{-1} was used.

The glycerol 3-phosphate space was determined with the silicone oil centrifugation technique [8]. Mitochondria (6 μl of 11.1 nmol cytochrome *a*/ml) were suspended for 15 s at 22 °C in 240 μl sucrose medium which contained 10 mM potassium phosphate and 0.5 mM EGTA, pH 7.2. To this were added: 60 μl H_2O, 4 μl 0.53 M D,L-glycerol 3-phosphate, 6 μl 77 mM ADP, 5 μl ^3H_2O (5 μCi), 6 μl L-[U-^{14}C]glycerol 3-phosphate (0.3 μCi) or 6 μl [U-^{14}C]sucrose (0.3 μCi). The $CaCl_2$ samples contained also 12 μl of 10 mM $CaCl_2$. The mitochondria were then centrifuged in a Beckman Microfuge B through 50 μl of Versilube F50 into 50 μl of 1.5 M perchloric acid. Respiration rates were determined in parallel experiments in the absence of the radioisotopes.

Sodium DL-glycerol 3-phosphate and oligomycin were obtained from Sigma Chemical Co. 5-Chloro-3-*tert*-butyl-2′-chloro-4′-nitrosalicylanilide (S-13) was a gift from Dr. P. C. Hamm, Monsanto Co., St Louis, Mo. Nagarse was obtained from the Enzyme Development Corporation, New York, N.Y. Radioisotopes were obtained from New England Nuclear, Boston, Mass.

RESULTS

Activation of glycerol 3-phosphate respiration at high substrate concentrations by Ca^{2+}

The mitochondria respire rapidly and at a constant rate on glycerol 3-phosphate in the presence of EGTA, calcium, ADP and phosphate (Fig. 1a). When calcium is omitted, the respiration rate is initially slower and decreases progressively (Fig. 1b). Upon addition of calcium, the respiration rate increases to the control rate. When ruthenium red is present at a concentration sufficient to inhibit calcium uptake [9], the decrease of the respiration as well as the stimulation by calcium still occurs (Fig. 1c). The addition of a small amount of calcium causes a transient increase in respiration (Fig. 1d) which is also insensitive to ruthenium red inhibition. S-13 stimulates the respiration rate to a small extent over the state 3 rate (Fig. 2a). The decrease in respiration rate that occurs in the presence of EGTA is completely prevented by the initial presence of S-13 (Fig. 2c). High concentrations of S-13 added after the decrease has set in provide only partial restoration of respiration (Fig. 2d). In the presence of oligomycin, the calcium stimulated rate equals the state 4 rate and is well below the state 3 rate (Fig. 2b). This is consistent with the observation in Fig. 1c and

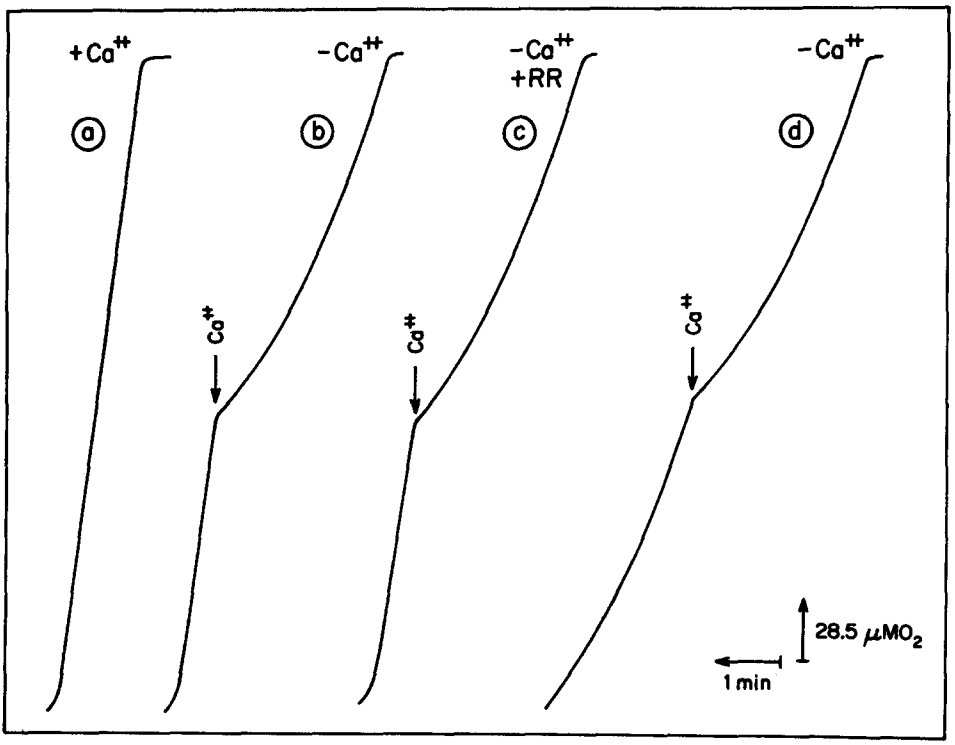

Fig. 1. Stimulation of glycerol 3-phosphate respiration by calcium. Respiration was measured in sucrose medium in the presence of 40 mM sodium DL-glycerol 3-phosphate/10 mM potassium phosphate/4 mM EGTA/2 mM ADP. (a), plus 4 mM $CaCl_2$; (b), plus 4 mM $CaCl_2$ at arrow; (c), plus 1.3 nmol ruthenium red/mg protein and 4 mM $CaCl_2$ at the arrow; (d), plus 400 μM $CaCl_2$ at arrow. 0.26 mg of mitochondrial protein/ml was present in all of the above reaction media.

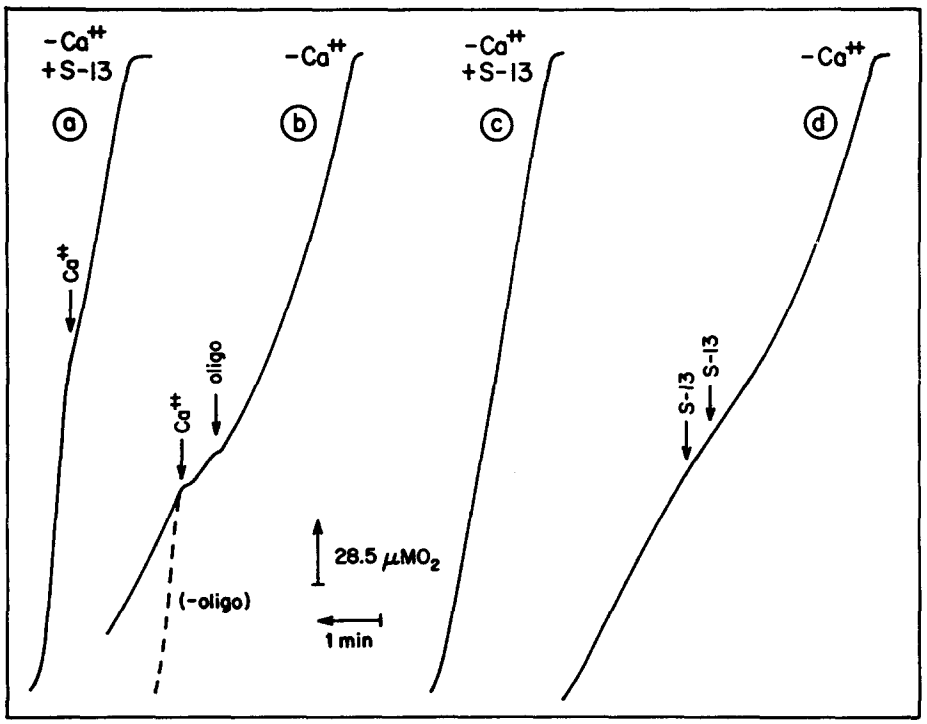

Fig. 2. The effect of uncouplers and inhibitors of energy transduction on the calcium-glycerol 3-phosphate respiration. Reaction conditions as in Fig. 1. (a), 3.5 mol S-13/mol cytochrome *a* and 4 mM $CaCl_2$ was added at the arrow; (b), 7.7 μg oligomycin/nmol cytochrome *a* was added and 4 mM $CaCl_2$; (c), 1.75 mol S-13/mol cytochrome *a*; (d), 1.75 mol S-13/mol cytochrome *a* and 3.5 mol S-13/mol cytochrome *a* were added. 0.17 nmol cytochrome *a*/ml was present in all of the above reaction media.

indicates that the response to calcium is actually an increase in state 3 and is not due to energy-linked calcium accumulation.

The striking requirement for divalent cations is most clearly shown in Fig. 3. The respiration rates were determined from experiments of the type in Fig. 1b by varying the glycerol 3-phosphate concentration. Rates in the absence of calcium were determined 2.5 min after initiation of respiration, i.e. just before the addition of calcium.

Effect of EGTA on the coupled glycerol 3-phosphate respiration

Estabrook and Sacktor [3] made the original observation that EDTA inhibits glycerol 3-phosphate respiration and suggested that EDTA might be directly responsible for the inhibition instead of indirectly, through the removal of divalent cations from the respiratory chain. Donnellan and Beechey [5] observed that Ca^{2+} and Sr^{2+} and to a smaller extent Mg^{2+} could overcome the inhibition by EDTA of uncoupled respiration. Fig. 4 shows that under conditions similar to those of Fig. 3, Sr^{2+}, Ca^{2+} and Mn^{2+} can overcome the inhibition by EGTA. I have plotted the total amount of cations added. This type of presentation could be misleading since, as shown by

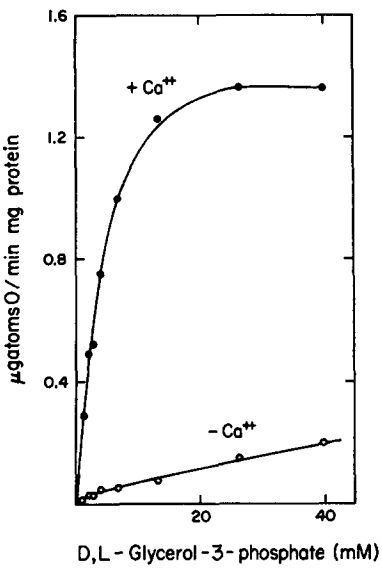

Fig. 3. Dependence of the respiration rate on glycerol 3-phosphate concentration. Experimental conditions as Fig. 1(b).

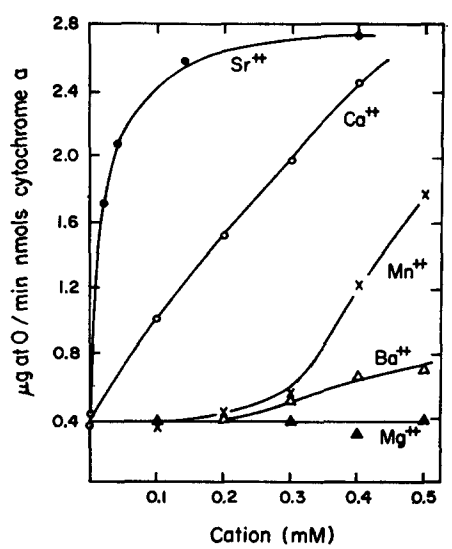

Fig. 4. Dependence of the glycerol 3-phosphate respiration on divalent cations. Conditions as in Fig. 1(a) except that 6.6 mM Na DL-glycerol 3-phosphate and 400 μM EGTA were used and calcium was replaced by the ions indicated.

TABLE I

THE EFFECT OF FREE EGTA CONCENTRATIONS ON THE GLYCEROL 3-PHOSPHATE RESPIRATION RATE

The calculations were carried out with the data from Fig. 5. The following log K_i (log of equilibrium constant) values were used: 12.3 (Mn^{2+}), 10.9 (Ca^{2+}), 7.9 (Sr^{2+}) [10]. The respiration rates (glycerol 3-phosphate) are in μg atoms O/min nmol cytochrome *a*. The EGTA concentrations are in μM.

Cation (total added)	EGTA (free)	Respiration rate
Mn^{2+} (460 μM)	< 1	1.6
Ca^{2+} (197 μM)	202	1.6
Sr^{2+} (14.5 μM)	387	1.6

Donnellan and Beechey [5], for uncoupled respiration at low substrate concentrations, free Ca^{2+} is able to overcome the EDTA inhibition at a lower concentration than free Sr^{2+}. Utilizing the results of Fig. 4 for the coupled mitochondria and the known log of the equilibrium constants [10], one can calculate the half maximal respiration rates as a function of free EGTA (Table I). There is no correlation between the free EGTA concentration and the coupled respiration rate.

Localization of the calcium effector site

Evidence has been presented in the past that suggests that the mitochondrial inner membrane is impermeable to glycerol 3-phosphate [2] and that the glycerol 3-phosphate oxidation site is located on the outside of the inner membrane [1, 2]. The present experimental conditions are different. However, Table II shows that coupled mitochondria in the presence or absence of calcium are impermeable to glycerol 3-phosphate, even at high concentrations. Since no evidence has as yet been presented excluding a glycerol 3-phosphate oxidation site on the matrix side of the inner membrane, we consider this experiment relevant to eliminate Ca^{2+} mediated glycerol 3-phosphate transport into the matrix as a factor in explaining these data.

Fig. 5 demonstrates that the mitochondrial cytochromes undergo a slow oxidation during the inhibition of respiration and that they all become more reduced

TABLE II

EFFECT OF CALCIUM ON THE GLYCEROL 3-PHOSPHATE IMPERMEABLE MITOCHONDRIAL MATRIX

V is the volume of radioisotope solution that has been centrifuged into the perchloric acid (see Materials and Methods for details of the conditions). Volumes are ±S.E.M. with $N = 4$.

		+$CaCl_2$
$V(H_2O) - V$(glycerol 3-phosphate) (μl/nmol cytochrome *a*)	2.6±0.1	2.8±0.1
$V(H_2O) - V$(sucrose) (μl/nmol cytochrome *a*)	2.3±0.2	2.3±0.2
Glycerol 3-phosphate respiration (μg atoms O/min nmol cytochrome *a*)	0.21	1.95

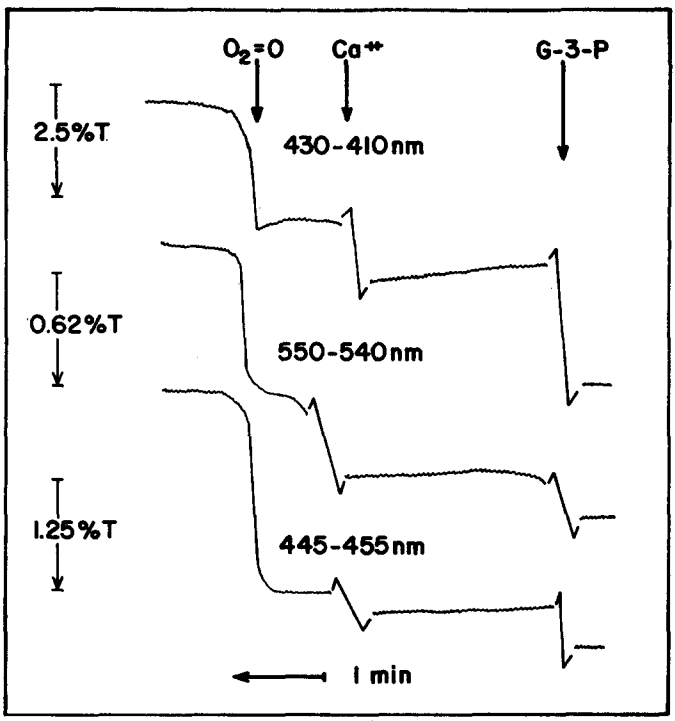

Fig. 5. Absorbance changes of the mitochondrial cytochromes. The medium (1.1 ml sucrose medium) contained 10 mM potassium phosphate/4 mM EGTA/2 mM ADP/0.11 μM cytochrome *a*. The respiration was started with 24 mM sodium DL-glycerol 3-phosphate and was allowed to proceed for about 2.5 min. 4 mM $CaCl_2$ was added and the suspension eventually became anaerobic. Parallel experiments were carried out for cytochrome *b* (430–410 nm), cytochrome *c* (550–540 nm), and cytochrome aa_3, (445–455 nm).

after the addition of calcium. The experiments suggest that the effector site is localized on the substrate side of cytochrome *b*.

Hansford and Chappell [4] observed that pyruvate (state 3 and state 4) respiration was not affected by the calcium concentrations that affect glycerol 3-phosphate respiration. Donnellan and Beechey [5] extended this observation to proline respiration and NADH oxidation by submitochondrial particles.

We have carried out a similar experiment in the presence of lower calcium concentrations to determine whether the membrane potential generated during state 3 pyruvate-proline respiration is as effective as the glycerol 3-phosphate state 3 respiration in removing calcium from the effector site. Table III shows that state 3 pyruvate-proline respiration almost completely inhibits glycerol 3-phosphate respiration without at the same time affecting its own state 3 rate. These experiments place the calcium effector site on the substrate side of coenzyme Q, which is the first redox center common to NADH and glycerol 3-phosphate respiration. At higher calcium concentrations (Table III, Expt. 3) the pyruvate-proline respiration, but not the glycerol 3-phosphate respiration, is inhibited. This may be due to the inhibition of the isocitrate dehydrogenase by calcium [11].

TABLE III

EFFECT OF THE COUPLED PYRUVATE-PROLINE RESPIRATION IN THE PRESENCE AND ABSENCE OF EGTA ON GLYCEROL 3-PHOSPHATE RESPIRATION

Arithmetic average of two experiments. The respiration rate (pyruvate-proline, with or without EGTA and $CaCl_2$) was determined 1.5 min after the addition of ADP and is expressed as μg atoms O/min nmol cytochrome *a*. The conditions: 0.5 ml of 0.2 M sucrose/40 mM Mops, pH 7.2, 22 °C/ 10 mM potassium phosphate/20 mM proline/4 mM pyruvate (neutralized with $NaHCO_3$)/320 μM ATP, the mitochondria were added and after 2 min, 2 mM ADP was added. 1.5 min later 0.1 μM rotenone was added, then 26.4 mM sodium DL-glycerol 3-phosphate. Where indicated, 4 mM EGTA and 4 mM $CaCl_2$ were present from the beginning or the $CaCl_2$ was added when indicated.

Experiment		Respiration Rate
1	Pyruvate + proline (state 3)	3.6
	+ rotenone	0
	+ glycerol 3-phosphate	3.4
2	Pyruvate + proline + EGTA (state 3)	3.6
	+ rotenone	0
	+ glycerol 3-phosphate	0.1
	+ $CaCl_2$	2.9
3	Pyruvate + proline + EGTA + $CaCl_2$	0.9
	+ rotenone	0
	+ glycerol 3-phosphate	3.0

The effect of calcium on the glycerol 3-phosphate linked reduction of ferricyanide and phenazine methosulfate

Calcium has been shown to affect the rate of phenazine methosulfate and ferricyanide reduction by glycerol 3-phosphate in the presence of cyanide and low glycerol 3-phosphate concentration [2, 4, 5]. These experiments leave open a possible interaction of the artificial electron acceptors at or near cytochrome *c* and thus do not eliminate a Ca^{2+} effector site near cytochrome *c*.

We have shown (Fig. 2) that S-13 overcomes only to a small extent the energy-linked inhibition of glycerol 3-phosphate respiration. Therefore, the calcium activation should be detectable in the glycerol 3-phosphate: ferricyanide or phenazine methosulfate reaction in the presence of Antimycin A. Table IV shows supporting data; the calcium activation can be detected and it is much smaller with the S-13 uncoupled respiration.

The activation is about the same at the two phenazine methosulfate (PMS) concentrations (Table IV, Expt. 2). The extent of stimulation, however, is much smaller than in the respiration assays. Even state 3 pyruvate-proline respiration is only able to inhibit the glycerol 3-phosphate linked reduction to an extent that permits a 3- to 4-fold stimulation by calcium (Table IV, Expt. 3). The possibility that PMS is contaminated with traces of calcium was considered; however, PMS can also be added together with pyruvate-proline and the resulting respiration rates with or without Ca^{2+} by glycerol 3-phosphate are the same as in Table IV, Expt. 3.

Thus, the reduction site for the artificial electron acceptors is not as strongly affected by the calcium as that of the first redox center common to the NAD and glycerol 3-phosphate respiratory chains.

TABLE IV

THE EFFECT OF ENERGY-LINKED CALCIUM TRANSPORT ON THE GLYCEROL 3-PHOSPHATE-LINKED REDUCTION RATE OF FERRICYANIDE AND PHENAZINE METHOSULFATE (PMS)

Reactions were carried out at 22 °C; they are averages of duplicate experiments from two mitochondrial preparations.

Experiment		Reaction rate
1a	Ferricyanide[a]	3.6
	$+CaCl_2$	6.4
b	Ferricyanide+S-13	5.1
	$+CaCl_2$	5.9
2a	PMS[b]	0.92 (1.23)
	$+CaCl_2$	1.76 (2.59)
b	PMS+S-13	1.45 (2.17)
	$+CaCl_2$	1.69 (2.66)
3a	PMS[c]	0.66
	$+CaCl_2$	2.12
b	PMS	2.6

[a]The ferricyanide assays (μmol ferricyanide/min nmol cytochrome *a*) conducted in 0.2 M sucrose/40 mM Mops, pH 7.2/10 mM potassium phosphate/4 mM EGTA/2 mM ADP/mitochondria (53 nM cytochrome *a*)/26.4 mM sodium DL-glycerol 3-phosphate; 2.5 min respiration, then 0.42 μM Antimycin A/0.71 mM NaCN. Reaction was started with 240 μM ferricyanide. Absorbance changes were monitored at 450–460 nm with an Aminco-Chance Dual wavelength Spectrophotometer and a Texas Instrument strip chart recorder. Parallel experiments were carried out by adding 4 mM $CaCl_2$ just before ferricyanide or adding 62.5 nM S-13 immediately after the mitochondria. [b]The PMS assays (μg atoms O/min nmol cytochrome *a*) were carried out in an oxygen electrode vessel. Reactants and conditions were identical to the ferricyanide assays, except the concentration of mitochondria was doubled, NaCN was omitted, the final reaction was started with 280 μM PMS or 560 μM PMS (parentheses). Calcium was added during the PMS respiration. [c]The conditions were as in Table III, experiment 2; however, 0.8 μM Antimycin A was added after rotenone, then 280 μM PMS and 26.4 mM sodium DL-glycerol 3-phosphate. EGTA was not present in experiment (b).

DISCUSSION

It has been known for some time that the calcium requirement for uncoupled glycerol 3-phosphate respiration of blowfly mitochondria is eliminated [4], or almost eliminated [5], at high substrate concentrations. Results from the present investigation demonstrate that coupled state 3 respiration removes calcium from a binding site (effector site) on the glycerol 3-phosphate: coenzyme Q reductase system. Since this site has high affinity for calcium and since calcium is only slowly removed from the effector site (faster with pyruvate-proline respiration) the inhibited glycerol 3-phosphate oxidation rate at high substrate concentrations can most readily be detected in the presence of EGTA.

Rossi and Lehninger [12] demonstrated that when ADP and calcium are both present in a phosphate containing medium, calcium is transported into the matrix space of mammalian mitochondria before ADP is phosphorylated. In other words, the membrane potential is sufficiently high during state 3 to permit transport of calcium into the matrix. I wish to propose that in the present case with the blowfly mitochon-

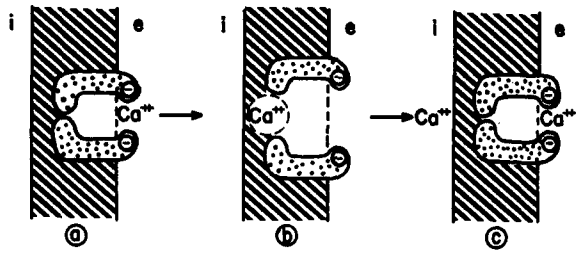

Fig. 6. Diagrammatic representation of glycerol 3-phosphate dehydrogenase subunits in the mitochondrial inner membrane. (a), the active dehydrogenase; (b), the state 3 (EGTA to lower the medium free calcium concentration) inhibited dehydrogenase; (c), the calcium reactivated dehydrogenase. "i" is for the matrix and "e" for the external or cytoplasm side of the inner mitochondrial membrane.

dria, Ca^{2+}, the heterotropic effector of the glycerol 3-phosphate oxidation, is moved during state 3 respiration towards the mitochondrial matrix space and thus away from its effector site (Fig. 6) resulting in the inhibition of respiration. The present results do not permit a differentiation between (a) a dehydrogenase complex with its affinity for calcium (Fig. 6b) lowered to make the medium free calcium concentration insufficient for activation and (b) the removal of free calcium from the medium towards the matrix (only to a small extent reversed by S-13) by the dehydrogenase complex results in a calcium concentration insufficient for activation. The latter situation could be achieved by continuously cycling through the steps of Fig. 6 in the absence of a change in affinity for calcium.

When an uncoupler is added to the inhibited mitochondria (Fig. 2), a small fraction of the calcium ions within the hydrophobic space of the membrane is still close to the effector sites and thus reoccupy them upon elimination of the membrane potential by the uncoupler. A small activation of the respiration (Fig. 2) is thus observed.

The transport of calcium across the mitochondrial membrane (or towards the matrix space) as indicated by the inhibition of glycerol 3-phosphate respiration, occurs very likely also during the active state 3 glycerol 3-phosphate respiration, i.e. in the presence of higher free Ca^{2+} concentrations. Fig. 1 suggests that a p-Ca^{2+} of more than 10 stimulates the respiration to a small extent. The calcium movement as observed in this system is thus small in comparison to the ruthenium red sensitive mitochondrial calcium transport [9].

An actual mechanism of calcium transport across these mitochondrial membranes in the presence of ruthenium red could occur via dehydrogenase associated lipids, i.e. a calcium catalyzed and energy-linked flip-flop of lipids. Tyson and co-workers [13] suggest that cardiolipin, which occurs in blowfly mitochondria at about the same concentration as in mammalian mitochondria [14], can catalyze the transport of Ca^{2+} across hydrophobic barriers. The K_m for this transport process, however, appear to be very high. This value cannot yet be readily compared to our ruthenium red insensitive transport system.

Fisher and co-workers [15] observed significant stimulation at high glycerol 3-phosphate concentrations of the respiration of lung mitochondria that had been prepared in the presence of high EDTA or EGTA concentrations. We have prepared the blowfly mitochondria in the presence of 20 mM EGTA without significantly

changing the properties of the mitochondria as reported in the present communication. The lung mitochondria of Fisher and co-workers [15] possessed relatively low respiratory control ratios and very low respiration rates and it is possible that a perturbation of the membrane structure reduces the affinity of the dehydrogenase effector site for calcium and makes it more accessible to the cation chelator of the medium.

The present experiments suggest indirectly that the rate of coenzyme Q reduction by the dehydrogenase is more critically dependent on Ca^{2+} than the reduction of the more hydrophilic artificial electron acceptors.

The results of Fisher and co-workers [15] suggest also that the glycerol 3-phosphate respiration is stimulated to a larger extent by Ca^{2+} than the rate of reduction of artificial electron acceptors. They, however, used only KCN as an inhibitor and, as discussed earlier, some of the redox dyes may have interacted with redox centers near cytochrome *c* and their reaction rates may not so well represent the dehydrogenase activity as the reductions in the presence of Antimycin A, as carried out in the present study. A calcium effector site closely associated with the phospholipids near the coenzyme Q reduction site could explain some of the results of the present investigation.

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