THE EFFECT OF Ca^{2+} ON THE OXIDATION OF GLYCEROL PHOSPHATE BY BLOWFLY FLIGHT-MUSCLE MITOCHONDRIA

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The oxidation of glycerol-1-phosphate (GP) by isolated blowfly flight-muscle mitochondria is inhibited by addition of EDTA. This is reversed by addition of 5mM-Mg^{2+}, Ca^{2+} and a number of other bivalent metal ions (Estabrook & Sacktor, 1958). It was concluded that EDTA itself may be responsible for inhibition of the dehydrogenase. In this paper it is shown that GP oxidation by isolated flight-muscle mitochondria is markedly stimulated by low levels of Ca^{2+}. The dehydrogenase in intact and sonicated mitochondria shows allosteric kinetics with regard to substrate. Ca^{2+} acts by lowering the K_m for substrate. When the enzyme was extracted with Triton Ca^{2+}-activation was lost, but some measure of allosteric behaviour with regard to substrate concentration was retained at low Triton to mitochondrial concentration ratios. In the presence of stabilized low levels of Ca^{2+}, achieved by using ethylene-glycol-bis-(aminoethyl)tetracetate (EGTA)-Ca^{2+} buffers described by Portzehl, Caldwell & Rüegg (1964), the oxidation of GP was stimulated 3-4 fold on addition of ADP and phosphate. A dual physiological role of Ca^{2+}, released on initiation of contraction in flight-muscle is suggested, the first in activating the myosin ATPase, the second in allowing rapid rates of GP oxidation and therefore ATP synthesis.

METHODS AND MATERIALS. Mitochondria were prepared from the thoraces of 30-40 Caliphora vomitora 10-20 days after emergence. The heads, wings and

abdomens were removed from the pre-cooled flies and the muscle squeezed from the thoraces and placed in 20-30ml of 0.25M-sucrose containing 5mM-Tris-Cl pH 7.4 and 1mM-EGTA at 0^{o}. 5Mg of crystalline bacterial proteinase (Nagarse) dissolved in 5ml of preparation medium was added. The suspension was then briefly homogenized manually, in a loose-fitting Dounce homogenizer, left at 0^{o} for 10min and then re-homogenized. The homogenate was passed through cheese-cloth and centrifuged at 9000g for 5min. The mitochondrial pellet was resuspended in 40ml of preparation medium and centrifuged at 4000g for 5min. The dark-red homogeneous pellet was resuspended in 2ml of medium. A low speed spin was not used since this considerably decreased the yield of mitochondria and apparently there were no myofibrils left after enzymatic digestion.

Sonicated particles were prepared by subjecting the mitochondrial suspension to 6 separate periods of 30sec of sonication in an MSE 60 watt ultrasonic disintegrator. The tube was immersed in an NH_4Cl-ice mixture. The sonicate was centrifuged at 9000g for 5min to remove any intact mitochondria and the particles were obtained by centrifugation at 100,000g for 20min.

The dehydrogenase was solubilized by treating either mitochondria or particles with Triton X-114. Centrifugation at 100,000g for 40min yielded a cytochrome-rich pellet virtually devoid of GP dehydrogenase activity.

Oxygen consumption was followed using a Clark-type electrode as described by Chappell (1964). Approximately 1mg of mitochondrial protein was added to an incubation mixture containing 100mM-KCl, 10mM-Tris-Cl pH 7.1 and other additions as indicated in legends to figures. The total volume was 4ml and temperature 20^{o}.

RESULTS. In confirmation of the findings of Van den Bergh & Slater (1962) intact blowfly flight-muscle sarcosomes isolated as described above oxidized

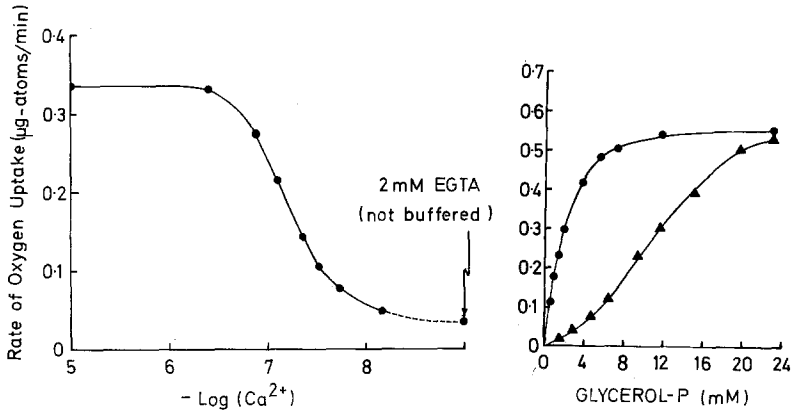

Fig. 1A. The effect of low levels of free Ca^{2+} on the rate of oxidation of GP. The mitochondria (approximately 1mg protein) were added to a medium containing 3.5mM-DL-GP, 0.5µM-carbonylcyanide-p-trifluoromethoxyphenylhydrazone (FCCP) and 2mM-EGTA to which Ca^{2+} had been added to give the free Ca^{2+} concentration indicated (Portzehl et al., 1964).

B. The effect of GP concentration on the rate of oxidation. ● — ● 10^{-5}gm-ion of free Ca^{2+}; ▲ — ▲ 2mM-EGTA, no Ca^{2+}. 0.5µM-FCCP was present.

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only GP and pyruvate at observable rates. However it was found that when EDTA (see Estabrook & Sacktor, 1958) or EGTA was present in the incubation mixture the rate of oxidation of 2mM-DL-GP was severely inhibited. An investigation of the inhibitory effect of EGTA revealed that Ca^{2+} was required for the oxidation of GP when the EGTA-Ca^{2+} buffers described by Portzehl et al. (1964) were used (Fig. 1A). At concentrations of free Ca^{2+} less than 10^{-8}gm-ion/l the rate of oxidation of 3.5mM-DL-GP was one-tenth the rate at 10^{-5}gm-ion/l or greater concentrations of free Ca^{2+}. It should be noted that in these experiments the concentration of free EGTA did not vary significantly and so could not be responsible for the effects observed.

At high concentrations of GP, Ca^{2+} had no effect. Systematic investigation of this phenomenon revealed that Ca^{2+} acted by lowering the apparent K_m for GP oxidation (Fig. 1B). Further it was also apparent that v versus S plots, especially at low free Ca^{2+} concentrations, were not rectangular

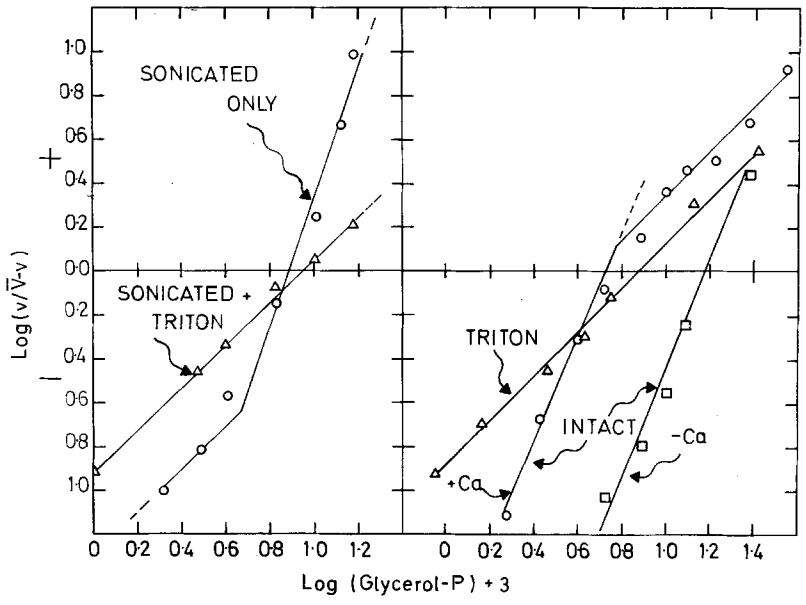

Fig. 2. Hill plots of the kinetics of GP oxidation by mitochondria, particles and solubilized dehydrogenase. In all cases, 2.2mM-phenazine methosulphate and 1.2mM-HCN were present.

A. O — O, particles; △ — △ particles with 0.6mg Triton X-114/ml.
B. O — O, intact mitochondria with 10^{-6}gm-ion/l free Ca^{2+};
□ — □, intact with 10^{-8}gm-ion/l free Ca^{2+};
△ — △, mitochondria with 0.6mg Triton X-114/ml.

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hyperbolae. The allosteric nature of the kinetics of GP oxidation are revealed by the Hill plots shown in Fig. 2. At both 10^{-5}gm-ion Ca^{2+}/l and in the presence of excess EGTA the slope of the log (v/V - v) versus log (S) plot was 2-3 in a wide range of experiments. At high (Fig. 2B) and at low (Fig. 2A) concentrations the slope had a value of approximately unity.

These experiments did not reveal whether the allosteric kinetics were due to and the Ca^{2+} effect was on (1) the penetration of the mitochondrial membrane by substrate or product, (2) the dehydrogenase or (3) the respiratory chain and energy conservation system. Ca^{2+} at the levels used in these experiments (less than 10^{-6}gm-ion/l) did not affect the rate of oxidation of pyruvate either in the presence or absence of ADP and phosphate. This would

appear to rule out possibility (3). Treatment of the mitochondria with high levels of Triton X-114 abolished the allosteric behaviour of the system (Fig. 2B) whereas sonication did not (Fig. 2A). The treatment of sonicated mitochondria with Triton also led to classical kinetics. However when the enzyme was extracted from the mitochondria or sonicated particles at low Triton to protein concentration ratios (1mg/ml) some measure of allosteric behaviour was observable with a Hill plot slope of approximately 1.3-1.4. With the extracted enzyme no reproducible effect of Ca^{2+} or of EGTA was observable.

In contrast to the findings of Lennie & Birt (1967) with the fly Lucilia cuprina the activity of the enzyme bound to mitochondria, sonicated particles and after extraction with Triton was unaltered after periods of 24hr or more.

In the presence of Ca^{2+}-buffers giving free ion concentrations of 10^{-6} gm-ion/l, respiratory control ratios of 3-5 were observed with 3.5mM-DL-GP (Fig. 3). At low Ca^{2+} concentrations (less than 10^{-8}gm-ion/l) no stimulation of respiration on addition of ADP and phosphate was observed. Increasing Ca^{2+} from 10^{-8} to 10^{-6}gm-ion/l caused a progressive increase in the ADP-stimulated (state 3) rate, without an increase in the resting (state 4) rate.

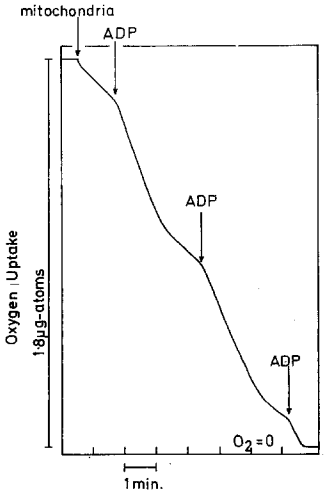

Fig. 3. The effect of ADP on the rate of GP oxidation. Mitochondria were added to a medium containing 3.5mM-DL-GP, 3mM-phosphate and 1.2mg of bovine plasma albumin/ml. Where indicated 1.0µmole of ADP was added.

Above 10^{-6} gm-ion/l some measure of uncoupling occurred. At 20-40 mM-DL-GP Ca^{2+} was not necessary for the observation of respiratory control. Uncoupling agents (e.g. FCCP and 2,4-DNP) caused rates of oxidation equivalent to those observed with ADP. With 1mM-pyruvate respiratory control ratios of 8 were routinely observed.

DISCUSSION. Ca^{2+} is required both for the activation of the Mg^{2+}-ATPase and oscillatory contractions of glycerinated insect flight-muscle myofibrils (Rüegg & Tregear, 1966). Activity was low at 10^{-8} and maximal at approximately 10^{-5} gm-ion/l. The implication of these findings is that Ca^{2+} is responsible for the initiation of the contractile process in insect as in other muscles. Further, in insect flight-muscles Ca^{2+} is required for maximal rates of ADP-stimulated GP oxidation. In this case increased mitochondrial metabolism is elicited not only by availability of ADP but also of Ca^{2+}, both of which are made available on contraction.

The control of GP oxidation depends on both the lowering of K_m by Ca^{2+} and also on the allosteric nature of the enzyme kinetics. At physiological levels of GP (approximately 2mM of the L-isomer) (Sacktor & Wormser-Shavit, 1966) a ten-fold increase in uncoupled - and ADP-stimulated oxidation occurred on increasing the Ca^{2+} concentration from 10^{-8} to 10^{-5} gm-ion/l.

It appears that Ca^{2+} stimulation occurs only when the dehydrogenase is bound to the mitochondrial structure. When the enzyme is solubilized with Triton, Ca^{2+}-activation disappears, the K_m is high but a measure of allosteric behaviour remains. The Hill plot slopes of 2-3 observed with the structurally-bound enzyme indicate possibly that the dehydrogenase is composed of four sub-units which show co-operative binding effects (see Monod, Changeaux & Jacob, 1963). Ca^{2+} increases the affinity of the structurally-bound enzyme for substrate.

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