THE ENERGY-DEPENDENT ACCUMULATION OF PHOSPHATE BY BLOWFLY MITOCHONDRIA AND ITS EFFECT ON THE RATE OF PYRUVATE OXIDATION

R. G. Hansford and J. B. Chappell
Department of Biochemistry, University of Bristol.
England.

Received February 14, 1968

During an investigation of the kinetic properties of the NAD-linked isocitrate dehydrogenase (NAD-IDH) of mitochondria derived from the thorax of Caliphora vomitora (a species of blowfly) it became apparent that this enzyme was inhibited by ATP and $NADH_2$ and was activated by ADP, Mg^{2+}, isocitrate and phosphate. A particularly striking feature was the dependence on phosphate. A plot of activity versus concentration resulted in a sigmoidal curve. At 5mM phosphate the enzyme produced 4-5% of its maximal activity and at 30mM 80%. Sacktor & Hurlbut (1966) have reported that at rest the phosphate content of whole blowfly thorax is 6.8μmoles/gm. wet wt. and in activity 7.5μmoles/gm.

In this paper it is shown that isolated blowfly mitochondria are able to accumulate phosphate in a reversible energy-dependent fashion. The concentration in the intramitochondrial water can be as high as 4 times that in the suspending medium. The significance of this finding is illustrated in terms of the kinetic and steady-state response of O_2 uptake and respiratory carriers of intact mitochondria to ADP, phosphate and uncoupling agents. The conclusion is drawn that NAD-IDH activity is the prime controlling factor in the rate of O_2 uptake during pyruvate oxidation by blowfly flight-muscle mitochondria.

METHODS AND MATERIALS

Mitochondria were isolated from blowfly thorax muscle as described by Hansford & Chappell (1967). Respiration was followed using an O_2-electrode (Chappell, 1964) in a medium containing 100mM-KCl, 20mM - tris-chloride pH 7.4 and other additions as indicated in the legends to Figs.

To assay the NAD-IDH activity of blowfly mitochondria the suspension was treated for 1 min. in 15 sec. bursts in an M.S.E. 60 W sonicator with the tube immersed in an $(NH_4)_2 SO_4$/ice mixture under the

conditions described by Goebell and Klingenberg (1964). The suspension was then centrifuged at 100,000g. for 20 min. at 4°. The supernatant was used for the spectrophotometric assay of NAD-IDH activity under the conditions described in legends to Figs.

RESULTS

The dependence of the NAD-IDH activity of blowfly mitochondria on added phosphate is illustrated in Fig. 1. This enzyme differs from that derived from rat heart-muscle in the absolute requirement for phosphate (or arsenate). The heart enzyme shows only a slight activation by phosphate (Goebell & Klingenberg, 1964).

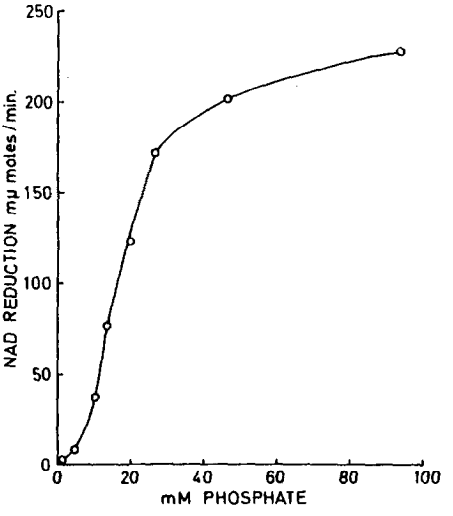

Fig. 1. The phosphate dependence of blowfly NAD-IDH activity. An aliquot of the supernatant obtained after sonication of the mitochondria was added to a medium containing 0.5mM-L(+)-isocitrate, 2.5mM-NAD, 1.3mM-ADP and 10mM-$MgCl_2$. The total volume was 3 ml., the temperature 23° and the final pH 7.2. The phosphate concentration was varied as shown and the ionic strength kept constant at 0.26 by adding varying amounts of triethanolamine-chloride and KCl.

The rate of ADP-stimulated pyruvate oxidation is markedly influenced by phosphate. Thus more than 20mM-phosphate is required before maximal rates are achieved. In contrast only 2mM-phosphate is required to produce maximal rates of ADP-stimulated glycerolphosphate oxidation. This value is of the same order as that obtained by Chance & Williams (1955) for rat liver mitochondria oxidizing D-β-hydroxybutyrate. The requirement for high levels of phoshate for pyruvate oxidation by blowfly mitochondria is even more dramatic when trifluoromethoxycarbonylcyanide - phenylhydrazone (FCCP) is used to stimulate respiration. This effect is far more pronounced in the presence of oligomycin. This suggested that blowfly mitochondria may be capable of accumulating phosphate against a concentration gradient.

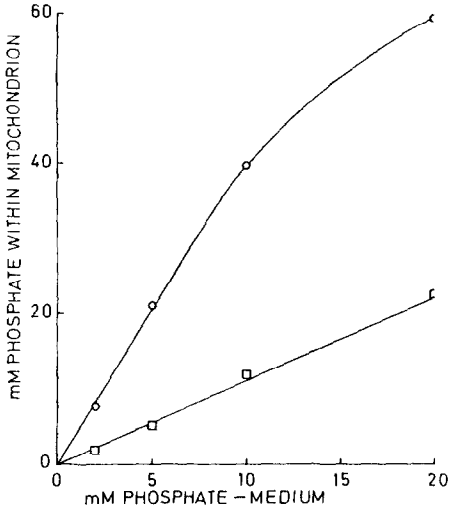

Fig. 2. The concentration of phosphate in intramitochondrial water as a function of the external phosphate concentration. Mitochondria (approximately 4 mg. of protein) were added to a medium containing 2mM-pyruvate, 4mM-proline, oligomycin (1µg./ml.) and varying amounts of phosphate as shown. ^{14}C-labelled sucrose and $^{3}H_2O$ were also present. After 5 min. at 20° the mitochondria were sedimented in an M.S.E.18 centrifuge accelerated maximally at 11,000 r.p.m. and then stopped (total time about 4 min.). Phosphate was determined in the supernatant and pellets after deproteinization and water and sucrose-spaces calculated after determination of ^{14}C and ^{3}H by dual-channel scintillation counting.

Direct support for this is given by the experiments shown in Fig. 2. In these experiments mitochondria were incubated with pyruvate and proline in the presence and absence of FCCP at different phosphate levels. $^{3}H_2O$ and ^{14}C-labelled sucrose were included to enable total water and "sucrose-spaces" to be determined. The mitochondria were then sedimented by rapid centrifugation. Phosphate, $^{3}H_2O$ and ^{14}C-labelled sucrose, were determined in the pellet and supernatant. In the presence of FCCP, phosphate equilibrated with the mitochondrial water, but in the absence of FCCP considerable concentration of phosphate occurred. The accumulation of phosphate at the higher phosphate levels rules out the possibility that the phosphate was derived from the hydrolysis of intramitochondrial ATP. In separate experiments it was shown that the ATP level in blowfly intramitochondrial water was approximately 3mM. Also oligomycin was present and this inhibits the hydrolysis of ATP.

Phosphate accumulation was supported not only by pyruvate but also by glycerolphosphate oxidation. The accumulation was both inhibited and reversed by FCCP with both substrates.

It is apparent that the accumulation of these large phosphate concentrations of up to 60mM must involve either the uptake of a counter cation, e.g. K^+, or exchange for an anion already contained within the

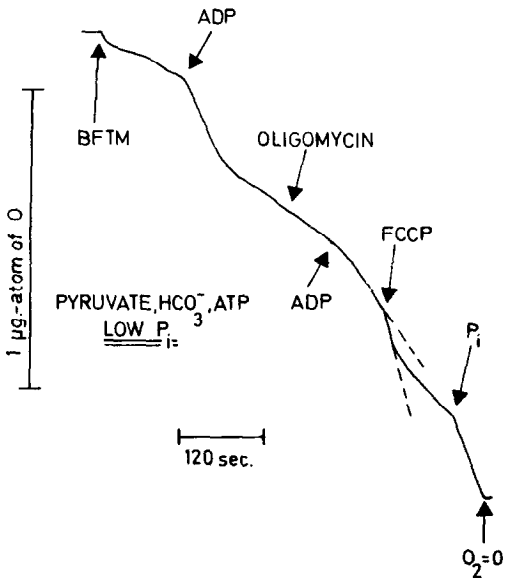

Fig. 3 The effect of phosphate on the rate of oxidation of pyruvate in the presence of oligomycin and FCCP. Blowfly thorax mitochondria (BFTM) (approximately 1 mg. of protein) were added to 3.4 ml. of a medium containing 2mM-pyruvate, 0.5mM-HCO_3^-, 1mM-ATP and 3mM-phosphate. Other additions were as indicated; ADP (0.29mM, final concentration), oligomycin (1.5µg./ml.), FCCP (0.6µM), phosphate (15mM). Temperature 25°.

---

mitochondrion. The first alternative is unlikely in that no swelling occurred under conditions where phosphate was accumulated. It is suggested that anion exchange occurs and that HCO_3^- may be involved.

This ability to concentrate phosphate in an energy-dependent process is reflected in several aspects of the kinetics of pyruvate oxidation. Thus, in Fig. 3 an oxygen-electrode experiment is shown in which FCCP was added to mitochondria suspended in the presence of pyruvate, ATP, HCO_3^-, (to "spark" pyruvate oxidation), ADP, oligomycin and a low level of phosphate (3mM). Respiration was rapid initially but then fell to a low rate. The initial high rate was re-instated on addition of a high level of phosphate (25mM). These results are most easily interpreted in terms of the proposed energy-dependent phosphate pump and the requirement for phosphate for the NAD-IDH activity. It is unlikely that the requirement for phosphate is primarily for the succinyl-CoA synthetase reaction since the K_m for this enzyme is low with other mitochondria.

Studies of the redox-state of NAD (by fluorimetry) and of cytochrome c (by double-beam spectrophotometry at 550-540mµ) also indicate the

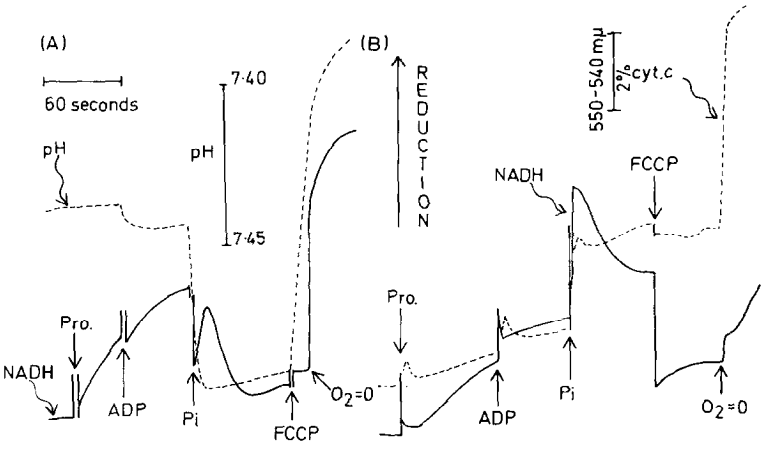

Fig. 4A. The effect of phosphate on the kinetics of NAD oxidation and reduction. ADP phosphorylation was followed by the associated alkaline pH change. Mitochondria (2.5mg. of protein) were added to a medium containing 1mM-ATP and 2mM-pyruvate. Total volume 2.0ml., temperature 25°. Where indicated proline (10mM, final concentration), ADP (0.5mM), phosphate (12.5mM) and FCCP (1μM) were added. The medium was saturated with O_2. Fig. 4B The effect of oligomycin on the response of NAD and cytochrome c to the addition of phosphate. The conditions were as described in the legend to Fig. 4A except that 1.5μg. of oligomycin/ml. was present.

---

presence of a phosphate-pump which exerts an energy-demand on the respiratory chain energy-conservation system. Thus when phosphate (10mM) is added to mitochondria in the presence of glycerolphosphate, with no added ADP present, the NAD which is extensively reduced (by reversed electron transport) is extensively oxidized. Oligomycin does not prevent this effect, indicating that this is not due to interaction with the phosphorylation system. This result is easily accounted for in terms of an energy-dependent pump. In Fig. 4A the effect of adding phosphate to mitochondria treated with pyruvate, proline and ADP is shown. Immediately after addition of phosphate there is a rapid oxidation of $NADH_2$ followed by a reduction. After the ADP has been phosphorylated, indicated by cessation of the alkaline change due to ATP synthesis, the $NADH_2$ becomes extensively oxidized. Subsequent additions of ADP cause reduction of NAD (not shown). In the presence of oligomycin (Fig. 4B) phosphate causes a rapid and extensive reduction (most probably due to activation of NAD-IDH) followed by a slow oxidation due to the energy demand of phosphate accumulation. The experiment shown in Fig. 4A can best be interpreted as follows:-

(a) The initial oxidation of $NADH_2$ is due to interaction of phosphate and ADP

with the respiratory chain phosphorylation system in the conventional fashion, (b) the subsequent slow reduction is due to (i) partial exhaustion of ADP decreasing the activity of the respiratory chain and (ii) activation of NAD-IDH by the higher phosphate level which has been created inside the mitochondria, and (c) the final slow oxidation is due to (i) decrease of the NAD-IDH activity by lack of ADP and (ii) the energy-demand caused by phosphate accumulation.

ACKNOWLEDGEMENTS

We would like to thank the Medical Research Council for a research grant and the Science Research Council for financial support for R.G.H.

REFERENCES

Chance, B. & Williams, G.R. (1955) J. biol. Chem. 217, 409.
Chappell, J.B. (1964) Biochem. J. 90, 225.
Goebell, H. & Klingenberg, M. (1964) Biochem. Z. 340, 441.
Hansford, R.G. & Chappell. J.B. (1967) Biochem. biophys. res. Comm. 27, 686
Sacktor, B. & Hurlbut, E.C. (1966) J. biol. Chem. 241, 632.