ROLE OF Ca^{2+} IN THE OXIDATION OF EXOGENOUS NADH BY PLANT MITOCHONDRIA

J.O.D. COLEMAN and J.M. PALMER*
Department of Botany, University of London, King's College
68 Half Moon Lane, London SE24 9JF, England

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1. Introduction

Intact plant mitochondria readily oxidise externally added, reduced nicotinamide-adenine dinucleotide (NADH) [1–5]. This oxidation has been shown to be insensitive to inhibition by rotenone, amytal, and piericidin A [5–7], and shows respiratory control by oxidative phosphorylation, yielding a P/O ratio $\simeq 2$ [3–5, 7].

In contrast, oxidation of NADH, generated endogenously by NAD-linked dehydrogenases, was markedly sensitive to inhibition by rotenone, amytal and piericidin A, and was coupled to phosphorylation with a P/O ratio $\simeq 3$ [4–7].

From recent studies [5], it has been suggested that the inner membrane of plant mitochondria was impermeable to NADH, and that oxidation of exogenous NADH took place via a dehydrogenase located on the outer surface of the inner membrane, and which was connected to the cytochrome chain after bypassing the first site of phosphorylation and as a result missing the sites of inhibition of rotenone and piericidin A.

Earlier studies [1, 8] revealed that the oxidation of NADH by plant mitochondria was stimulated by divalent cations. Recently Miller et al. [9] have shown Ca^{2+} and Sr^{2+} to be the most effective, and that stimulation was limited to the oxidation of exogenous NADH and could not be observed when malate-pyrvuvate or succinate were used as electron donors.

* Present address: Department of Botany, Imperial College, Prince Consort Road, London SW 7, England

Interpretation of the observed cation effects was based on the assumption that NADH oxidation occurred on the inside of the mitochondria and in the absence of added cation the rate of NADH oxidation was limited by a slow rate of entry of NADH into the mitochondria. Hackett [1] suggested a possible action of the cation was to increase the permeability of the mitochondria to NADH. Miller et al. [9] concluded that the cation could act by activating a specific NADH transport system located in the mitochondrial membrane. Alternatively, it was suggested that the cation could cause the release of a rate-limiting step in the electron-transport pathway involved in oxidation of NADH [1, 9].

In view of the suggestion that exogenous NADH is oxidised via a dehydrogenase located on the outer face of the inner membrane [5], this study was initiated to investigate the effect of divalent cations on the oxidation of NADH via this external pathway.

It is shown that the oxidation of NADH by Jerusalem artichoke mitochondria was specifically and markedly inhibited by ethylene glycol bis(β-aminoethyl)-*N,N*^{1} tetraacetic acid (EGTA). This inhibition could be reversed by adding Ca^{2+}, Sr^{2+} or Mn^{2+} in a manner which suggested that there is a divalent cation requirement for the oxidation of exogenous NADH.

The site of cation action was found to be between NADH dehydrogenase and cytochrome *b*. A possible role of Ca^{2+} has been suggested as part of a mechanism which controls the oxidation of exogenous NADH by plant mitochondria, and as a result can regulate the cytosolic NAD^+/NADH ratio.

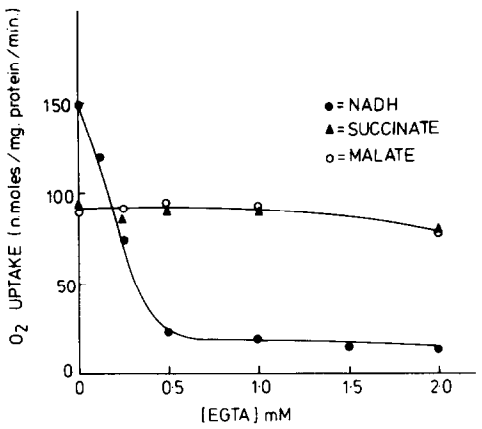

Fig. 1. The effect of EGTA on the oxidation of NADH 1 mM, malate 100 mM, and succinate 100 mM. Oxygen uptake was measured in an assay medium containing 1 mM ADP and 25 mM KH_2PO_4.

2. Methods

Mitochondria were prepared from tubers of the Jerusalem Artichoke (*Helianthus tuberosus*) by the method of Palmer [10], using an extracting solution consisting of sucrose 0.5 M, *N*-tris(hydroxymethyl)-methyl-2-aminoethane sulphonic acid (TES) 5 mM, EGTA 1 mM, bovine serum albumin (BSA) 0.1%, at pH 8.0.

Oxygen uptake was measured in a Gilson "Oxygraph" oxygen electrode, using a standard assay medium containing sucrose 0.4 M, TES 5 mM, BSA 0.1%, at pH 7.4. Approximately 2 mg mitochondrial protein were added, other additions are as indicated in legends to figures. The total reaction volume was 1 ml and the temperature 25°.

ADP was assayed by the method of Adam [11] using the enzymes supplied by Boehringer Corporation Ltd.

NADH was determined enzymatically according to Klingenberg [12]. Protein estimation was carried out using biuret reagent, after solubilisation in deoxycholate [13].

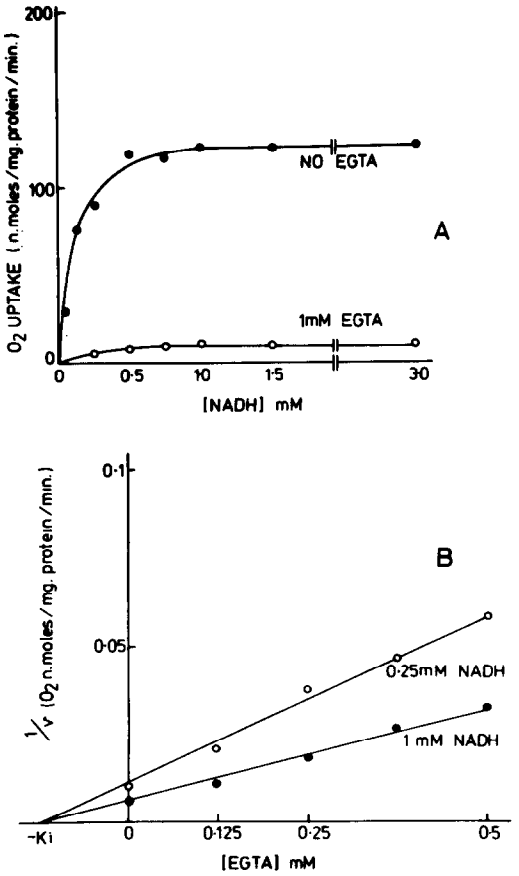

Fig. 2. A: The effect of NADH concentration on the rate of oxidation, in the presence and absence of EGTA. ADP 1 mM and KH_2PO_4 25 mM were present. B: Dixon plots of the kinetics of NADH oxidation in the presence of EGTA.

3. Results

3.1. *Inhibition of NADH oxidation by EGTA*

Mitochondria, isolated from Jerusalem artichoke tubers, rapidly oxidised externally added NADH. The oxidation was insensitive to rotenone and to piericidin A, and linked to phosphorylation with a P/O $\simeq$ 2. These observations confirmed previous findings [5].

However, it was found that when EGTA was present in the assay medium, the rate of oxidation of NADH was severely inhibited, 1 mM EGTA causing almost complete inhibition (fig. 1). The inhibition was specific for exogenous NADH, malate and succinate being unaffected.

Table 1

The concentration of chelator necessary to result in 50% inhibition of NADH oxidation.

Compound	I_{50} (mM)
Ethyleneglycol bis (β-aminoethyl)-*N*,N^1-tetraacetic acid (EGTA)	0.25
Ethylenediamine tetraacetic acid (EDTA)	2.0
N-hydroxyethylenediamine triacetic acid (HEDTA)	1.5
Diethylenetriamine penta-acetic acid (DPTA)	1.5

The concentrations of chelator causing 50% inhibition (I_{50}) were calculated by plotting the rate of oxygen uptake against chelator concentration. The protein concentration was the same in all cases. Oxygen uptake was stimulated by FCCP 10 nmole, with NADH 1 μmole as substrate.

The inhibition was independent of NAD concentration as shown in fig. 2A. A Dixon plot (fig. 2B) confirms the inhibition to be non-competitive with respect to NADH, with a K_i of 0.125 mM, thus indicating inhibition of NADH oxidation by EGTA does not involve alteration of the apparent K_m for NADH.

3.2. *Effects of other chelating compounds*

In an attempt to determine whether the inhibition was specific for EGTA, other known chelators analogous to EGTA were tested. Table 1 shows the relative effectiveness of EGTA, ethylenediamine tetraacetic acid (EDTA), *N*-hydroxyethylenediamine triacetic acid (HEDTA), and diethylenetriamine penta-acetic acid (DPTA). The results are presented as the concentration of chelator to produce 50% inhibition of the *p*-trifluoromethoxyphenylhydrazone (FCCP) stimulated rate of oxygen uptake.

The results show that although EGTA was by far the most effective, EDTA, HEDTA, and DPTA would also cause inhibition, but at significantly higher concentrations.

3.3. *Reversal of EGTA inhibition by divalent cations*

It was found that if Ca^{2+} ions were added after EGTA inhibition, the original oxidative activity could be restored. Fig. 3A shows concentration curves for the Ca^{2+} reactivation of EGTA-inhibited

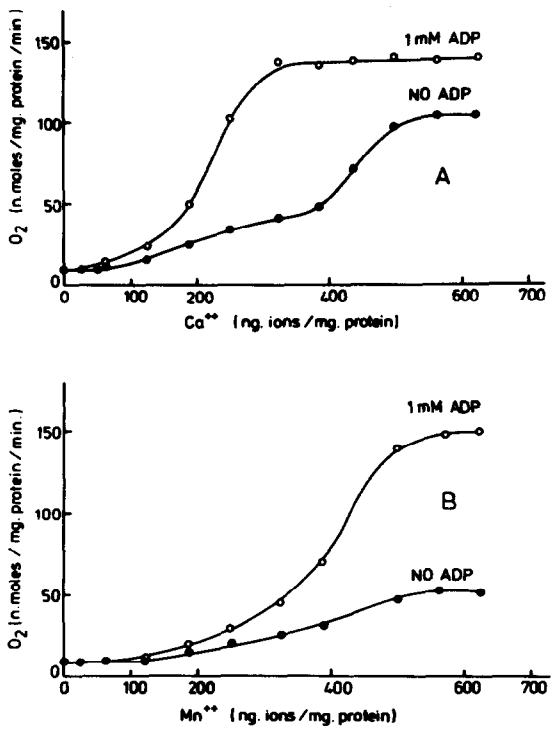

Fig. 3. The effects of divalent cations on the oxidation of NADH in mitochondria in an assay medium containing 1 mM EGTA. KH_2PO_4, 25 mM was also present in the assay medium. A: The effects of Ca^{2+}. B: The effect of Mn^{2+}.

NADH oxidation. From these graphs it is apparent that two types of Ca^{2+} effects can be distinguished. The first effect is obtained around 300 ng ions/mg protein, at this level of Ca^{2+}, there was complete restoration of the original NADH oxidative activity, respiration could be stimulated by ADP giving rates of oxygen uptake, ADP:O ratios and respiratory control ratios equal to those obtained with untreated mitochondria (see fig. 4).

At concentrations of Ca^{2+} in excess of 400 ng ions/mg protein, there is an additional stimulation of respiration by Ca^{2+}. Under these conditions there is little or no respiratory control (see fig. 4(d)), this may be accounted for by the active accumulation of Ca^{2+}, uncoupling oxidative phosphorylation.

A similar biphasic effect was obtained when Sr^{2+} was used, over the same concentration range.

Mn^{2+} (fig. 3B), shows only one effect corresponding to the effect produced by low levels of Ca^{2+} or

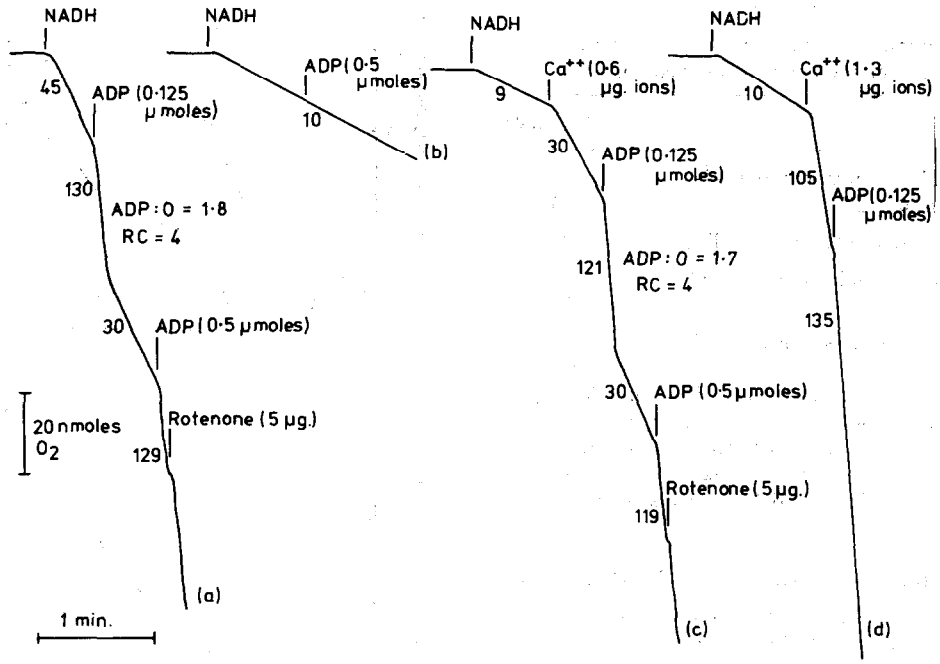

Fig. 4. Oxygen electrode traces of NADH (1 mM) oxidation, showing the inhibitory effects of EGTA and reversal of the inhibition by Ca^{2+}. (a) Mitochondria in standard assay medium. (b) (c) and (d) Mitochondria in assay medium containing 1 mM EGTA.. Numerals under traces represent the rate of oxygen uptake in nmole of oxygen/mg protein/min.

Sr^{2+}. However the Mn^{2+} concentration required was twice that of Ca^{2+}. No reactivation could be obtained with Mg^{2+} over a similar concentration range.

Fig. 4(a) shows an oxygen electrode trace of NADH oxidation in untreated mitochondria, showing an ADP: $O \simeq 2$, and insensitivity to rotenone, at a concentration in excess of that required to cause complete inhibition of oxidation of endogenously produced NADH [5]. Fig. 4(b) shows the low rates of NADH oxidation when mitochondria were treated with 1 mM EGTA. Fig. 4(c) shows restoration of NADH oxidation in EGTA treated mitochondria with low levels of Ca^{2+}, with rates of oxidation, ADP:O ratios, respiratory control values, and insensitivity to rotenone, as shown in untreated mitochondria. Fig. 4(d) shows the lack of respiratory control obtained when higher levels of Ca^{2+} were added.

From these results it is evident that the pathway of NADH oxidation after EGTA-Ca^{2+} treatment, has similar characteristics to the pathway of NADH oxidation in untreated mitochondria. This indicates that the reversal of EGTA inhibition by Ca^{2+} does not involve an increase in permeability of the mitochondria to NADH, and thus permitting oxidation on the internal dehydrogenase but rather the reactivation of EGTA-inhibited oxidation of NADH via the externally located dehydrogenase.

3.4. *The locus of divalent cation action*

Direct assay of the NADH dehydrogenase followed by the reduction of ferricyanide as described in [5], showed NADH-ferricyanide reductase activity to be insensitive to EGTA. Spectrophotometric studies of the electron transport chain components were therefore carried out to determine the site of divalent cation action.

Fig. 5 demonstrates the change in steady state reduction of cytochrome *b*, as measured by absorption of its Soret band at 434 nm, using 415 nm as a reference of wavelength. Fig. 5(b) shows that addition of NADH to untreated mitochondria caused a partial reduction of cytochrome *b*, addition of Ca^{2+}

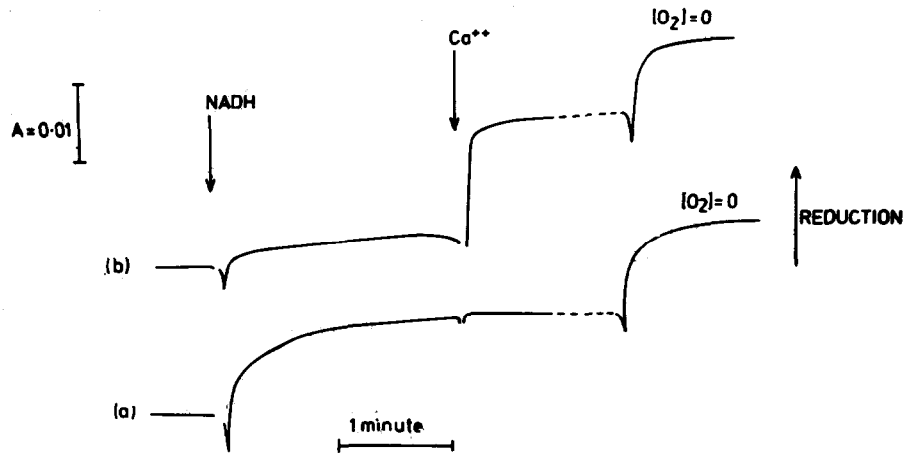

Fig. 5. Steady state levels of cytochrome *b* reduction of mitochondria in (a) the absence of EGTA and (b) the presence of 1 mM EGTA. 2 mg of mitochondrial protein were suspended in assay medium 2.5 ml, and measurements were carried out using the wavelength pair 434 and 415 nm, in a dual wavelength spectrophotometer. Additions were NADH 2 μmole, Ca^{2+} 600 g ions.

produced no change in this steady state level of reduction, finally complete reduction of *b* was observed at the onset of anaerobiosis. Fig. 5(a) shows that the addition of NADH to mitochondria treated with EGTA, the steady state level of reduction of cytochrome *b* was significantly less than that seen in untreated mitochondria. Addition of Ca^{2+} to these EGTA-treated mitochondria caused a further reduction of cytochrome *b*, and finally complete reduction with anaerobiosis.

4. Discussion

The finding that in plant mitochondria the oxidation of exogenous NADH is specifically inhibited by chelators of divalent cations, and the complete reversal of this inhibition by Ca^{2+}, Sr^{2+} and Mn^{2+} suggests there is a divalent cation requirement for the oxidation of exogenous NADH, a requirement which is not shared by endogenously produced NADH.

Spectrophotometric analysis suggests that the locus of cation action appears to be on the dehydrogenase complex at a point between the site of NADH-ferricyanide reduction and a cytochrome *b* component of the electron transport chain.

The physical localisation of the NADH dehydrogenase on the outer surface of the inner membrane may represent a physiological mechanism whereby reducing equivalents originating in the cytosol are made available to the respiratory chain. This localisation also allows the dehydrogenase to respond to changes of Ca^{2+} concentration in the intermembrane space, which is probably in equilibrium with the cytosol. Thus NADH dehydrogenase activity and hence the cytosolic $NAD^+/NADH$ ratio could be controlled by the Ca^{2+} levels of the cytosol.

The findings reported here have also been observed in mitochondria from other plant sources. It has also been observed that associated with the external NADH dehydrogenase in the outer mitochondrial compartment is a NAD-linked L-malate oxidoreductase (decarboxylating) "malic enzyme", EC 1.1.1.39 [14].

The localisation of these enzymes, and their divalent ion requirements, could be part of a mechanism regulating the transport and utilisation of reducing equivalents between mitochondria and cytosol, a mechanism corresponding in principle to the proposed "shuttle systems" of animal cells.

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