THE MECHANISM OF K^+-STIMULATED EXOGENOUS NADH OXIDATION IN PLANT MITOCHONDRIA

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1. Introduction

Early work by Hackett [1] showed that the oxidation of extra-mitochondrial reduced nicotinamide adenine dinucleotide (NADH) by plant mitochondria was stimulated by high concentrations of monovalent cation salts (10–100 mM) and lower concentrations of divalent cation salts (1–10 mM). It was suggested that salt solutions increased the permeability of the mitochondria to NADH which was assumed to be oxidised internally as in mammalian mitochondria [2].

Further work has concentrated exclusively on the divalent cation stimulation which is specific to exogenous NADH and not found with succinate or malate-pyruvate [3]. Coleman and Palmer [4] have shown that the oxidation of exogenous NADH is non-competitively inhibited by ethylene glycol bis(β-aminoethyl)-*N,N*^{1} tetraacetic acid (EGTA). The inhibition could be reversed by Ca^{2+}, Sr^{2+} or Mn^{2+} and a specific site of divalent cation action was located on the respiratory chain between NADH dehydrogenase and cytochrome *b*.

Recent evidence [5,6] suggesting that extra-mitochondrial NADH oxidation occurs via a dehydrogenase located on the outer surface of the inner membrane necessitates a re-examination of the monovalent cation stimulation.

The results presented suggest that K^+ acts by releasing membrane-bound endogenous Ca^{2+} thus increasing Ca^{2+} activity at the divalent cation-stimulated site of exogenous NADH oxidation.

2. Methods

Mitochondria were isolated from 6-day-old etiolated shoots of corn (*Zea mays* L. var. Kelvedon Glory) using the basic procedure of Kenefick and Hanson [7]. The isolation medium contained 400 mM sucrose, 50 mM *N*-tris(hydroxymethyl)-methyl-2-aminoethane sulphonic acid (TES), 5 mM ethylenediamine tetraacetic acid (EDTA), adjusted to pH 7.6 with tris(hydroxymethyl)aminomethane (Tris). The washing medium consisted of 400 mM sucrose, 10 mM TES, 1 mM EDTA, adjusted to pH 7.6 with Tris.

The standard assay medium contained 200 mosM sucrose–KCl, 20 mM tris(hydroxymethyl)methylglycine (Tricine)–Tris (pH 7.5), 25 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCP), 1 mg/ml bovine serum albumin (BSA). The reaction vol was 3.1 ml containing approximately 0.2 mg mitochondrial protein/ml and the temperature 25°C. Mitochondria were incubated for 3 min before the addition of exogenous NADH (1 mM).

Oxygen uptake was measured using a Clark oxygen electrode. Metabolism-independent binding of externally added Ca^{2+} was determined in the presence of $^{45}CaCl_2$ in the assay medium following a 5 min incubation period in the presence of NADH. The mitochondria were collected by Millipore filtration and $^{45}Ca^{2+}$ determined as described previously [8]. External and internal Ca^{2+} binding were determined by incubating an additional sample for 1 min with a four-fold excess of EGTA prior to

filtration [9]. Endogenous mitochondrial Ca^{2+} was determined using an EEL 240 atomic absorption spectrophotometer. Mitochondrial protein was estimated using the procedure of Lowry et al. [10] with BSA as a standard.

3. Results and discussion

The corn mitochondria used in these experiments, when placed in a standard respiratory control assay medium, oxidised extra-mitochondrial NADH with respiratory control ratios of 3–4 and ADP:0 ratios of 1.6–1.8 (data not shown). These values are typical for plant mitochondria [4,6].

The addition of KCl to an inorganic cation-free assay medium produced an approximate two-fold stimulation of NADH oxidation with a K_M for KCl of 20 mM (fig.1). Incorporation of EGTA in the assay medium only partially inhibited NADH oxidation in the absence of KCl but completely inhibited KCl-stimulated oxidation.

Fig.2A shows that, in the presence of 50 mM KCl and 1 mM EGTA, complete recovery of NADH oxidation occurred at an approximate Ca^{2+} concentra-

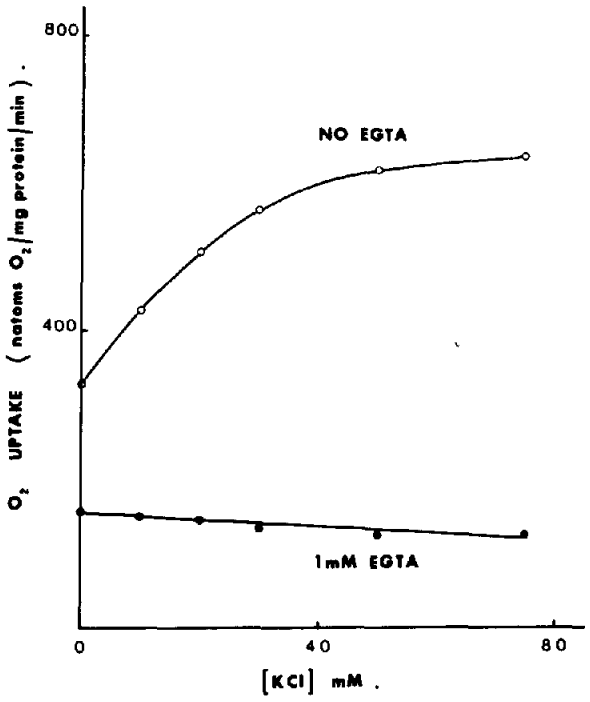

Fig.1. The effect of KCl on NADH oxidation, in the presence and absence of EGTA.

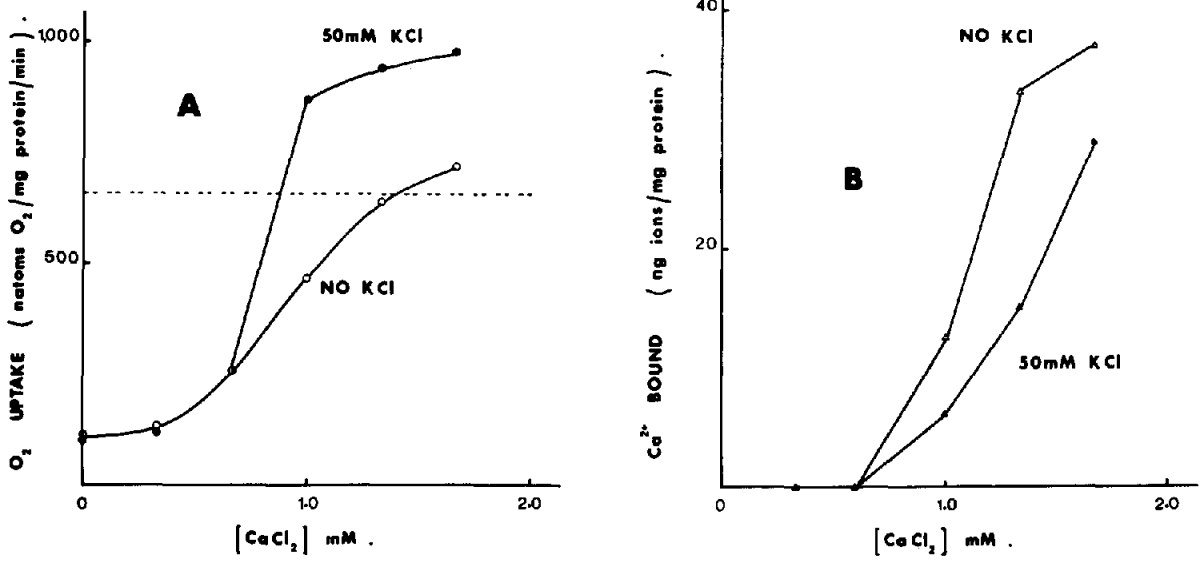

Fig.2. (A) The effect of KCl on Ca^{2+}-stimulated NADH oxidation in the presence of 1 mM EGTA. The dotted line represents NADH oxidation in an assay medium containing 50 mM KCl in the absence of both EGTA and Ca^{2+}. (B) The effect of KCl on metabolism-independent Ca^{2+}-binding in the same experiment.

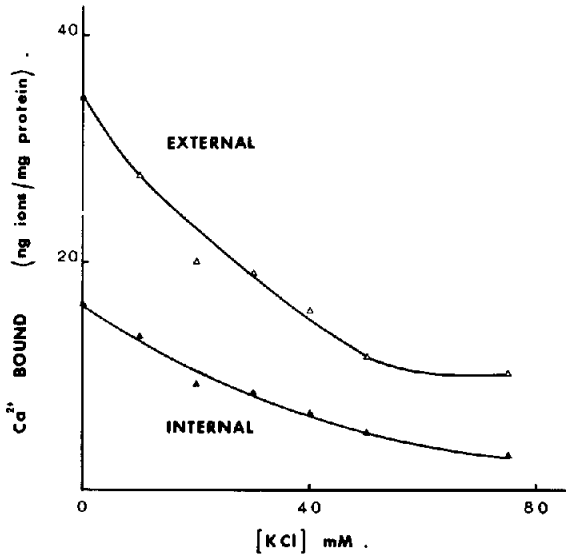

Fig.3. The effect of KCl on external and internal Ca^{2+}-binding in the absence of EGTA. $^{45}CaCl_2$ 0.5 mM was present.

tion of 0.9 mM. However, in the absence of KCl, approximately 1.4 mM Ca^{2+} was required to attain the same rate. Metabolism-independent Ca^{2+} binding was not detected at Ca^{2+} concentrations < 0.6 mM but, at higher Ca^{2+} concentrations, Ca^{2+} binding was markedly inhibited by KCl (fig.2B). Apparently, the promotion by K^+ of Ca^{2+}-activated NADH oxidation in EGTA-treated mitochondria is associated with reduced Ca^{2+} binding.

Fig.3 shows that corn mitochondria, in the absence of KCl, bound externally added Ca^{2+} in both the outer and inner mitochondrial spaces. Approximately 70% of the total CCP-insensitive Ca^{2+} binding was external and 30% internal. Addition of KCl strongly inhibited Ca^{2+} binding in both mitochondrial spaces. A similar inhibition of external Ca^{2+} binding by K^+ occurs in rat liver mitochondria but internal Ca^{2+} binding is inhibited by K^+ only in the presence of valinomycin or gramicidin [11,12]. However, corn mitochondria are passively permeable to external K^+ in the absence of an ionophore [13] and internal Ca^{2+} binding is presumably inhibited by spontaneously penetrating K^+ (fig.3). Work with submitochondrial particles has shown that K^+ and Ca^{2+} bind competitively to phospholipid binding sites [14].

The location of the activating Ca^{2+} is obviously crucial to understanding the promotion by K^+ of Ca^{2+}-activated NADH oxidation (fig.2A). Fig.4A shows that a late addition of EGTA, a non-penetrant with virtually instantaneous quenching of external Ca^{2+} [9], only slightly inhibited Ca^{2+}-activated NADH oxidation. The promotion of NADH oxidation by K^+ could, therefore, involve displacement of internal membrane-bound Ca^{2+} leading to an increase in free internal Ca^{2+} and resulting in activation of the external NADH dehydrogenase.

A similar late addition of EGTA had little effect on K^+-stimulated NADH oxidation in the absence of added Ca^{2+} (fig.4B). Since the prior addition of EGTA prevents K^+-stimulated NADH oxidation (fig.1), it seems likely that the effect of K^+ involves the participation of the externally-bound component of endogenous Ca^{2+} (12 ng ions Ca^{2+}/mg protein). Accordingly, it is suggested that K^+-stimulated NADH

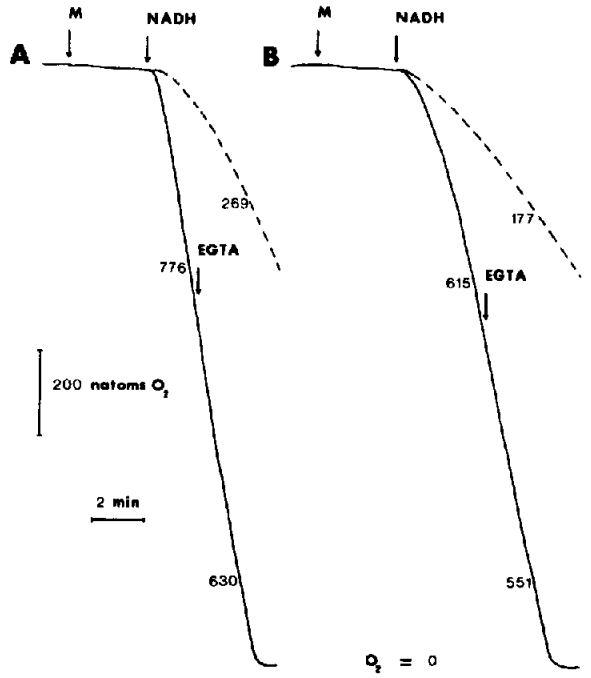

Fig.4. The effect of time of addition of EGTA (3 mM) on NADH oxidation in the presence of; (A) 50 mM KCl, 1 mM $CaCl_2$, 1 mM EGTA. (B) 50 mM KCl. Dotted lines represent EGTA in the assay medium at the start of the experiment. Numerals refer to the rate of oxygen uptake in natoms O_2/mg protein/min.

oxidation is a result of: (a) displacement of external membrane-bound endogenous Ca^{2+} by K^+ and (b) inward movement of this displaced endogenous Ca^{2+} across the inner membrane, as well as, (c) enhancement of free internal endogenous Ca^{2+} by occupation of the internal cation binding sites by penetrating K^+.

It is of interest that mobilisation of endogenous membrane-bound Ca^{2+} is thought to account for the inhibition by halothane of NAD^+-linked State 3-(or uncoupler-)stimulated respiration in mammalian mitochondria [15]. However, in this case, the release of Ca^{2+} results in inhibition of endogenous NADH oxidation probably by binding to the internal NADH dehydrogenase.

The proposal that free internal Ca^{2+} controls the rate of exogenous NADH oxidation in plant mitochondria is apparently similar in principle to the activation by Ca^{2+} and Mg^{2+} of intramitochondrial malic enzyme in adrenal cortex mitochondria [16]. In addition, this paper suggests that the concentration of free internal Ca^{2+} is a function of both transport of external cations across the inner membrane and interaction between intramitochondrial cations and their internal phospholipid binding sites.

References

[1] Hackett, D. P. (1961) Plant Physiol. 36, 445–452.
[2] Lenninger, A. L. (1951) J. Biol. Chem. 190, 345–359.
[3] Miller, R. J., Dumford, S. W., Koeppe, D. E. and Hanson, J. B. (1970) Plant Physiol. 45, 649–653.
[4] Coleman, J. O. D. and Palmer, J. M. (1971) FEBS Lett. 17, 203–208.
[5] Palmer, J. M. and Passam, H. C. (1970) Biochem. J. 122, 16P.
[6] Douce, R., Mannella, C. A. and Bonner, W. D. (1973) Biochim. Biophys. Acta 292, 105–116.
[7] Kenefick, D. G. and Hanson, J. B. (1966) Plant Physiol. 41, 1601–1609.
[8] Earnshaw, M. J., Madden, D. M. and Hanson, J. B. (1973) J. Exp. Bot. 24, 828–840.
[9] Reed, K. C. and Bygrave, F. L. (1974) Biochem. J. 140, 143–155.
[10] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, H. J. (1951) J. Biol. Chem. 193, 265–275.
[11] Scarpa, A. and Azzone, G. F. (1968) J. Biol. Chem. 243, 5132–5138.
[12] Rossi, C., Azzi, A. and Azzone, G. F. (1967) J. Biol. Chem. 242, 951–957.
[13] Kirk, B. I. and Hanson, J. B. (1973) Plant Physiol. 51, 357–362.
[14] Scarpa, A. and Azzi, A. (1968) Biochim. Biophys. Acta 150, 473–481.
[15] Grist, E. M. and Baum, H. (1974) FEBS Lett. 48, 41–44.
[16] Pfeiffer, D. R. and Tchen, T. T. (1975) Biochemistry 14, 89–96.