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THE EFFECT OF CALCIUM ON THE RESPIRATORY RESPONSES OF MUNG BEAN MITOCHONDRIA

ANTHONY L. MOORE and WALTER D. BONNER Jr.

Johnson Research Foundation, Department of Biochemistry and Biophysics, University of Pennsylvania, Philadelphia, Pa. 19174 (U.S.A.)

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SUMMARY

Purified mung bean hypocotyl mitochondria were examined for their capacity to carry out respiration-dependent accumulation of calcium. The addition of 0.1–1.0 mM calcium to mung bean mitochondria supplemented with succinate gave no stimulation of state 4 respiration even in the presence of inorganic phosphate and the ionophoretic antibiotic A-23187. Even at high calcium concentrations, no transient changes in the respiratory activity occurred and subsequent addition of ADP initiated a further state 3 response. Although the additions of calcium resulted in a rapid H^+ ejection, it was insensitive to lanthanum and uncoupling agents. Similarly, additions of calcium failed to initiate any transient changes in the oxidation-reduction states of either pyridine nucleotides or cytochrome *b*. Direct spectrophotometric recordings of absorbance changes of murexide revealed no respiration-linked calcium transport. It is proposed that although mung bean mitochondria possess a respiration-linked electrochemical potential gradient it would appear that this potential cannot be expressed as calcium transport even at high ion concentrations, probably due to a low calcium membrane permeability.

INTRODUCTION

The mechanism by which Ca^{2+} and other divalent cations are accumulated by mammalian mitochondria has been studied extensively [1–4]. It is well established that the accumulation of Ca^{2+} by animal mitochondria is an energy-dependent process, in which the energy can be derived from electron transport or ATP hydrolysis [1–4]. The addition of Ca^{2+} to isolated mammalian mitochondria which oxidize endogenous or added substrates in phosphate-supplemented media is accompanied by a stimulation of electron transport, the steady states of the respiratory carriers jump to new and characteristic oxidation-reduction levels, light scattering changes are initiated, and rapid H^+ ejection occurs [1]. More recently attention has been focused

Abbreviations: RC, respiratory control; 1799, bis-(hexafluoroacetonyl)acetone.

on the existence of a Ca^{2+} carrier localized in the inner membrane of the mitochondria. The Ca^{2+} binding properties of the proposed carrier [5], its inhibition by lanthanides [6], its kinetic behavior [7] and the nature of the driving force have been studied in detail [8]. It would therefore appear that according to the considerable literature available from mammalian studies that the active accumulation of Ca^{2+} by mammalian mitochondria is intimately associated with and characterized by an extremely high affinity for Ca^{2+}. Even in the group of organelles which exhibit a low affinity for Ca^{2+} such as yeast mitochondria [9], distinct changes in steady-states levels of respiratory carriers have been detected, provided the ion concentration is sufficient to cause some movement across the membrane. Notable in this group are plant mitochondria. It has been reported that mung bean mitochondria demonstrate a lack of Ca^{2+} stimulation of respiration [10]. The comprehensive studies of Hanson and his colleagues [11–17], however, suggest that plant mitochondria do actively accumulate Ca^{2+}, and that this accumulation is seen as a secondary event arising via the formation of a phosphorylated intermediate of oxidative phosphorylation. Chen and Lehninger [20] have also indicated that several plant species do accumulate Ca^{2+} during respiration. Most of these studies of in vitro mitochondrial Ca^{2+} transport have, however, involved incubation of mitochondria with isotopically labelled Ca^{2+} and subsequent analysis of Ca^{2+} uptake after centrifugation or filtration. Although they have provided a substantial amount of information on Ca^{2+} accumulation in plant mitochondria very little data has been presented as to the effect of Ca^{2+} on the respiratory carriers. Since it is well documented that the response of the respiratory carriers to calcium is a very sensitive indicator of the interaction of Ca^{2+} with the respiratory chain [1] and in view of the suggestion that the transport of Ca^{2+} may be an alternative to oxidative phosphorylation [21], it is particularly important to investigate this response.

A detailed study has, therefore, been carried out on the effect of calcium on the respiratory responses of purified plant mitochondria. In this study we have not considered the dependency of exogenous NADH oxidation on divalent cations, explanations for which are varied and well documented [16–19]. The results presented in this report demonstrate important differences with respect to the parameters of active calcium transport usually observed with vertebrate mitochondria. In constrast to mammalian mitochondria and previous reports on plant mitochondria [1, 11–16] the results of these studies are interpreted as an indication that calcium does not interact with the electron transport chain of purified mung bean hypocotyl mitochondria probably due to a low permeability of the inner membrane to calcium.

Preliminary results of these studies have been presented orally [22].

MATERIALS AND METHODS

Plant material. The plant material used in these experiments was etiolated mung bean hypocotyls (*Phaseolus aureus*). Bean seedlings were grown for 5 days in a dark room maintained at 28 °C and 60 % relative humidity.

Preparation of mitochondria. Mitochondria were prepared as described by Douce et al. [23]. The plant tissue was homogenized for 15 s with a Moulinex mixer 66 (Alençon, France). The mitochondria were then separated from broken cell material and nuclei by filtration through a fine cloth followed by two cycles of differen-

tial centrifugation and were purified further by sedimentation to equilibrium on discontinuous sucrose gradients (SW 25.1 rotor, 20K, 1 h). Isolation media contained 1 mM K_2 EDTA. Mitochondria prepared in this manner, in addition to being highly purified, were also tightly coupled; average ADP : O ratios for malate and succinate were 2.6 and 1.6, respectively, and RC ratios for the same substrates were approx. 5 and 3–4. Integrity assays of the inner and outer membranes were evaluated as indicated by Douce et al. [23].

Reaction media. Mitochondrial reactions and spectrophotometric measurements were carried out in 0.3 M mannitol, 5 mM $MgCl_2$, 10 mM KCl and 5 mM potassium phosphate buffer (pH 7.2).

Analytical and spectral measurements. Mitochondrial respiration was measured polarographically using a Clark oxygen electrode (Yellow Springs Instruments Co., Cleveland, Ohio). Measurements were carried out in a closed Lucite cuvette at 25 °C. Mitochondria were added to 3.0 ml of the reaction media with 10 mM sodium succinate as respiratory substrate. The sequence of additions and the concentration of additional reagents are indicated in the legends.

Simultaneous measurements of changes in pH and oxygen consumption were performed in a closed lucite cuvette at 25 °C, a Beckman Expandomatic pH meter with a combination pH electrode connected to a 10 mV strip chart recorder being used. The H^+ ejection in each experiment was determined quantitatively by back titration of the reaction mixture with standard NaOH or HCl.

Spectrophotometric measurements were carried out with a dual wavelength spectrophotometer (Johnson Research Foundation) as described by Chance [24]. The dual wavelength instrument was used to determine the time course and extent of reduction and oxidation of cytochrome *b*-566 (566–575 nm). Experiments were conducted without stirring in a 3.0 ml open cuvette with a 1.0 cm light path. Minimum time for achieving complete anaerobiosis was approx. 5 min for all conditions.

Pyridine nucleotide fluorescence was observed in an Eppendorf fluorimeter with excitation at 366 nm and a 400–3000 nm guard filter in front of the photomultiplier. Light scattering measurements were made in a Zeis PMQ II spectrophotometer at 520 nm.

Ca^{2+} uptake was measured directly by the murexide technique, by recording the absorption changes of the metallo-chromic indicator murexide through a dual wavelength spectrophotometer (540–507 nm). In the presence of murexide, Ca^{2+} uptake by mitochondria (or Ca^{2+} binding by chelating agents) produces an increase in light absorbance at 540 nm by reducing the concentration of Ca^{2+} available for formation of calcium · murexide complex. Free murexide absorbs more strongly than the complex [25, 26].

Protein determination. Protein was determined by the method of Lowry et al. [27] using crystalline bovine serum albumin (Miles Labs) as the standard.

Reagents. All reagents were of the highest grade available commercially. Antimycin A (Calbiochem), oligomycin (Sigma), bis-(hexafluoroacetonyl)acetone (1799) (obtained from Dr. P. Heytler, E. I. DuPont de Nemours Co., Wilmington, Del.) and A-23187 (donated by Dr. A. Scarpa, Johnson Foundation, University of Pennsylvania) were dissolved in absolute ethanol. All other reagents were dissolved in distilled water and adjusted to pH 7.2.

RESULTS

Fig. 1A shows typical changes in the mitochondrial respiratory activity upon additions of low concentrations of ADP. In this case, purified mung bean hypocotyl mitochondria supplemented with succinate as substrate are employed. It is evident from Fig. 1A that plant mitochondria purified by the sucrose density gradient technique [23] showed good capacity for oxidative phosphorylation and were tightly coupled (see Materials and Methods).

The addition of 100 μM $CaCl_2$ (Fig. 1B) did not evoke any change in the rate of oxygen consumption, and further addition of ADP initiated a state 3 response. Under conditions comparable to those employed here, addition of a small amount of Ca^{2+} to vertebrate mitochondria evokes a distinct jump in the respiratory rate with a stoichiometric return to state 4 after the accumulation of Ca^{2+} is complete [1]. Moreover, in most vertebrate mitochondria the respiratory rates observed during calcium-activated respiration are usually significantly higher than those obtained in the presence of ADP and phosphate [1]. When Ca^{2+} was added at much higher concentrations (Figs. 1C and 1D) (500 μM–1 mM) no transient changes in the respiratory activity occurred even in the presence of phosphate and Mg^{2+} (cf. ref. 20).

The lack of any Ca^{2+}-stimulated state 4 respiration was further demonstrated by the use of the calcium-specific ionophoretic antibiotic A-23187 [28, 29]. In contrast to its uncoupling effect [29] on mammalian mitochondria and as observed in Figs.

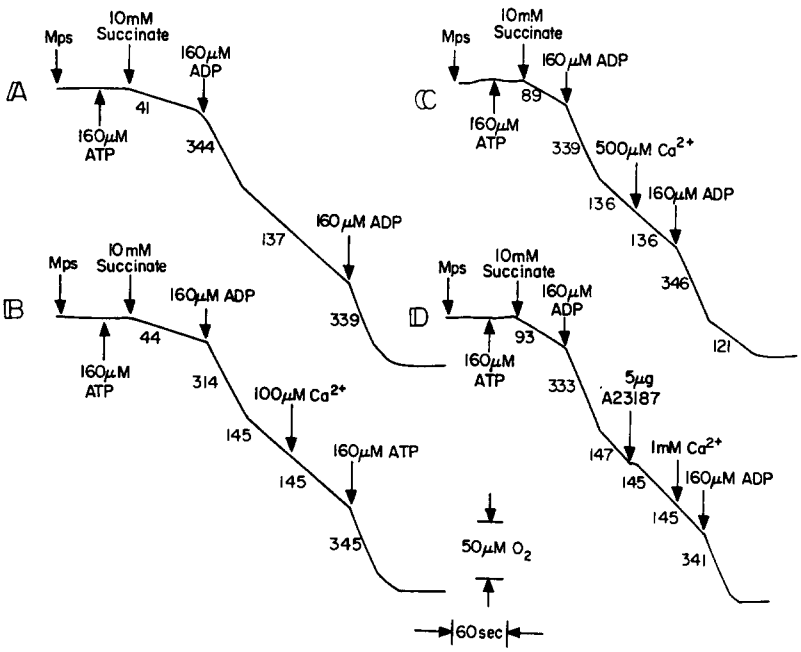

Fig. 1. Effect of calcium addition on the respiratory activity of purified mung bean mitochondria. The incubation medium (3.0 ml) contained 0.3 M mannitol, 10 mM potassium phosphate, 10 mM KCl, 5 mM $MgCl_2$ adjusted to pH 7.2 and purified mitochondria (Mps, 0.7 mg protein) at 25 °C. The rates of oxygen uptake are in nmol/min per mg protein.

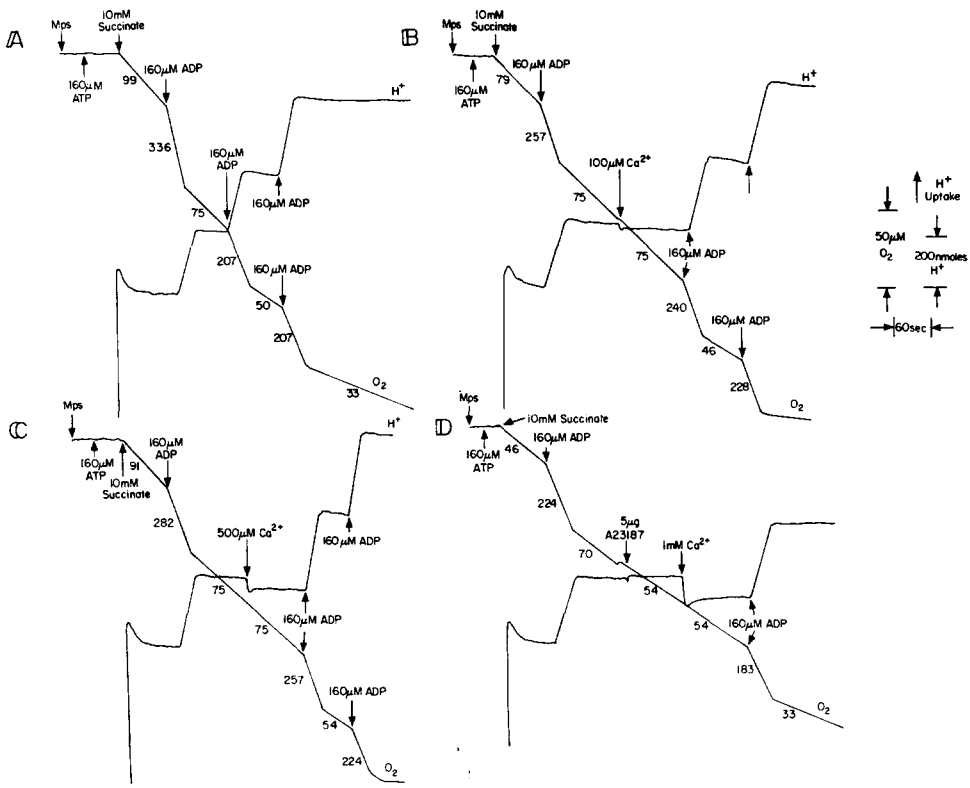

Fig. 2. Effect of calcium additions on H^+ movements. Experimental conditions were as described in Fig. 1. Purified mung bean mitochondria were added to 3.0 ml of the reaction media. Calibration of H^+ ejection was determined by back-titration with standard NaOH or HCl.

1A–1C, cyclic stimulation by ADP was not abolished.

It is well recognized in mammalian mitochondria, that rapid H^+ release accompanies the activation of respiration by calcium in a stoichiometric manner [1, 32]. Simultaneous measurements of oxygen and pH (Fig. 2) indicate an uptake of H^+ during the phosphorylation of ADP. The addition of low concentrations of Ca^{2+} (100 μM) resulted in a rapid H^+ ejection, but in contrast to mammalian mitochondria it did not appear to be synchronized with any stimulation of respiration nor were its extent and kinetics in any way comparable to the H^+ uptake observed during phosphorylation of ADP. Of special significance was the finding that the Ca^{2+}-induced H^+ ejection in no way interfered with either the extent or rate of any subsequent ADP-induced H^+ uptake. Again the addition of A-23187 (Fig. 2D) had no effect on the H^+ efflux associated with Ca^{2+} addition or on any subsequent ADP additions.

The traces in Fig. 3 reveal that the H^+ ejection observed upon Ca^{2+} addition is insensitive to uncoupling agents such as 1799. Although the addition of 1 μM 1799 released respiration from phosphorylation (marked by an increase in O_2 consumption), it had no effect on the Ca^{2+}-induced H^+ ejection. Similarly the H^+ ejection is also insensitive to 1 μM lanthanum chloride as further H^+ ejection is observed upon subsequent Ca^{2+} additions. The deflection of the pH electrode trace upon addition of

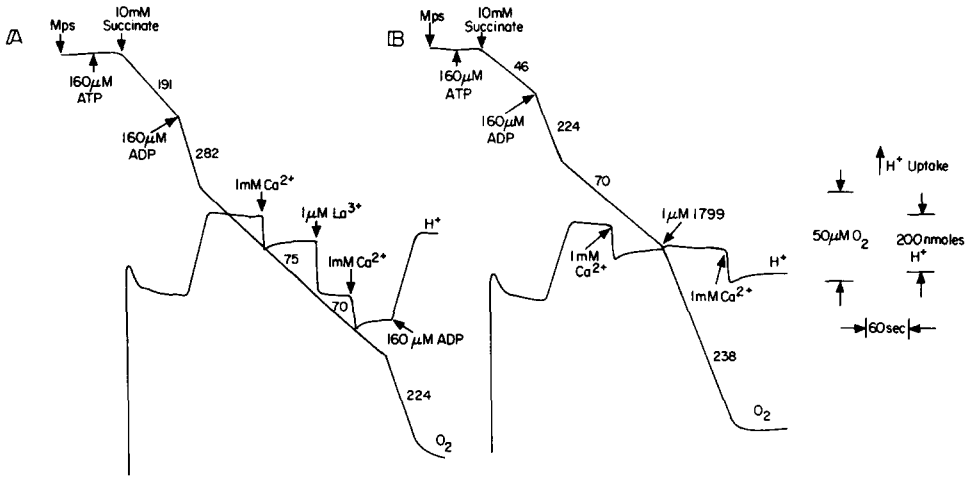

Fig. 3. Effect of Ca^{2+} on H^+ movements and its sensitivity to uncoupling agents and inhibitors. Reaction media as described in Fig. 1. Approx. 0.8 mg of mitochondrial protein was added. Calibration of H^+ ejection determined by back-titration with standard HCl or NaOH. Rates of oxygen uptake are in nmol/min per mg protein.

lanthanum chloride is due to an inequality of the pH of the solution and the reaction medium. Ruthenium red also does not affect the Ca^{2+}-induced H^+ ejection.

Since it is possible that transient changes in the respiratory carriers can occur in the absence of measurable changes in the respiratory rates [1], it was important to examine the response of the respiratory carrier steady states to Ca^{2+} addition. Active Ca^{2+} transport in animal mitochondria is reflected by rapid changes in the redox states of the carriers [1]. According to Vinogradov et al. [7] the fluorescence of mitochondrial pyridine nucleotides can be used as an intrinsic probe for the study of intramitochondrial divalent cation transport.

Fig. 4A shows a typical response of the pyridine nucleotides of purified mung bean mitochondria to ADP additions when succinate is the substrate. Addition of succinate results in an increase in fluorescence corresponding to a reduction of the pyridine nucleotides. Upon addition of ADP, the pyridine nucleotides are rapidly oxidized (respiratory rate in state 3) returning to the initial reduced state as ADP is exhausted.

In Figs. 4B–4D the second ADP addition is replaced by Ca^{2+}. In each of these cases no transient changes in the steady-state levels of the pyridine nucleotides can be detected, even with high Ca^{2+} concentrations and in the presence of 5 μg A-23187.

Similarly Ca^{2+}-induced oxidation of cytochrome *b* has been used as a sensitive indicator of Ca^{2+} interaction with the respiratory chain [1]. In the experiment illustrated in Fig. 5 changes in the oxidation-reduction states of cytochrome *b*-566 of purified mung bean mitochondria have been monitored. Upon addition of 10 mM succinate, cytochrome *b*-566 is rapidly reduced, ADP additions resulting in a rapid oxidation returning to the reduced state following ADP exhaustion. As observed in Fig. 4 it is readily apparent that additions of Ca^{2+} were without effect on the steady-state levels of cytochrome *b*-566.

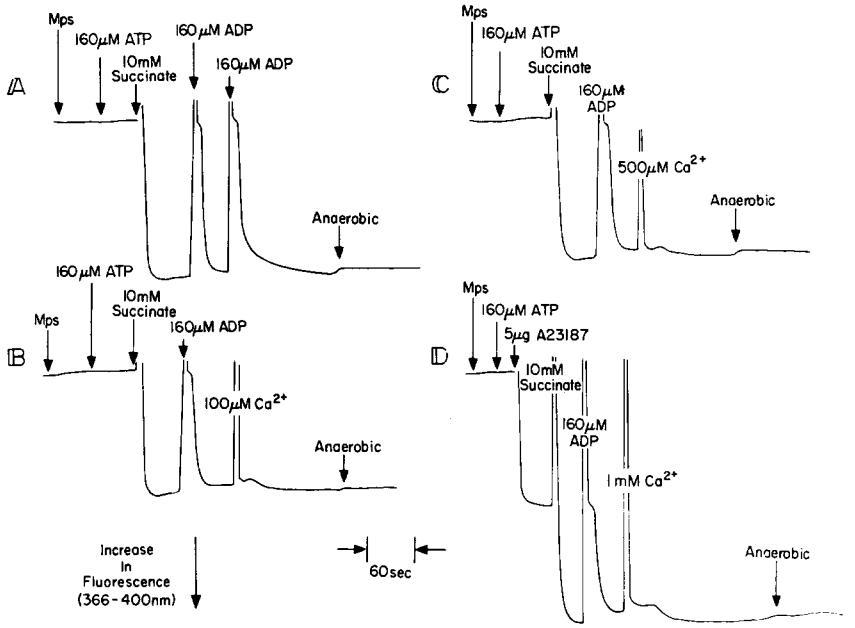

Fig. 4. Effect of Ca^{2+} on pyridine nucleotide fluorescence. Reaction medium as in Fig. 1. Fluorescence of reduced pyridine nucleotides, measured as described in Materials and Methods, increases in the direction indicated. 0.89 mg of purified mung bean mitochondria were added to 3.0 ml of the reaction media.

In mammalian mitochondria energy-linked Ca^{2+} accumulation is closely associated with low amplitude light scattering changes of the suspension [1]. Similar changes were looked for in mung bean mitochondria as illustrated in Fig. 6. Mitochondria oxidizing succinate (Fig. 6A) show a decrease in light scattering correspon-

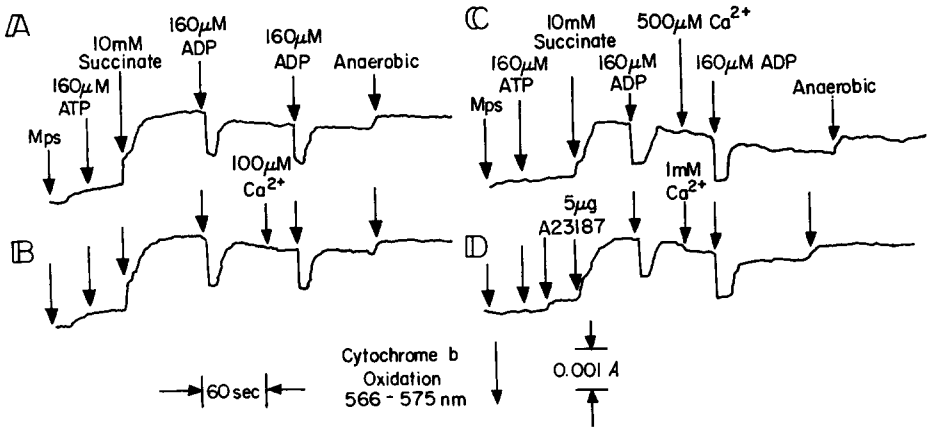

Fig. 5. Effect of Ca^{2+} on redox levels of cytochrome *b*. Changes of oxidation-reduction states of cytochrome *b*-566 were monitored as indicated in Materials and Methods. 1.7 mg of purified mung bean mitochondria were suspended in 3.0 ml of the reaction media described in Fig. 1.

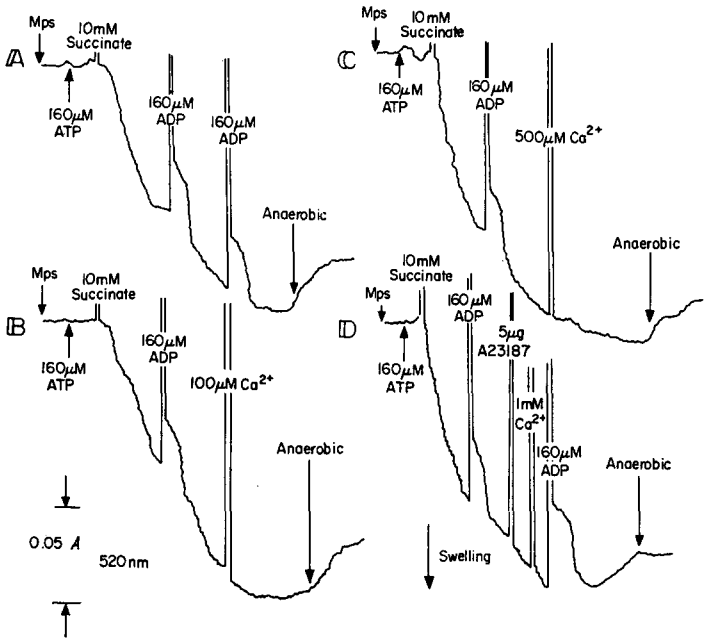

Fig. 6. Effect of Ca^{2+} on light scattering of mitochondrial suspension. 1.2 mg of purified mung bean mitochondria were suspended on 3.0 ml of the reaction media (see Fig. 1). A decrease in absorbance corresponds to mitochondrial swelling. Changes in light scattering were monitored as indicated in Materials and Methods.

ding to low amplitude swelling, whereas in the active phase of respiration (state 3) a transient increase in light scattering is observed. Ca^{2+} again appears to have no effect on low amplitude light scattering changes in contrast to its effect on mammalian mitochondria [1].

Since respiratory effects are only indirect indicators of mitochondrial Ca^{2+} uptake, direct binding studies were undertaken. The use of a calcium-sensitive agent such as murexide permitted the direct measurement of mitochondrial Ca^{2+} uptake [25, 26].

The addition of 200 μM Ca^{2+} to mung bean mitochondria in the absence of succinate or any other permeant anion (Fig. 7a) resulted in a rapid decrease in absorbance at 540 nm due to the formation of the calcium · murexide complex. A similar absorbance decrease is observed in the presence of 10 mM succinate (Fig. 7b), however, in contrast to mammalian studies no subsequent increase in absorbance occurs, corresponding to Ca^{2+} accumulation, even in the presence of nigericin [8]. It is also apparent from Fig. 7 that although there appears to be some accumulation (note different levels in presence or absence of succinate) it is insensitive to valinomycin, 1799 or A-23187. Even in the presence of a permeant anion such as P_i (Figs. 7c and 7d), no active Ca^{2+} accumulation was detected in contrast to previous workers [11–13]. Unlike intact liver or heart mitochondria [8, 9], mung bean mitochondria also do not accumulate Ca^{2+} in an ATP-driven process (not shown). Sufficient Mg^{2+} (5 mM) was present within the media to prevent formation of appreciable Ca · ATP complex.

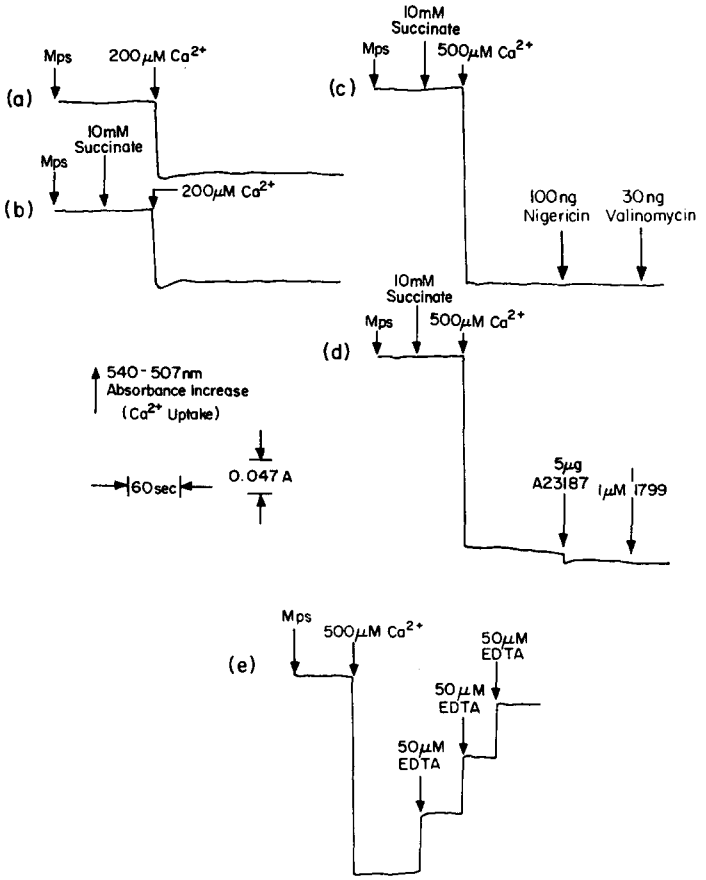

Fig. 7. Spectrophotometric measurements of Ca^{2+} uptake in mung bean mitochondria. The reaction mixture contained 0.3 M mannitol, 10 mM morpholinepropane sulfonate (pH 7.2) 5 mM $MgCl_2$ and 10 mM KCl, 100 μM murexide and 0.8 mg/ml of mitochondrial protein; 0.5 mM potassium phosphate present in c and d. The other additions are indicated in the figure. Temperature, 25 °C.

Increasing concentrations of EDTA dissociated the calcium · murexide complex as indicated by the upward deflections (Fig. 7e).

DISCUSSION

An important point which has emerged from the experiments presented in this report is that purified mung bean mitochondria differ from mammalian mitochondria with respect to active calcium transport.

The stimulation of respiration by the addition of calcium is one of the most common characteristics associated with active calcium transport, and it is readily apparent that mung bean mitochondria do not show any Ca^{2+} stimulation of state 4 respiration even in the presence of high calcium concentrations (up to 1 mM) in agreement with earlier reports [10, 20]. Similarly no stimulation of succinate support-

ed respiration was observed even in the presence of phosphate and following a cycle of respiratory control (cf. ref. 15). The ionophoretic antibiotic A-34187 in the presence of calcium failed to release state 4 respiration in contrast to its respiratory stimulatory effect on vertebrate mitochondria [29]. However, this was not surprising since it has been proposed that A-23187 catalyses an electroneutral exchange of extramitochondrial protons for intramitochondrial calcium [28]. The uncoupling effect observed in vertebrate mitochondria being due to the reaccumulation of Ca^{2+} probably in response to the electrochemical gradient across the inner mitochondrial membrane [8, 28, 29]. Thus even though A-23187 probably equilibrated Ca^{2+} across the plant mitochondrial membrane, the Ca^{2+} was not reaccumulated possibly due to the lack of an active uptake system. It has been suggested that during state 4 respiration when the phosphate potential is high, salt accumulation by plant mitochondria is favored, whereas when the phosphate potential is low (state 3), the conserved energy is diverted into ATP synthesis which not only inhibits further salt transport but also leads to the discharge of previously accumulated divalent cations [15]. In purified mung bean this is obviously not the case since even under high phosphate potential conditions (state 4) no stimulation of respiration or interaction with the respiratory carriers was detected. It is of particular interest that although the accumulation of calcium by plant mitochondria observed by previous workers [15] was claimed to be energy dependent, no release of the accumulated cation was observed upon anaerobiosis.

The ejection of H^+ upon Ca^{2+} addition was not synchronized to any stimulation of respiration and was found to be insensitive to low concentrations of uncoupler and the specific calcium transport inhibitor lanthanum chloride (cf. ref. 20). It is therefore readily apparent that the Ca^{2+}-induced H^+ ejection observed in these experiments is clearly an energy-independent process. The H^+ ejection is possibly attributable to an energy-independent binding, in agreement with Mitchell and Moyle [30] and supporting an earlier finding of Rossi et al. [31] that metabolism-independent Ca^{2+} uptake is accompanied by a H^+ release. However, Wenner and Hackney [33] are not in complete agreement with the findings of Rossi et al. [31] and experiments are currently in progress to clarify this point.

A major difference between vertebrate and plant mitochondria is the observation that even at high Ca^{2+} concentrations there was no Ca^{2+}-induced transient changes in the oxidation-reduction states of either cytochrome *b*-566 or the pyridine nucleotides. These results, as well as the lack of any Ca^{2+}-induced low amplitude swelling, correlate well with the absence of any respiratory stimulation upon Ca^{2+} addition.

The plant mitochondria used in these studies showed very good capacity for oxidative phosphorylation as characterized by high respiratory control, intact inner and outer membranes and rapid change in the oxidation-reduction states of the respiratory carriers and pyridine nucleotides upon ADP addition. It is therefore apparent that the lack of activity in energy-linked Ca^{2+} transport was not due to any deficiency in the primary energy-conserving mechanism, coupled to electron transport, but rather due to deficiences in the Ca^{2+} transport system per se. This interpretation is further supported from the studies with the calcium-sensitive agent murexide which permitted the direct measurement of any mitochondrial calcium uptake by spectrophotometric means. This technique allows a rapid monitoring of uptake and release of calcium by the mitochondria. In contrast to vertebrate and yeast mitochondria [8, 9],

however, the addition of calcium to purified mung bean mitochondria oxidizing succinate in the absence of any other permeant anions failed to induce any increase in the absorbance of the calcium · murexide complex. If the driving force in the uptake of divalent cations into energized mitochondria is the membrane potential (cf. ref. 8), then under the conditions outlined in Fig. 7, the addition of an uncoupler should result in a release of any accumulated Ca^{2+} whereas nigericin should stimulate accumulation. This is obviously not the case in this study, since there was no change in absorbance of the murexide complex in the presence of either of these ionophores.

From the results presented it is therefore readily apparent that although purified mung bean mitochondria possess a respiration-linked electrochemical gradient, it would appear that this potential cannot be expressed as calcium transport even at high ion concentrations. This can be accounted for either by a mitochondrial inner membrane which is impermeable to Ca^{2+} or due to the lack of a specific Ca^{2+} carrier.

It is possible, however, that mitochondria can accumulate Ca^{2+} in an energy-dependent manner if the inner membrane has become permeable to Ca^{2+} through preparative damage (Moore, A. L., unpublished observation). The high respiratory control ratios observed in these experiments, however, indicate that this is not the case. Thus plant mitochondria would appear to differ in a fundamental way from the mitochondria of vertebrate tissues in that they lack an active Ca^{2+} transport system.

The absence of an active Ca^{2+} transport system in plant mitochondria is of potential importance for future studies. For example, plant mitochondria may be used to investigate the stoichiometric relationship between energy-dependent proton ejection and electron transport in the absence of any energy-dependent calcium transport.

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