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Transport of NAD$^+$ in Percoll-Purified Potato Tuber Mitochondria

INHIBITION OF NAD$^+$ INFLUX AND EFFLUX BY *N*-4-AZIDO-2-NITROPHENYL-4-AMINOBUTYRYL-3′-NAD$^+$

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ABSTRACT

A mechanism by which intact potato (*Solanum tuberosum*) mitochondria may regulate the matrix NAD content was studied *in vitro*. If mitochondria were incubated with NAD$^+$ at 25°C in 0.3 molar mannitol, 10 millimolar phosphate buffer (pH 7.4), 5 millimolar $MgCl_2$, and 5 millimolar α-ketoglutarate, the NAD pool size increased with time. In the presence of uncouplers, net uptake was not only inhibited, but NAD$^+$ efflux was observed instead. Furthermore, the rate of NAD$^+$ accumulation in the matrix space was strongly inhibited by the analog *N*-4-azido-2-nitrophenyl-4-aminobutyryl-3′-NAD$^+$. When suspended in a medium that avoided rupture of the outer membrane, intact purified mitochondria progressively lost their NAD$^+$ content. This led to a slow decrease of NAD$^+$-linked substrates oxidation by isolated mitochondria The rate of NAD$^+$ efflux from the matrix space was strongly temperature dependent and was inhibited by the analog inhibitor of NAD$^+$ transport indicating that a carrier was required for net flux in either direction. It is proposed that uptake and efflux operate to regulate the total matrix NAD pool size.

Stimulation of respiration in isolated mitochondria by exogenous NAD$^+$ is well known (2–4). NAD$^+$ is accumulated by plant mitochondria via a specific carrier (13) which is inhibited by the NAD$^+$ analog NAP_4-NAD$^+$ (15) and it is now generally agreed that stimulation of O_2 uptake by added NAD$^+$ is due to its stimulation of matrix-localized enzymes (5, 13, 17, 18). Not all plant mitochondria, however, respond to added NAD$^+$, and those that do not generally have high endogenous NAD$^+$ contents and rapid rates of respiration (18).

Recently, it has been shown that highly purified mitochondria which are stable for several hours, in terms of membrane properties and enzyme activities, slowly lose their NAD$^+$ to the suspending medium (15). In the present paper, we examine the nature of the NAD$^+$ influx and efflux during *in vitro* storage of mitochondria.

MATERIALS AND METHODS

Plant Material and Chemicals. Potato (*Solanum tuberosum* L. var Urgenta) tubers were purchased from a local market. Silica sol was obtained under the name of Percoll from Pharmacia Fine Chemicals, Uppsala, Sweden. *N*-4-Azido-2-nitrophenyl-4-aminobutyryl-3′NAD$^+$ (NAP_4-NAD$^+$) was synthesized as previously described (15). [adenosine^{14}C]NAD$^+$ and [U-^{14}C]sucrose were purchased from New England Nuclear, and ^3H_2O was from CEA (Saclay, France). All other reagents were of analytical grade.

Preparation of Mitochondria. Mitochondria from potato tubers were prepared as rapidly as possible by the method of Bonner (1). Mitochondria thus obtained (washed mitochondria) were then purified by isopycnic centrifugation in density gradients of Percoll as previously described (14). The mitochondrial fraction was carefully removed by siphoning from the bottom of the tube and pelleted, after diluting 10-fold with medium B (0.3 M mannitol, 10 mM phosphate buffer (pH 7.2) and 0.1% defatted BSA), by centrifuging at 15,000*g* for 15 min. The pellet was resuspended in a small volume of medium B (final concentration, 60 to 70 mg mitochondrial protein/ml).

O_2 Uptake Measurement. O_2 uptake was measured at 25°C with a Clark-type O_2 electrode purchased from Hansatech Ltd., King's Lynn, Norfolk, U.K. The reaction medium (medium A) contained 0.3 M mannitol, 5 mM $MgCl_2$, 10 mM KCl, 10 mM phosphate buffer, 10 mM MOPS[2] buffer (pH 7.2), 0.1% defatted BSA, and known amounts of mitochondrial protein (final volume, 1 ml).

Assay of NAD$^+$. NAD$^+$ was determined by the method of Klingenberg (8). It is important to maintain the total NAD pool in its oxidized state (NAD$^+$) since any NADH present is destroyed by the subsequent acid treatment leading to an underestimation of total NAD. Consequently, before acid extraction of NAD$^+$, a portion of the mitochondrial suspension (1.5–2 mg protein) was suspended in 2 ml of medium B and centrifuged at 4°C for 5 min at 12,000*g*. The supernatant was removed and the pellet was resuspended in 0.5 ml of medium A containing 200 μM oxaloacetate which has been shown previously to enter plant mitochondria and oxidize NADH (6). NAD$^+$ concentrations were measured with a fluorimeter assembled in our laboratory, with a Xenon (Hg) lamp (75 w) and a Jobin-Yvon monochromator (H-25). Excitation wavelength was 365 nm. Emitted light was detected at 90° by a R 376 Hamamatsu photomultiplier through quartz lenses and filtered through a Balzer K45 interference filter (peak transmission, 450 nm; bandwidth, 50 nm). The high voltage photomultiplier power supply, signal amplifier, and processor were original components developed by Metrologic (Grenoble, France).

NAD$^+$ Uptake. The uptake of [^{14}C]NAD$^+$ by intact purified

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[2] Abbreviations: MOPS, morpholinopropanesulfonic acid; FCCP, carbonylcyanide-*p*-trifluoromethoxyphenyl hydrazone.

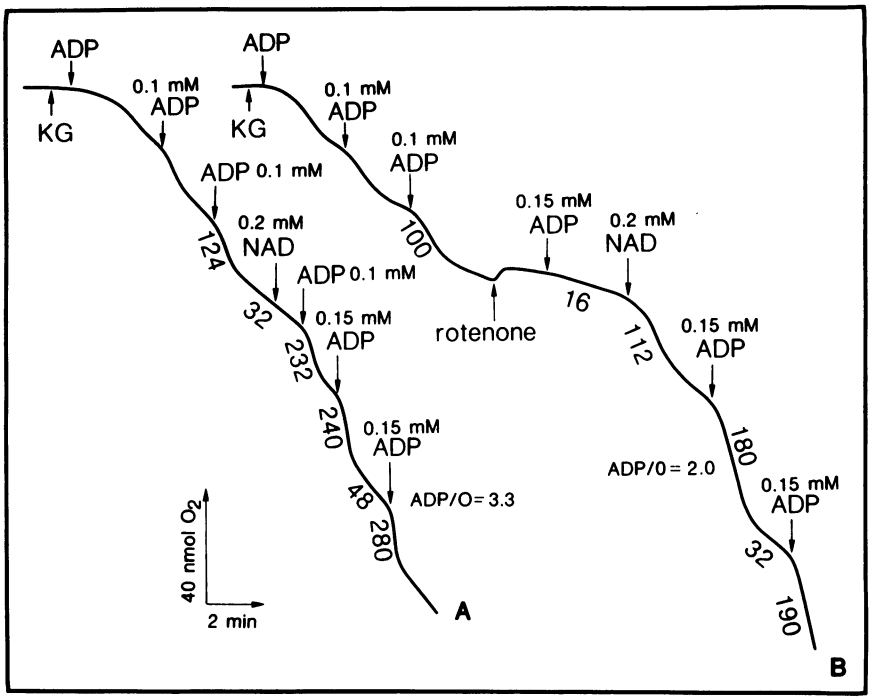

FIG. 1. α-Ketoglutarate oxidation by potato mitochondria. Mitochondria were purified from tubers which had been stored for 65 d at 10°C and O_2 consumption measured as described in "Materials and Methods." The pH of the reaction medium was 7.2 and 2.5 mM malonate and 0.2 mM thiamin pyrophosphate were included in the medium. Where indicated, 5 mM α-ketoglutarate, 0.1 mM ADP (except where indicated otherwise), and 12 μM rotenone were added. Numbers on traces refer to nmol of O_2 consumed/min·mg protein.

N-4-azido-2-nitrophenyl-4-aminobutyryl-3'-NAD$^+$
(NAP_4NAD^+)

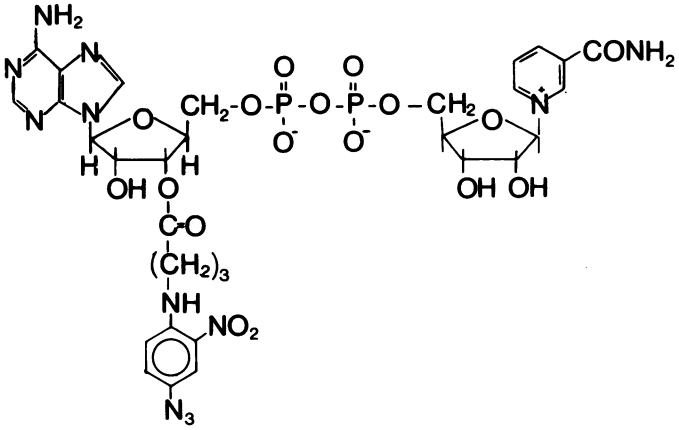

FIG. 2. Structure of the inhibitor (NAP_4-NAD^+) of the NAD^+ transporter.

mitochondria was initiated by adding 5 μl of the mitochondrial suspension (about 0.4 mg protein) to 100 μl of medium A containing 5 mM citrate or α-ketoglutarate, 100 μM thiamin pyrophosphate, 1 mM ADP, 5 mM glucose, 10 μg of hexokinase, and labeled compounds in a 400 μl capacity polypropylene tube. The uptake was stopped by rapid centrifugation (Beckman Microfuge B) of the mitochondria through a layer of silicone oil (50 μl of Versilube F50) into 50 μl of 1.6 M $HClO_4$ (the silicone oil has a density of 1.065 g/ml; the density of 1.6 M $HClO_4$ is 1.08 g/ml). Furthermore, the strong inhibition of NAD^+ transport by the NAD^+ analog NAP_4-NAD^+ (see "Results") provided an opportunity to include 100 μM NAP_4-NAD^+ routinely as an inhibitor-stop at the end of the incubation time. This procedure was used to increase the accuracy of the timed measurements of NAD^+ uptake. In order to be sure that accumulated ^{14}C radioactivity was still in NAD^+ and not, for instance, in AMP, the endogenous NAD^+ content was also measured by an enzymic method (see "Assay of NAD^+" above) at the end of each experiment. For details on the silicone layer filtering centrifugation technique and on the evaluation of the uptake into the sucrose-impermeable space, which is the space surrounded by the mitochondrial inner membrane, see Klingenberg and Pfaff (9). The intramitochondrial volume was estimated with [^{14}C]sucrose and ^3H_2O (19).

NAD^+ Loading. Mitochondria suspended in medium A were loaded with NAD^+ by incubating them at 25°C with 10 mM citrate, 1 mM ADP, 0.3 mM thiamine pyrophosphate, 5 mM glucose, 20 μg hexokinase, and [^{14}C]NAD^+ (0.2 mM, 19.7 GBq·mmol^{-1}). At equilibrium (*i.e.* after 7 min), external [^{14}C]NAD^+ was eliminated by rapid centrifugation (10,000*g*, 5 min; rotor SM24, Sorvall) through a layer of Percoll (5% v/v Percoll, 0.3 M mannitol, 10 mM phosphate buffer [pH 7.2], 0.1% BSA, and 10 mM citrate). The presence in the medium of an energy source (citrate) allows the maintenance of the protonmotive force during the course of centrifugation. The centrifugation was carried out at 0°C. The supernatant was removed by aspiration and the mitochondrial pellet (NAD^+-loaded mitochondria) was gently resuspended in medium B at a final protein concentration of 5 mg/ml. At various times, aliquots were taken for the measurement of α-ketoglutarate-dependent O_2 consumption rates, efflux of [^{14}C]NAD^+ as determined by silicone oil centrifugal filtration, and NAD^+ content of the matrix space (see above).

Enzymes and Other Assays. Outer membrane integrity was estimated using Cyt *c*-dependent O_2 uptake (14). Mitochondrial protein was estimated by the method of Lowry *et al.* (10).

RESULTS

Effect of NAD^+ on Substrate Oxidation. Figure 1 depicts typical O_2 electrode traces obtained with potato mitochondria oxidizing α-ketoglutarate, which show the different effects of NAD^+ on rotenone-sensitive and -insensitive electron transport. The amount of NAD^+ present in these mitochondria (1.2 nmol/mg protein) was sufficient to allow state 3 rates (O_2 uptake in the presence of ADP) of approximately 50% of maximal rate to be sustained; that is, adding NAD^+ stimulated state 3 rates by about 2-fold (Fig. 1A). The rate of O_2 uptake in the presence of rotenone, however, was almost completely dependent on added NAD^+ (Fig. 1B). Rotenone is an inhibitor of electron flow

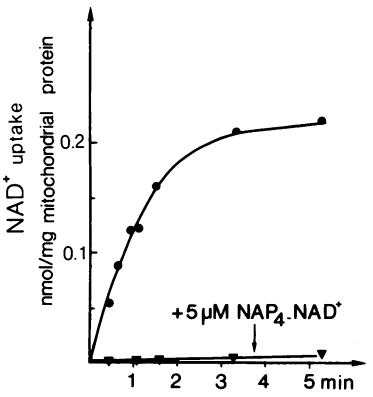

FIG. 3. Effect of NAP_4-NAD^+ on [^{14}C]NAD^+ uptake by potato mitochondria. [^{14}C]NAD^+ uptake was measured by silicone oil filtration (see "Materials and Methods"). The pH of the assay medium was 7.2 and the temperature 25°C. Labeled NAD^+ was added at 10 μM and NAP_4-NAD^+ at 5 μM. α-Ketoglutarate was added as substrate to drive NAD^+ uptake (see "Materials and Methods"). Note that at equilibrium the concentration of [^{14}C]NAD^+ in the matrix space (0.2 nmol/mg mitochondrial protein) was found to be higher than in the medium. When 200 μM [^{14}C]NAD^+ was provided externally, the endogenous [^{14}C] NAD^+ content reached at the equilibrium was 8 nmol/mg mitochondrial protein (not shown).

through complex I of the respiratory chain but plant mitochondria have the ability to bypass this site during oxidation of endogenous NADH (16). This bypass is coupled to only two sites of energy transduction (note the difference in ADP/O ratios between Fig. 1, A and B) and may be catalyzed by a second internal NADH dehydrogenase. This second system has been shown to have a much lower affinity for NADH than its rotenone-sensitive counterpart in submitochondrial particles (12), and the results in Figure 1 suggest that this is also the case with intact potato mitochondria. The rates of O_2 uptake upon addition of rotenone is thus a sensitive indicator of the level of matrix NAD^+.

Uptake of NAD^+ by Isolated Mitochondria. The stimulation of O_2 uptake by added NAD^+ implies that NAD^+ can be accumulated by mitochondria (18). This process was investigated further using an aryl-azido derivative of NAD^+ (NAP_4-NAD^+) (Fig. 2) synthesized in our laboratory (15). Figure 3 shows that [^{14}C]NAD^+ added at 10 μM to the medium was accumulated by isolated potato mitochondria against a concentration gradient and that this process was severely inhibited by NAP_4-NAD^+ (Fig. 3). In the dark, NAP_4-NAD^+ acts as a competitive inhibitor of NAD^+ uptake (15). Figure 4 shows that even low concentrations (30 μM) of added NAD^+ stimulated O_2 uptake, but at these low concentrations an appreciable lag was observed prior to the stimulation (approximately 3 min at 30 μM NAD^+; Fig. 4). This lag decreased as the external NAD^+ increased and vice versa (results not shown) and represents the time required to accumulate NAD^+ in the matrix space (Fig. 3), *i.e.* to saturate the matrix dehydrogenase. Stimulation of O_2 uptake by added NAD^+ at concentrations below 0.5 mM was inhibited by NAP_4-NAD^+ (Fig. 4C) and by prior addition of the uncoupler FCCP (Fig. 4B). Parenthetically, net uptake was not only inhibited by FCCP, but NAD^+ efflux was observed instead (results not shown). The latter result suggests that NAD^+ uptake is an energy-dependent process. The results in Figures 2 and 3, together with those in References 13 and 18, demonstrate that NAD^+ uptake by isolated plant mitochondria is a carrier-mediated process.

Efflux of NAD^+ from Isolated Mitochondria. Most of the time, potato tuber mitochondria contained low amounts of endogenous NAD^+ as indicated by the very low rate of O_2 uptake in the presence of rotenone which was stimulated by adding NAD^+ (Fig. 1). Consequently, with the aim of investigating the nature of NAD^+ efflux from the matrix space, mitochondria suspended in medium A were at first loaded with NAD^+ (see "Material and Methods"). Figure 5 demonstrates that once isolated NAD^+-loaded mitochondria progressively lose NAD^+ in the suspending medium insofar as the medium does not contain an energy source (tricarboxylic acid cycle substrates). At 0°C, the rate of passive diffusion of NAD^+ out of the mitochondria was extremely low (*i.e.* 0.2–0.3 nmol/h·mg protein during the first 10 h of aging; Fig. 5A). However, this low rate was sufficient to empty the mitochondria of their NAD^+ content in 30 to 40 h as shown in a previous publication (15). In contrast, the initial rate of 'passive diffusion' of NAD^+ observed at 25°C was much higher than that observed at 0°C and was found to be in the range of 3 to 5 nmol/h·mg protein (Fig. 5A). Consequently, at 25°C, the endogenous NAD^+ content decreased to 10 to 20% of the control (freshly NAD^+-loaded mitochondria) within only 2 h. Under these circumstances, when NAD^+-linked substrates were used,

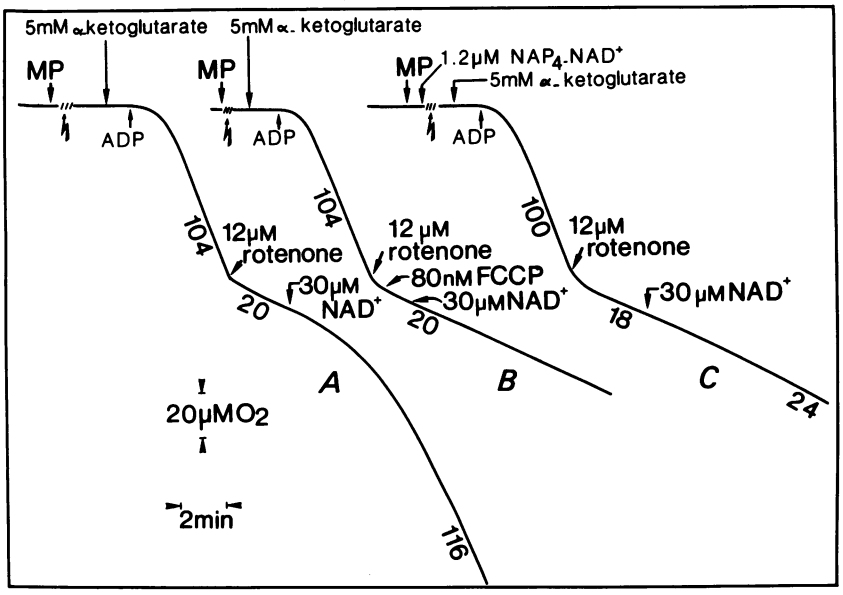

FIG. 4. Effect of FCCP and NAP_4-NAD^+ on the rates of rotenone-resistant oxidation of α-ketoglutarate by purified mitochondria isolated from potato tubers. The electrode medium is described in the text. The numbers along the traces refer to nmol of O_2 consumed/min·mg protein. Note that addition of exogenous NAD^+ strongly stimulates the rotenone-resistant oxidation of α-ketoglutarate (trace A) and that this stimulation is inhibited by uncoupler (FCCP) (trace B) and NAP_4-NAD^+ (trace C). Note that the full rate of rotenone-insensitive α-ketoglutarate oxidation (trace A) is recovered 3 min after addition of 30 μM NAD^+. The zigzag arrow indicates that mitochondria were subjected to light for 3 min prior to addition of substrate (see text).

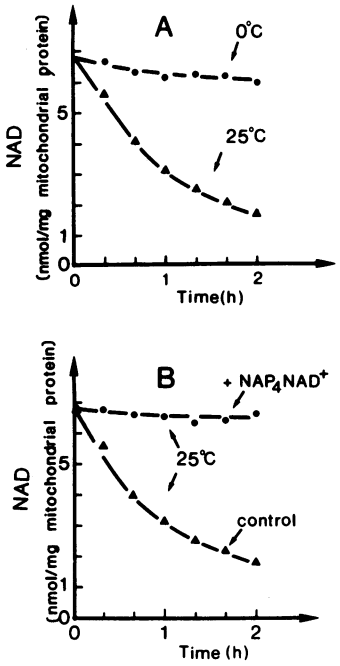

FIG. 5. Effect of temperature (A) and NAP_4-NAD^+ (B) on NAD^+ efflux by NAD^+-loaded mitochondria isolated from potato tubers. [^{14}C] NAD^+ efflux was determined by silicone oil centrifugal filtration as described in "Materials and Methods." Assuming a volume of 2.5 μl/mg mitochondrial protein (15), the concentration of NAD^+ found in NAD^+-loaded mitochondria was approximately 2.8 mM. NAP_4-NAD^+ was added at 90 μM and the medium containing NAP_4NAD (●, +NAP_4NAD^+) or not (▲, control), was subjected to strong, white light as described in the text. Note that NAP_4NAD^+ prevents NAD^+ efflux from NAD^+-loaded mitochondria.

O_2 uptake became rapidly dependent on added NAD^+ (Table I). Subsequent uptake of NAD^+ via a specific carrier restores electron transport fully (Table I). Separate measurements of KCN-sensitive ascorbate Cyt *c*-dependent O_2 uptake (14) showed that the apparent percentages of intact mitochondria remained constant during the incubation at 25°C (see Fig. 6 legend). These data suggested that the net efflux of NAD^+ observed during the first 2 h of the experiments could not be attributed to nonspecific leakage due to the membrane damage. It is clear therefore that when the temperature of suspending medium increased, the rate of NAD^+ leakage increased. Interestingly, at a given temperature, we have observed that the initial rate of NAD^+ efflux from the mitochondria is dependent upon the intramitochondrial NAD^+ concentration (result not shown).

With the aim of demonstrating that the leakage of NAD^+ from the matrix occurs on the NAD^+ carrier, and not through the hydrophobic core of the membrane as previously discussed (15), experiments were carried out to examine NAD^+ leakage and its inhibition with NAP_4-NAD^+. For this purpose, NAD^+-loaded mitochondria were divided into two equal aliquots; to one batch, 90 μM NAP_4-NAD^+ was added and both aliquots were subjected at 0°C to strong white light (Tungsten Halogen Projector lamp, Sylvania, 250 w, 24 v, 3 × 2 min). In the light (see 7), NAP_4-NAD^+ caused a full nonreversible inhibition of NAD^+ transport, as it presumably binds covalently to the active site of the carrier (15). After this treatment, both batches showed high rates of O_2 uptake, which were only slightly inhibited by rotenone and were not stimulated by adding NAD^+ (Figs. 6, A and B) indicating that the mitochondria which had been loaded with NAD^+ retained much of this when they were subjected to strong white light insofar as the experiment is carried out at 0°C (Fig. 5A). The mitochondria then were stored at 25°C for 2 h and O_2 consumption again was measured. The mitochondria which had been treated with NAP_4-NAD^+ retained high rates of O_2 uptake both in the presence and absence of rotenone, and adding NAD^+ did not stimulate (Fig. 6D). The mitochondria which had not been treated with the transport inhibitor, on the other hand, were very dependent on added NAD^+ (Fig. 6C) showing that they had lost their endogenous NAD^+. These results strongly suggest that the leakage of NAD^+ from the mitochondrial matrix which occurs during storage is strongly inhibited by the analog inhibitor of NAD^+ transport, NAP_4-NAD^+. Figure 5B demonstrates that NAP_4-NAD^+ was indeed a potent inhibitor of net NAD^+ efflux observed at 25°C.

DISCUSSION

The results presented here confirm that plant mitochondria from storage tissues possess a specific NAD^+ carrier since NAD^+

Table I. *Effect of Aging at 25°C on the State-3 Rate of O_2 Uptake by NAD^+-Loaded Mitochondria Oxidizing Various Substrates*

NAD-loaded mitochondria were suspended at 25°C in medium B (5 mg protein/ml). At intervals, aliquots of the suspension (corresponding to 0.15 mg mitochondrial protein when NADH and succinate were used as substrates, and 0.3 mg when α-ketoglutarate was used as substrate) were taken for O_2 uptake measurements (see "Materials and Methods"). Other additions, as indicated were: 1 mM NADH, 0.6 m-eq. Ca^{2+}, 1 mM ADP; 5 mM succinate, 150 μM ATP, and 1 mM ADP; 5 mM α-ketoglutarate, 0.3 mM thiamin pyrophosphate, 0.5 mM CoA, 3 mM malonate, and 1 mM ADP; 12 μM rotenone; 0.2 mM NAD (final volume, 1 ml). Note that dilution of leaked NAD^+ (dilution factor 17) precludes its reabsorption (K_m (NAD) for carrier: 0.3 mM) which explains the need to add external NAD^+ to restore the initial rate of respiration.

Aging Period	Rates of Substrate Oxidation					
	NADH	Succinate	α-Ketoglutarate			
			− Rotenone		+ Rotenone	
			− NAD^+	+ NAD^+	− NAD^+	+ NAD^+
min	*nmol O_2/min·mg protein*					
0	511	420	385	400	321	347
30			320	360	190	290
60			246	355	92	290
90	524	420	210	355	52	320
120	500	426	98	350	26	295
180			26	373	6	295

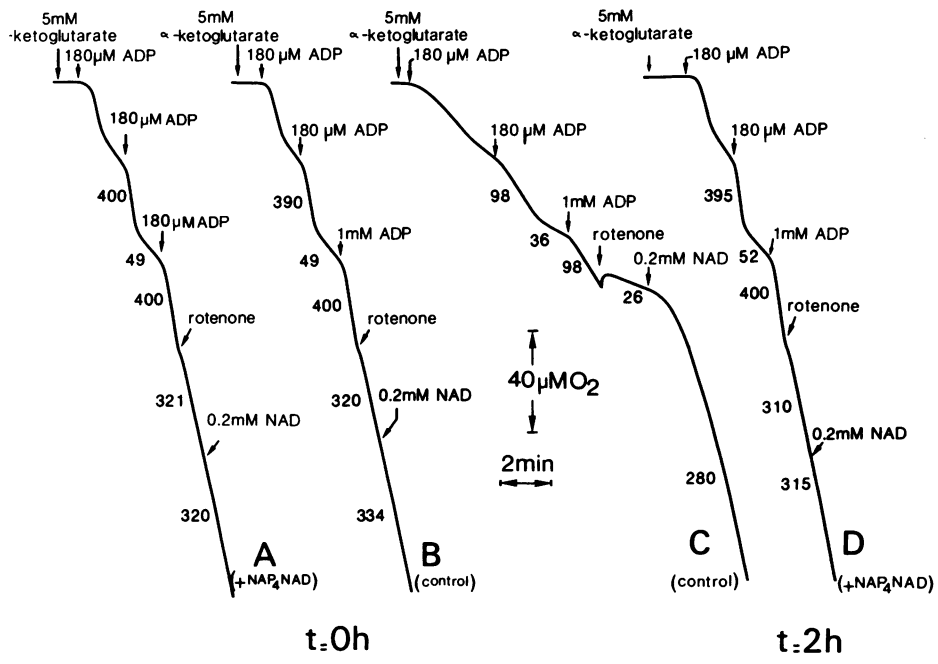

FIG. 6. Rates of α-ketoglutarate oxidation during aging of purified mitochondria isolated from potato tubers. NAD+-loaded mitochondria (see "Materials and Methods") were diluted with medium B (see text) to approximately 5 mg protein·ml. They were then divided into two equal aliquots; to one aliquot, 90 μM NAP_4-NAD+ was added and both aliquots were subjected to strong white light (see text). The mitochondria were then stored at 25°C for 2 h and O_2 consumption measured. For each time period, the percentages of mitochondria integrity were t = 0 h, 96%; t = 2 h, 95%. The values along the traces refer to nmol of O_2 consumed /min·mg protein. A, Mitochondria loaded with NAD+ and then treated with the NAD+-transport inhibitor, NAP_4-NAD+; B, mitochondria loaded with NAD+; C, as for B but after 2 h of storage at 25°C; D, as for A but after 2 h of storage at 25°C. The total amounts of NAD+ present in freshly prepared NAD+-loaded mitochondria (A,B) and 2-h-aged mitochondria (C,D) were, respectively, 6.7, 6.7, 0.8 and 6.5 nmol/mg protein. Note the marked decline of state 3 rates of α-ketoglutarate oxidation during mitochondrial aging in the absence of NAP_4-NAD+ and the recovery of the initial rate when the suspending medium contains 200 μM NAD+ (C).

uptake, which is an energy-dependent process, exhibits Michaelis-Menten kinetics (13, 18) and is almost completely inhibited by the analog NAP_4-NAD+. Furthermore, the results demonstrate that the passive diffusion of NAD+ out of the mitochondria which is strongly temperature dependent occurs via this NAD+ carrier because the analog NAP_4-NAD+ almost completely prevents leakage of NAD+ out of the mitochondria during their storage. It is clear, therefore, that a protein was required for net flux in either direction. In fact, direct passage of NAD+ through the membrane lipid bilayer is most unlikely since the passage of ionic or polar molecules through the hydrophobic core of the membrane is thermodynamically a highly unfavorable process. It seems likely, therefore, that plant mitochondria have the ability to manipulate their matrix NAD+ concentration in a controlled fashion.

Since the intramitochondrial concentration of NAD+ has such a profound influence on matrix enzyme activity and O_2 uptake via the rotenone-insensitive pathway by isolated mitochondria (see Figs. 1 and 6; Ref. 15), it is potentially a very powerful regulator of plant respiration *in vivo* and could play an important role in the coarse control of metabolism, particularly during transition from a dormant stage to a stage of active growth (and vice versa). In this context, the mitochondria from young growing tissues, including the shoots of sprouting potato tubers, have higher matrix NAD+ contents than those from storage tissues (18). It is also interesting to note that preliminary experiments carried out in our laboratory indicate that, over the time of potato tuber storage, the endogenous NAD+ content of the mitochondria first declined from an original value of 3 nmol/mg protein to 0.3 nmol after 90 d of storage, and thereafter rose again during sprouting. It is possible, therefore, that the rates of respiration in these tissues would be affected by the concentration of NAD in the extramitochondrial cytoplasm and this concentration might differ significantly from one tissue to the other, or even between different physiological situations of the same tissue. Finally, Matthews *et al.* (11) have clearly demonstrated that *Helminthosporium maydis* race T toxin induces leakage of NAD+ from T cytoplasm corn mitochondria. The data presented here show that it is very likely that this leakage of NAD+ occurs via the NAD+ carrier and is triggered by the collapse of membrane potential in toxin-treated mitochondria.

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