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Regulation of succinate oxidation by NAD^+ in mitochondria purified from potato tubers

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NAD^+ supplied to purified *Solanum tuberosum* mitochondria caused progressive inhibition of succinate oxidation in State 3. This inhibition was especially pronounced at alkaline pH and at low succinate concentrations. Glutamate counteracted the inhibition. NAD^+ promoted oxaloacetate accumulation in State 3; supplied oxaloacetate inhibited O_2 uptake in the presence of succinate much more severely in State 3 than in State 4. NAD reduction linked to succinate oxidation by ATP-dependent reverse electron transport was likewise inhibited by oxaloacetate. We conclude that NAD^+-induced inhibition of succinate oxidation is due to an inhibition of succinate dehydrogenase resulting from increased accumulation of oxaloacetate generated from malate oxidation via malate dehydrogenase. The results are discussed in the context of the known regulatory characteristics of plant succinate dehydrogenase.

Introduction

Oxidation of succinate is mediated by the enzyme succinate dehydrogenase which is located on the inner mitochondrial membrane [1]. Succinate dehydrogenase normally passes reducing equivalents directly to the respiratory chain without the intervention of pyridine nucleotides. In State 4, succinate dehydrogenase may also oxidize succinate via reverse electron transport; in this case there is an ATP-dependent reduction of NAD^+ to NADH using electrons removed from succinate. Succinate dehydrogenase has been shown to be activated by ATP and reduced coenzyme Q, and deactivated by oxaloacetate [2,3], but the regulation of succinate dehydrogenase appears not to have received much detailed study in intact higher-plant mitochondria functioning as an integrated metabolic system. Regulation of succinate oxidation in animal mitochondria and its physiological significance have been extensively discussed [2,4,5].

The present investigation was prompted by the observation that the rate of succinate oxidation by potato mitochondria in State 3 is severely inhibited when NAD^+ is supplied in the incubation medium. Penetration of NAD^+ into the matrix of isolated intact plant mitochondria is rapid [6], and the mechanism of its penetration has been characterized in previous studies from this laboratory [7, 8].

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Abbreviation: Mops, 4,-morpholinepropanesulfonic acid.

Materials and Methods

Potato tubers (new potatoes, *Solanum tuberosum* L.) were stored in a cold room at 4°C. Mitochondria were isolated and purified by isopycnic centrifugation on Percoll density gradients as described by Neuburger et al. [9]. The purified mitochondria were stored at 0°C at a concentration of 50–100 mg protein per ml assayed according to Lowry et al. [10], with bovine serum albumin as the reference protein standard. NAD-depleted mitochondria were prepared by diluting the purified mitochondria into a large volume of reaction medium A [9] at 25°C for 1 h and then re-sedimenting the mitochondria by centrifugation [9]. NAD-loaded mitochondria were prepared according to Neuburger et al. [8].

The percentage intactness of the mitochondrial preparations was assessed by polarographic measurement of cyanide-sensitive ascorbate/cytochrome *c*-dependent O_2 uptake [9], an oxidation process completely restricted to broken mitochondria [11]. The percentage intactness of both the freshly purified and NAD-depleted mitochondria exceeded 90%. The NAD-depletion treatment had no adverse effect on intactness, coupling (as indicated by respiratory control ratios), or oxidation rates, apart from declines specifically attributable to cofactor depletion. A detailed study of total NAD pool sizes in freshly purified and NAD-depleted mitochondria has been published elsewhere [8].

Oxaloacetate determination were carried out by Ni-dependent decarboxylation and enzymic assay of pyruvate [6]. In experiments where the succinate dehydrogenase-mediated oxidation of succinate was studied under conditions of reverse electron transport, the level of mitochondrial NADH was monitored by measuring native fluorescence of NADH. Details of the fluorimeter employed have been published elsewhere [7]. NAD^+ and NADH were determined by the method of Klingenberg [12].

Oxygen uptake was measured polarographically at 25°C in a total volume of 1 ml in a Clark type of electrode (Hansatech, DW). In order to activate succinate dehydrogenase, ATP was routinely supplied in the reaction medium. The basic reaction medium contained 300 mM mannitol/5 mM $MgCl_2$/10 mM KCl/10 mM Mops/10 mM PO_4-buffer/0.1% defatted bovine serum albumin, at pH 7.2 (medium A) or 7.9 as indicated. Further details appear in the figure legends.

Results

Fig. 1 shows three O_2-electrode traces for freshly purified potato tuber mitochondria oxidizing 5 mM succinate at pH 7.2. Fig. 1A shows the response to ADP. The respiratory control ratio (State 3/State 4) was approx. 3. Fig. 1B shows a trace similar to 1A, except that ADP was added in excess, so that there was no transition to State 4 prior to anoxia; the State 3 rate remained virtually linear for about 3 min. Fig. 1C shows the same experiment as 1B, but with NAD^+ (2 mM) supplied in the reaction medium. In this case the state 3 rate of O_2 uptake was clearly non-linear and fell from an initial value of 392 nmol/mg protein per min to a value of 256 after 3 min.

The progressive nature of the inhibition in the

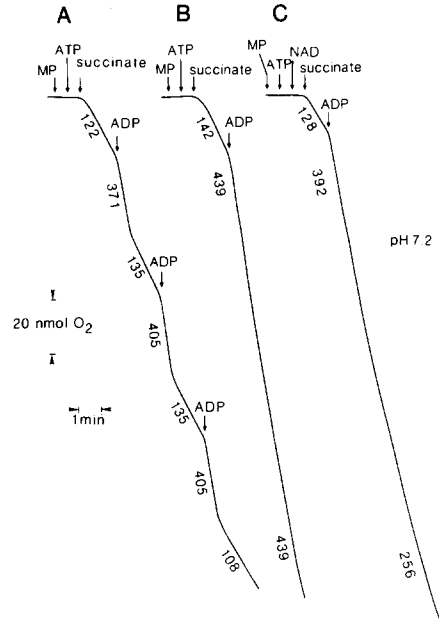

Fig. 1. Rates of succinate oxidation by purified potato mitochondria (MP) at pH 7.2. O_2 electrode recorder traces. Numbers alongside traces are nmol O_2 consumed/mg protein per min. Reaction medium = medium A. Protein conc. = 148 μg/ml. ADP additions: (A) 91 nmol per addition, (B) and (C) 455 nmol. ATP = 200 nmol; succinate, 5 μmol; NAD^+ (B), 100 nmol. Total vol. 1.0 ml.

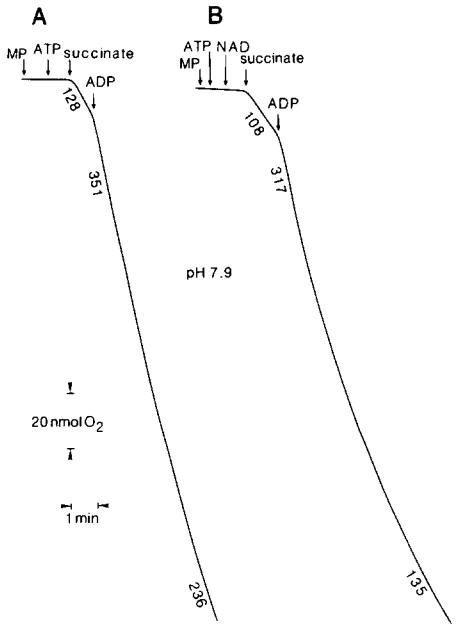

Fig. 2. Rates of succinate oxidation by purified potato mitochondria (MP) at pH 7.9. ADP = 728 nmol. Other components as in Fig. 1.

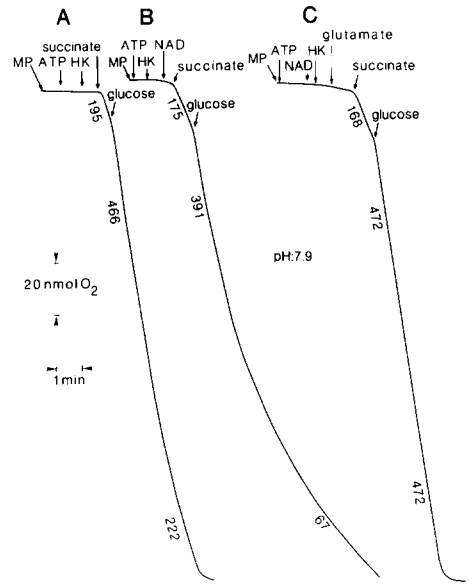

Fig. 3. Rates of succinate oxidation by purified potato mitochondria (MP) at pH 7.9. State 3 conditions obtained with ADP-generating system consisting of ATP (200 nmol) plus hexokinase (HK, 6 μg) plus glucose (5 μmol). Glutamate (C) = 10 μmol. Other components as in Fig. 1. Total vol. 1 ml.

presence of added NAD^+ suggests that NAD^+ brings about a gradual accumulation of an inhibitor or deactivator of succinate dehydrogenase. A distinct possibility would be that NAD^+ promotes the formation of oxaloacetate by malate dehydrogenase [2]. Malate derived from succinate oxidation, via fumarate [13], produces both oxaloacetate and pyruvate [6]; exogenously supplied NAD^+ which enters the matrix space [7] is known to stimulate oxaloacetate accumulation because of its effect in shifting the malate dehydrogenase equilibrium towards oxaloacetate production [6].

The above experiment was therefore repeated at pH 7.9, which strongly favors the operation of malate dehydrogenase (L-malate:NAD^+ oxidoreductase EC 1.1.1.37) over malic enzyme (L-malate:NAD^+ oxidoreductase (decarboxylating) EC 1.1.1.39) [6]. At pH 7.9 and at 5 mM succinate, the State 3 rate fell by 35% over a 3 min period with no added NAD^+ (Fig. 2A), and with added NAD^+ the State 3 rate fell by more than 50% (Fig. 2B) before respiration ceased, owing to anoxia. The same general trends were seen in a considerably more pronounced fashion when State 3 rates were measured in the presence of ADP generated on a continuous basis from ATP by the action of hexokinase and glucose. The use of hexokinase/glucose also serves to prevent a build-up of ATP, which is a potent activator of succinate dehydrogenase; here the rate fell by about 50% from the initial value in the absence of NAD^+ (Fig. 3A), and by over 80% when 2 μmol of NAD^+ were added (Fig. 3B). However, glutamate supplied along with NAD^+ completely prevented the decline in the State 3 rate, which now remained linear until anoxia (Fig. 3C). Glutamate transaminates oxaloacetate to aspartate in mitochondria [14]; thus, its prevention of the inhibition of succinate oxidation suggests that increased oxaloacetate accumulation at high NAD^+ levels is largely responsible for the inhibition. Moreover, it is evident that oxaloacetate is not so firmly bound to succinate dehydrogenase as to prevent its removal by transamination with glutamate. To ascertain whether there was any possibility that oxoglutarate produced by transamination or oxidative deamination of glutamate could contribute significantly to the oxygen uptake, we checked the rate of oxoglutarate oxidation in the absence

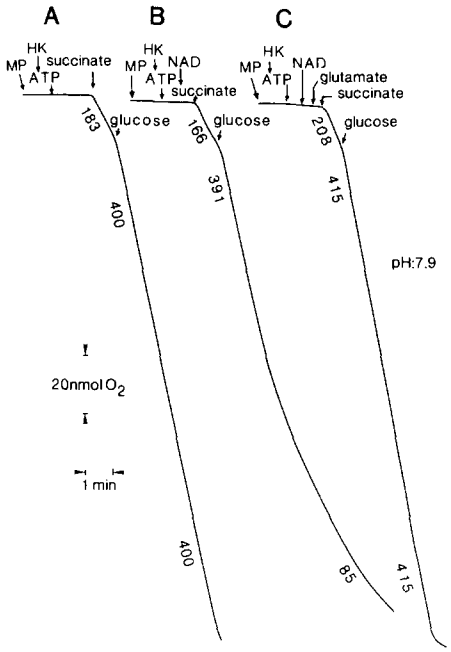

Fig. 4. Rates of succinate oxidation at pH 7.9 by purified mitochondria (MP) depleted of NAD^+. Protein conc. = 120 μg/ml. State 3 conditions obtained with ADP-generating system (as in Fig. 3). Total vol. 1 ml.

TABLE I

EFFECT OF INTRAMITOCHONDRIAL NAD^+ CONTENT ON ACCUMULATION OF OXALOACETATE DURING THE COURSE OF SUCCINATE OXIDATION IN STATE 3

The standard assay solution (medium A) was used with 1 mg mitochondrial protein/ml, 0.3 mM ATP, 5 mM glucose, 6 μg hexokinase/ml and 10 mM succinate. Final pH was 7.9 and final volume of the reaction medium was 10 ml. At various times, 1 ml aliquots were taken and added to 0.3 ml of cold 20% $HClO_4$ containing 1 mM EDTA. The samples were centrifuged for 10 min at 15000 × g. The supernatant was neutralised up to pH 6 with KOH and used for oxaloacetate determination as described by Tobin et al. [6]. Normal mitochondria, 2.4 nmol NAD/mg protein; loaded mitochondria, 9.0 nmol NAD/mg protein; depleted mitochondria, 0.6 nmol NAD/mg protein; depleted and loaded mitochondria were prepared according to Ref. 8. n.d., not detected.

Time (min)	Oxaloacetate formed (nmol)		
	normal mitochondria	depleted mitochondria	loaded mitochondria
0	n.d.	n.d.	n.d.
2	12	n.d.	57
4	21	2	93
6	30	4	115
8	39	8	144

of added thiamine pyrophosphate. This rate was found to be only about 5% of the rate of succinate oxidation with NAD^+ and glutamate present (data not shown). Moreover, the ADP/O ratio was found to be about 1.7 with succinate plus NAD^+ plus glutamate, a value typical of succinate oxidation. ADP/O values of more than 2.6 were determined for oxoglutarate oxidation with added thiamine pyrophosphate (not shown).

Fig. 4 shows comparable experiments with NAD-depleted mitochondria (see Materials and Methods). At pH 7.9, the State 3 rate of succinate oxidation with the ADP-generating system was linear (Fig. 4A), in contrast to the gradual decline observed with fresh mitochondria oxidizing succinate at this pH (Fig. 3A). Again, added NAD^+ caused a large inhibition (Fig. 4B). As with the fresh mitochondria, the NAD-induced inhibition of succinate oxidation by mitochondria previously depleted of NAD was completely abolished by glutamate (Fig. 4C), the final rate being 415 nmol/mg protein per min compared to 85 in the absence of glutamate (Fig. 4B). However, it should be noted that similar results were obtained when the mitochondria were washed by centrifugation after loading with NAD (not shown). Thus oxaloacetate is formed only by internal malate dehydrogenase.

The effect of intramitochondrial NAD content on accumulation of oxaloacetate is illustrated in Table I. We have observed that the higher NAD^+ concentration in the matrix space the higher the amount of oxaloacetate excreted in the external medium. Although the oxaloacetate transporter mechanism is known to export the bulk of mitochondrial oxaloacetate (and the overall measured oxaloacetate concentration is therefore not necessarily the same as the matrix concentration of oxaloacetate [15]), there is nevertheless an intramitochondrial oxaloacetate accumulation when NAD^+ is present in substantial amounts in the matrix space. The present data confirm the increase in oxaloacetate accumulation when NAD^+ is added, as has been shown earlier during the

course of malate oxidation [6].

Because oxaloacetate inhibition (deactivation) of succinate dehydrogenase may be significantly counteracted by the substrate, succinate, which is also an activator of the enzyme [16], we decided to study the effect of NAD^+ at a range of succinate concentrations. Fig. 5A and Fig. 5B present O_2-electrode data in which the effect of NAD^+ was tested at 5, 3 and 1.5 mM succinate. These experiments were done at pH 7.2. Fig. 5A shows traces in which State 3 conditions were obtained by means of the hexokinase/glucose/ATP system which generates ADP on a continuous basis. At 5 mM succinate, NAD^+ did not inhibit the initial State 3 rate, but caused a much more rapid decline compared to that in the absence of NAD^+. At 3 mM succinate, NAD^+ caused a still more pronounced fall-off in the State 3 rate compared to the minus-NAD control; the subsequent addition of glutamate largely restored the original State 3 rate. At a still lower succinate level (Fig. 5B) the decline in the State 3 rate was very rapid and severe, especially with added NAD^+. Again, the addition of glutamate abolished the inhibition.

The effect of exogenously- supplied oxaloacetate on succinate oxidation in States 3 and 4 was examined on the O_2 electrode. In State 4 succinate oxidation was assayed fluorimetrically by measuring reduction of NAD^+ by succinate under conditions of ATP-dependent reverse electron transport. Table II shows the effect of oxaloacetate on

TABLE II

EFFECT OF SUPPLIED OXALOACETATE ON O_2 CONSUMPTION BY POTATO TUBER MITOCHONDRIA IN STATE 3 AND STATE 4

Substrate, succinate (5 mM). ATP (3 mM) supplied to all raction mixtures. State 3 conditions obtained with hexokinase/glucose ADP-generating system. These data are from a representative experiment and have been repeated four times. Mean control values: State 3 = 236 nmol O_2 consumed/mg protein per min; State 4, 60 nmol O_2 consumed/mg protein per min.

Respiratory state	oxaloacetate concentration (μM)	Inhibition (%)
3	10	16
	20	43
	30	61
	40	65
	80	86
	80 + 10 mM glutamate	24
4	40	33
	80	33
	120	45
4 + NAD (0.2 mM)	30	0
	70	0
	130	39

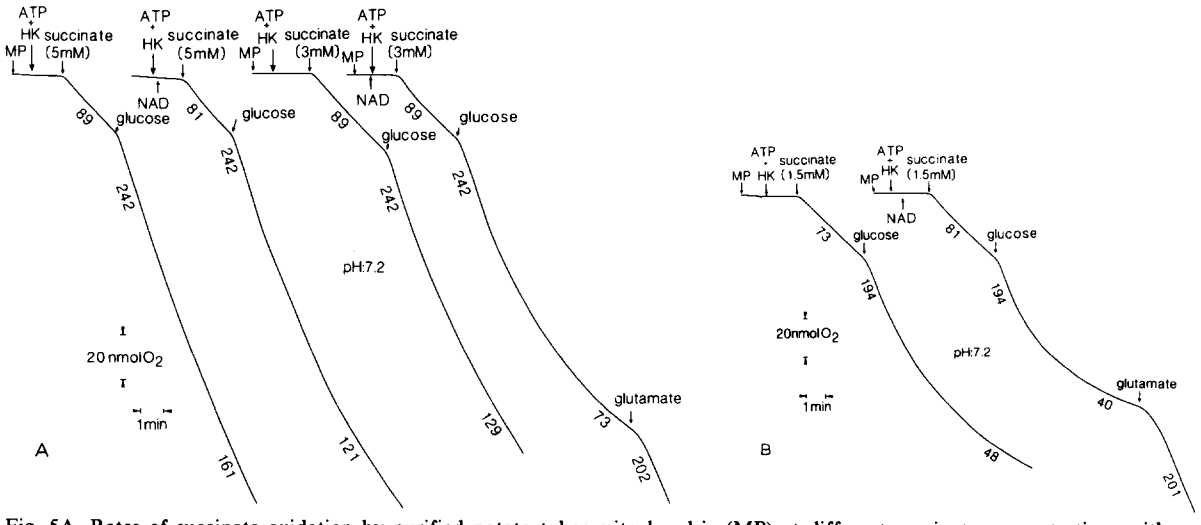

Fig. 5A. Rates of succinate oxidation by purified potato tuber mitochondria (MP) at different succinate concentrations with and without NAD^+, at pH 7.2. Succinate, 5 μmol and 3 μmol; protein = 124 μg/ml. ATP = 300 nmol; NAD^+ (as indicated) = 300 nmol; glutamate = 5 μmol. Total vol. 1 ml. B. Rate of succinate oxidation by purified potato tuber mitochondria with and without NAD^+ (pH 7.2)/succinate 1.5 μmol. Other components as in A.

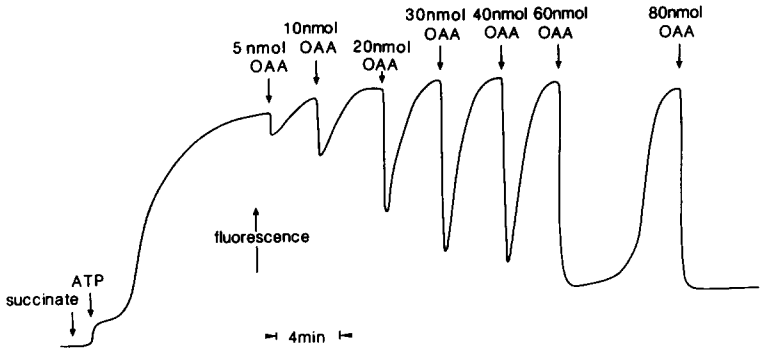

Fig. 6. NAD reduction linked to succinate oxidation by ATP-dependent reverse electron transport fluorimeter traces. Upward excursion of recorder pen indicates NADH formation (= increased fluorescence). The standard assay medium (medium A) contained 300 μM ATP, 10 mM succinate and 0.2 mg mitochondrial protein; the pH was 7.2 and the temperature 25°C. The final volume of the reaction mixture was 2 ml. The redox level of the endogenous NAD was monitored fluorimetrically with a fluorimeter assembled in our laboratory (see Ref. 7). OAA, oxaloacetate.

O_2 consumption at concentrations ranging from 10 to 130 μM oxaloacetate. Added oxaloacetate rapidly inhibited State 3 oxidation of succinate to a new steady-state rate, ranging from about 14% inhibition at 10 μM supplied oxaloacetate to over 85% at 80 μM oxaloacetate. This inhibition was strongly counteracted by glutamate. In State 4 (Table II) there was far less inhibition of succinate oxidation, with the addition of 80 μM oxaloacetate inhibiting O_2 uptake by only about 30%; the inhibition was even less with NAD^+ added in State 4.

Fig. 6 shows fluorimeter traces demonstrating the effect of oxaloacetate on NAD reduction brought about by succinate/ATP dependent reverse electron transport. In the presence of succinate, NAD reduction was initiated by addition of ATP. After completion of NADH formation, as indicated by the plateau in the fluorescence trace, addition of small amounts of oxaloacetate caused an immediate partial oxidation of the NADH (decrease in fluorescence) linked to reduction of the supplied oxaloacetate to malate by mitochondrial malate dehydrogenase. It is clear that even at the lowest concentration, oxaloacetate rapidly entered the matrix. Rapid penetration of plant mitochondria by oxaloacetate [17] is mediated by a special oxaloacetate transporter in plant mitochondira [18,19]. The oxaloacetate-induced rapid oxidation of NADH was followed by re-reduction of the NAD pool by further reverse electron transport from succinate. However, larger amounts of oxaloacetate (i.e., if the rate of oxaloacetate influx is higher than that of NADH production by reverse electron transport from suc-

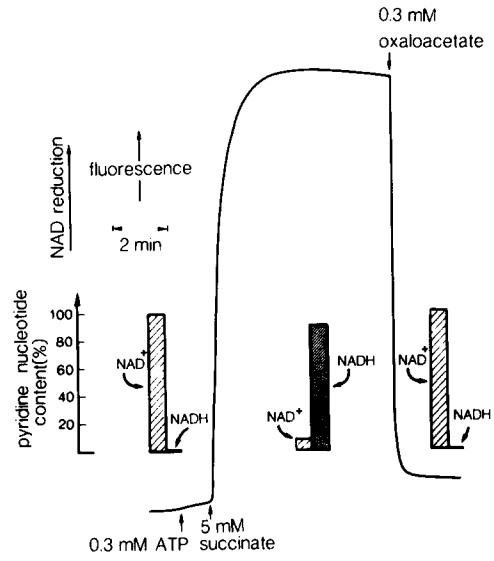

Fig. 7. Effect of exogenous oxaloacetate on the oxidation state of endogenous NAD and on pyridine nucleotide content (%) in purified potato tuber mitochondria oxidizing succinate. Fluorescence trace: the standard assay medium (medium A) contained 0.3 mM ATP, 5 mM succinate and 0.3 mg of mitochondrial protein. The pH was 7.2 and the temperature 25°C. The final volume of the reaction mixture was 2 ml. The redox level of the endogenous NAD was monitored fluorimetrically with a fluorimeter assembled in our laboratory [7]. Pyridine nucleotide assay: the standard assay medium (medium A) contained 0.3 mM ATP, 5 mM succinate and 3 mg of mitochondrial protein. The pH was 7.2 and the temperature 25°C. The final volume of the reaction mixture was 2 ml. Great care was taken to prevent anaerobiosis. As indicated, extraction and spectrophotometric assays of NAD and NADH were carried out according to Klingenberg [12]. The total amount of pyridine nucleotide (NAD + NADH) in freshly prepared potato mitochondria (NAD loaded, see Materials and Methods) was 4.5 nmol/mg protein.

cinate) could not be fully reduced to malate and therefore inhibited the subsequent reduction of NAD^+, since succinate dehydrogenase was severely inhibited. Subsequent addition of glutamate removed oxaloacetate by transamination and oxidation of succinate was reinitiated; i.e., succinate dehydrogenase was deinhibited, thus permitting further reverse electron transport (not shown). Interestingly, Fig. 7 indicates that, in the presence of succinate and ATP, the endogenous pool of NAD is almost entirely reduced, whereas in the presence of a large excess of oxaloacetate it is fully oxidized.

Discussion

The current investigation has demonstrated a strong regulatory effect of exogenously supplied NAD^+ on the oxidation of succinate by purified potato mitochondria under State 3 conditions. This regulation consists of an inhibition that is progressive with time. There is, however, no evidence that NAD^+ per se directly interacts with succinate dehydrogenase. The inhibition is abolished when glutamate is supplied either concurrently with NAD^+ or subsequently. Elevated NAD^+ levels favor oxaloacetate accumulation, and we confirmed that oxaloacetate severely inhibits succinate oxidation by intact potato mitochondria in State 3. Therefore, the probable cause of the inhibition brought about by NAD^+ is increased accumulation of oxaloacetate derived from malate. Glutamate removes oxaloacetate by transamination and thus prevents the inhibition. This explanation is in harmony with previous enzymological studies on the properties of plant succinate and malate dehydrogenases and with previous findings on the regulation of malate oxidation by NAD^+ [6,20]. During the course of succinate oxidation, malate thus formed can be oxidized by malic enzyme (which generates pyruvate) and/or by malate dehydrogenase (which produces oxaloacetate). Malate oxidation via malate dehydrogenase is particularly sensitive to the NADH/NAD^+ ratio in the mitochondrial matrix. This is largely because the equilibrium position of malate dehydrogenase strongly favors the reduction of oxaloacetate to malate rather than malate oxidation [15]; NAD^+ also counteracts ATP inhibition of malate dehydrogenase [21,22]

It is clear from Table II that the sensitivity of succinate oxidation to oxaloacetate is very much greater under State 3 than under State 4 conditions. The progressive inhibitory effect of NAD^+ on succinate oxidation is likewise very pronounced in State 3. In the absence of any metabolic reaction to remove oxaloacetate, oxaloacetate gradually accumulates in the matrix during succinate oxidation in State 3. (Here citrate synthase cannot remove oxaloacetate because of a lack of the co-substrate acetyl-CoA, since pyruvate dehydrogenase activity is low without added thiamine pyrophosphate.) This intramitochondrial oxaloacetate accumulation takes place even though much of the oxaloacetate is exported [23,24] via a specific carrier [18,19,24]. On the other hand, under State 4 conditions, oxaloacetate levels are low, because any accumulated oxaloacetate is reduced by malate dehydrogenase [15,24] using NADH derived either from succinate oxidation via reverse electron transport (Figs. 6 and 7) or (at pH 7.2) from the operation of malic enzyme. Thus, in State 4, added NAD^+ leads to a larger amount of NADH which in turn enables larger amounts of intramitochondrial oxaloacetate to be reduced to malate. In State 4 oxaloacetate is therefore less inhibitory to succinate oxidation in the presence of high matrix NAD content. (Table II). Moreover, in State 4 succinate dehydrogenase will be less oxaloacetate-sensitive than in State 3 because the highly reduced status of coenzyme Q in State 4 strongly activates succinate dehydrogenase, thereby counteracting the oxaloacetate inhibition [7]. Oxaloacetate accumulation has already been recognized as the principal cause of the decline in State 3 rates of malate oxidation under conditions (e.g., at pH > 7.6) where only the malate dehydrogenase enzyme can oxidize malate [23]. Clearly, oxaloacetate accumulation triggered by NAD^+ can also negatively regulate the oxidation of succinate.

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