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Comparison of the Kinetic Behavior toward Pyridine Nucleotides of NAD$^+$-Linked Dehydrogenases from Plant Mitochondria

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ABSTRACT

In this article we compare the kinetic behavior toward pyridine nucleotides (NAD$^+$, NADH) of NAD$^+$-malic enzyme, pyruvate dehydrogenase, isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, and glycine decarboxylase extracted from pea (*Pisum sativum*) leaf and potato (*Solanum tuberosum*) tuber mitochondria. NADH competitively inhibited all the studied dehydrogenases when NAD$^+$ was the varied substrate. However, the NAD$^+$-linked malic enzyme exhibited the weakest affinity for NAD$^+$ and the lowest sensitivity for NADH. It is suggested that NAD$^+$-linked malic enzyme, when fully activated, is able to raise the matricial NADH level up to the required concentration to fully engage the rotenone-resistant internal NADH-dehydrogenase, whose affinity for NADH is weaker than complex I.

Plant mitochondria seem to possess two internal NADH dehydrogenases on the inner surface of the inner membrane (10, 16). Complex I, the segment of the respiratory chain responsible for electron transfer from NADH to ubiquinone, and the rotenone-insensitive internal dehydrogenase operate in close relationship with all the NAD$^+$-linked TCA-cycle dehydrogenases, NAD$^+$-linked malic enzyme and glycine decarboxylase (in leaves), that share a common pool of NAD$^+$. When reduced by the matrix dehydrogenases, the pyridine nucleotide molecules diffuse to the inner membrane where they are oxidized. Under these conditions, the steric placement of the NAD$^+$-linked dehydrogenases within the matrix space (18), as well as their kinetic parameters (K_m[NAD$^+$], K_i[NADH]) (4) may be relevant in the outcome of the competition.

Because the rotenone-insensitive dehydrogenase, in contrast with complex I, exhibits a low affinity for internal NADH (16), the matrix concentration of NADH seems to play an important role in the regulation of the pathways responsible for endogenous NADH reoxidation. Furthermore, the rotenone-insensitive internal dehydrogenase is accessible to almost all matrix dehydrogenases, insofar as they can maintain a sufficient concentration of NADH (4). With the aim to understand the mechanism whereby the rotenone-insensitive pathway is engaged, in this paper we compare the kinetic behavior toward pyridine nucleotides of most of the matrix dehydrogenases extracted from pea leaves and potato tubers mitochondria: NAD$^+$-linked malic enzyme, pyruvate dehydrogenase, isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, and glycine decarboxylase from green tissues. Our results show that NAD$^+$-linked malic enzyme is less sensitive to NADH than the other matrix dehydrogenases, and lead us to discuss its eventual association with the cyanide-resistant electron pathway. It has been suggested that complex I may be associated with the normal cyanide-sensitive respiratory transport pathway, whereas the rotenone-insensitive internal NADH dehydrogenase is associated with the cyanide-resistant electron pathway providing a totally nonphosphorylating pathway for the oxidation of endogenous NADH (8, 16).

MATERIALS AND METHODS

Plant Material

Potato tubers (*Solanum tuberosum*) were obtained from a local market. Pea (*Pisum sativum* L. var Douce Provence) plants were grown from seeds in vermiculite for 15 d under a 18 h photoperiod of warm white light, at 25°C.

Preparation of Mitochondria

Mitochondria were isolated and purified from potato tubers as described by Neuburger *et al.* (12), using self-generating 28% (v/v) Percoll (Pharmacia) gradients. Mitochondria from pea leaves were isolated and purified as described by Douce *et al.* (3), using self-generating 28% (v/v) Percoll gradients and linear gradients of 0 to 10% (w/v; top to bottom) polyvinylpyrrolidone 25.

Measurement of Enzymic Activities

All assays were optimized with respect to the concentration of each component and to the pH of the reaction mixture. We verified that under these conditions, activities were linear with respect to the time for at least 2 min and were proportional to the amount of protein. The activity of each studied dehydrogenase was detected spectrophotometrically (Kon-

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tron, Uvikon 810) by following the reduction of NAD^+ to NADH at 340 nm. Incubation mixtures with a final volume of 0.5 mL were maintained at 25°C. The kinetic parameters of the dehydrogenases were calculated by linear regression on Lineweaver-Burk plots.

For NAD^+-linked malic enzyme (EC 1.1.1.39) the standard incubation mixture contained 30 mM Mops (pH 6.9), 200 μM $MnCl_2$, 5 mM β-mercaptoethanol, 200 μM coenzyme A, enzyme, and 50 mM malate (19). After 3 min incubation at 25°C, the reaction was initiated by the addition of NAD^+. Coenzyme A, a major activator of the plant malic enzyme, increases the affinity of the enzyme for NAD^+ (1, 19). For pyruvate dehydrogenase (EC 1.2.4.1), the standard incubation mixture contained 80 mM Mops (pH 7.5), 3 mM $MgCl_2$, 3 mM dithiothreitol, 400 μM coenzyme A, 400 μM thiamine pyrophosphate, 20 mM pyruvate, enzyme, and known amounts of NAD^+. For isocitrate dehydrogenase (EC 1.1.1.41), the standard incubation mixture contained 50 mM Hepes (pH 7.6), 1 mM $MgCl_2$, 20 mM isocitrate, enzyme, and known amounts of NAD^+. For α-ketoglutarate dehydrogenase (EC 1.2.4.2) the standard incubation mixture contained 20 mM KH_2PO_4 (pH 7), 10 mM $MgCl_2$, 10 mM dithiothreitol, 400 μM coenzyme A, 400 μM thiamine pyrophosphate, 1 mM ADP, 500 μM AMP (20), 20 mM α-ketoglutarate, enzyme, and known amounts of NAD^+. For glycine decarboxylase (EC 2.1.2.10) the standard incubation mixture contained 5 mM Mops, 5 mM Tris (pH 7.2), 1 mM β-mercaptoethanol, 1 mM $MgCl_2$, 1 mM EGTA, 20 μM pyridoxalphosphate, 350 μM H_4-folate (L-5,6,7,8-tetrahydropteroyl-L-glutamic acid, which had been prepared chemically by the catalytic hydrogenation of L-pteroylglutamic acid according to the method of Huennekens *et al.* [6]), enzyme, and known amounts of NAD^+. The medium and all the stock solutions were bubbled with argon to avoid the oxidation of H_4-folate to H_2-folate (L-7,8-dihydropteroyl-L-glutamic acid) in the presence of atmospheric O_2. The reaction, carried out under strict anaerobic conditions, was initiated by the addition of 20 mM glycine (2).

Solutions of NAD^+ (free acid, Boehringer), employed for the determination of kinetic constants were measured out by the method of Klingenberg (7).

Solubilization of Matrix Proteins

The purified mitochondrial pellets were suspended in a low osmolarity buffer, adapted to the further isolation of each concerned dehydrogenase and containing 4 μM leupeptin to protect the enzymes against endogenous proteases. Total release of the matrix proteins was achieved by three cycles of freezing and thawing. In the particular case of α-ketoglutarate dehydrogenase, 4 mM Chaps[2] were added to the medium in order to solubilize this complex. The mitochondrial suspension was frozen by placing it 2 min at liquid N_2 temperature. The frozen sample was then maintained at 30°C until thawed. This procedure breaks about 98% of the mitochondria. The disrupted membranes were sedimented by centrifugation for 1 h at 80,000*g*. The supernatant, which contained the matrix proteins, was concentrated by ultrafiltration on a XM 100 or XM 300 membrane (Diaflo, Amicon), using a stirring cell on a magnetic-stirring table (Amicon). At this stage, all the dehydrogenases could be stored at −80°C without deterioration for at least 2 months.

[2] Abbreviations: Chaps, cholamidopropyldimethylammoniopropanesulfonate; FPLC, fast protein liquid chromatography; LDS, lithium dodecyl sulfate.

Purification of the Matrix Dehydrogenases

All steps were carried out at 4°C. The methods were the same for both types of tissues.

NAD^+-Linked Malic Enzyme

The matrix proteins (280 mg), concentrated on a XM 100 membrane (Diaflo, Amicon) in a buffer A containing 10 mM Tris-HCl (pH 7.2), 1 mM β-mercaptoethanol, and 10 mM malate, were loaded on a DEAE-Trisacryl M (IBF) column (20 × 1 cm), equilibrated with the buffer A. The column was then washed with the same buffer to remove unbound proteins. This was followed by a 100 mL linear gradient of 10 to 100 mM Tris-citrate (pH 7.2), containing 10 mM malate and 1 mM β-mercaptoethanol (5). The NAD^+-linked malic enzyme was eluted when the Tris-citrate concentration was around 45 mM. Active fractions were pooled and concentrated by ultrafiltration on a PM 10 membrane (Diaflo, Amicon). The concentrated enzymatic extract (5–10 mg of protein) was loaded on a 30 × 1 cm Superose column (Pharmacia), equilibrated with buffer A. The column, connected to a Pharmacia FPLC system, was eluted with the same medium (flow rate 0.3 mL/min; fraction size 1 mL). Active fractions containing NAD^+-linked malic enzyme were combined and concentrated by ultrafiltration on a PM 10 membrane. The pooled fractions of NAD^+-linked malic enzyme (1–2 mg of protein) were applied to a 50 × 5 mm Mono-Q HR 5/5 column (Pharmacia), previously equilibrated with 10 mL of 10 mM Tris (pH 7.2) and 1 mM β-mercaptoethanol. The column, connected to a Pharmacia FPLC system, was eluted with a continuously increasing ammonium sulfate gradient (0–100 mM) (flow rate 0.3 mL/min; fraction size 0.6 mL). NAD^+-linked malic enzyme was eluted between 80 and 100 mM of ammonium sulfate, and active fractions were pooled and concentrated on a PM10 membrane. The sample was then stored at −80°C in the presence of 30 mM malate. The procedure resulted in a 250-fold purification. On the basis of LDS-PAGE, the NAD^+-linked malic enzyme that emerged from the ion-exchange column was more than 95% pure. In addition, the electrophoretic profile showed that the purified enzyme ran as two bands of equivalent proportions corresponding to molecular mass values of 59 and 62 kD, in agreement with Willeford and Wedding (21).

Pyruvate Dehydrogenase

The matrix proteins (80 mg), concentrated by ultrafiltration on a XM 300 Diaflo membrane in a buffer containing 5 mM Tris (pH 7.5), 3 mM $MgCl_2$, 3 mM dithiothreitol, 1 mM pyruvate, and 1 mM EDTA, were loaded on a 350 × 25 mm Sephacryl S-300 column (Superfine, Pharmacia), equilibrated with 5 mM Tris (pH 7.5), 1 mM pyruvate, 50 mM KCl, 2 mM

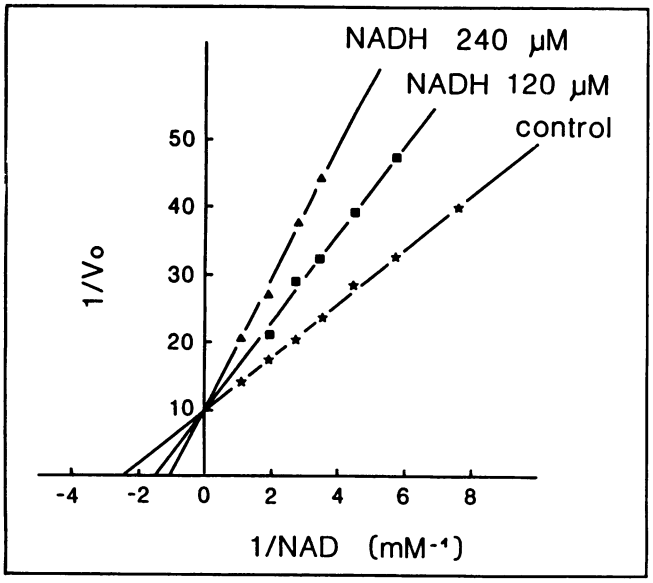

Figure 1. Initial velocity patterns of malate oxidation by purified NAD^+-linked malic enzyme isolated from potato tuber mitochondria. The reaction media and the preparation of the NAD^+-linked malic enzyme are described in the text. NAD^+ was the variable substrate and the concentration of NADH was fixed at zero (control) or at various concentrations as indicated. Malate oxidation was assayed at 25°C by measuring the formation of NADH at 340 nm, using a Kontron (Uvikon 810) spectrophotometer. V_o was the initial velocity of reaction and is expressed as nmol NADH·min^{-1}. The K_m for NAD^+ was 430 ± 10 μM; the K_i value for NADH was 190 ± 10 μM.

β-mercaptoethanol, and 1 mM EDTA. Proteins were eluted by the same buffer and the pyruvate dehydrogenase activity was completely recovered in the exclusion volume of the column. After concentration on a XM 300 Diaflo membrane, active fractions were stored at −80°C in the presence of 20 mM pyruvate and 0.4 mM thiamine pyrophosphate.

Isocitrate Dehydrogenase

The kinetic parameters of isocitrate dehydrogenase were measured directly on the matrix proteins (10 mg/mL), concentrated by ultrafiltration on a XM 100 Diaflo membrane in a buffer containing 5 mM KH_2PO_4 (pH 7.5), 2 mM $MgCl_2$, 0.1 mM EDTA, and 1 mM isocitrate. In this case, the matrix proteins were stored in the presence of 20 mM isocitrate and glycerol 5% (w/v), at −80°C.

α-Ketoglutarate Dehydrogenase

The matrix proteins (10 mg/mL), concentrated by ultrafiltration on a XM 100 Diaflo membrane in a buffer containing 10 mM KH_2PO_4 (pH 7), 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM EDTA, and 1 mM α-ketoglutarate, were precipitated by the addition of polyethylene glycol 6000 (Sigma), at a final concentration of 40 g/L. After stirring on ice during 1 h, the suspension was centrifuged for 20 min at 30,000*g*. The supernatant, devoid of α-ketoglutarate dehydrogenase activity, was discarded and the pellet was resuspended in a minimal volume of a solution containing 10 mM KH_2PO_4 (pH 7), 10 mM $MgCl_2$, 10 mM dithiothreitol, and 1 mM α-ketoglutarate. After centrifugation for 20 min at 10,000*g*, the supernatant contained 70% of the α-ketoglutarate dehydrogenase activity measured in the crude matrix extract. The sample was then stored at −80°C, in the presence of 1 mM α-ketoglutarate. Further purification of the complex of the α-ketoglutarate dehydrogenase led to a marked decrease in activity.

Glycine Decarboxylase

Glycine decarboxylase from pea leaf mitochondria was prepared according to Bourguignon *et al.* (2). Under these conditions, the matrix proteins suspended in a buffer containing 5 mM Mops, 5 mM Tris (pH 7), 1 mM β-mercaptoethanol, and 1 mM EGTA and concentrated by ultrafiltration on a XM 300 Diaflo membrane, contained all the proteins (P-, H-, T-, and L-proteins and serine hydroxymethyltransferase [SHMT]) involved in the conversion of glycine into serine. At this stage, the glycine cleavage/SHMT system could be stored at −80°C under N_2 without deterioration for several months (2).

RESULTS AND DISCUSSION

Each dehydrogenase showed perfect Michaelis behavior toward NAD^+ during kinetic experiments in the presence of the required cofactors and with saturating substrate concentrations. The K_m values for NAD^+ and the K_i values for NADH were determined by linear regression analysis of Lineweaver-Burk plots (Figs. 1, 2). Values obtained for the kinetic

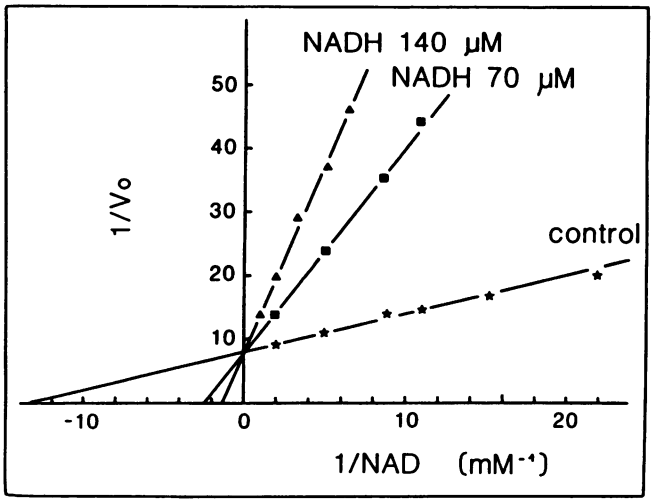

Figure 2. Initial velocity patterns of glycine oxidation by glycine decarboxylase isolated from pea leaf mitochondria. The reaction media and the preparation of the glycine decarboxylase are described in the text. NAD^+ was the variable substrate and the concentration of NADH was fixed at zero (control) or at various concentrations as indicated. Glycine oxidation was assayed at 25°C, in the presence of argon, by measuring the formation of NADH at 340 nm, using a Kontron (Uvikon 810) spectrophotometer. V_o was the initial velocity of reaction and is expressed as nmol NADH·min^{-1}. The K_m value for NAD^+ was 75 ± 10 μM and the K_i value for NADH was 15 ± 5 μM.

Table I. *Kinetic Parameters (K_m[NAD^+]; K_i[NADH]) of Various Matrix Dehydrogenases (Glycine Decarboxylase; NAD^+-Linked Malic Enzyme; NAD^+-Linked Tricarboxylic-Acid-Cycle Dehydrogenases) from Pea Leaf and Potato Tuber Mitochondria*

All enzyme preparations were purified or partially purified (see the text). Substrate oxidations (see the text) were assayed at 25°C, by measuring the formation of NADH at 340 nm, using a Kontron (Uvikon 810) spectrophotometer.

Dehydrogenases	K_m for NAD^+		K_i for NADH	
	Pea	Potato	Pea	Potato
NAD^+-linked malic enzyme	410 ± 10	430 ± 10	180 ± 20	190 ± 15
Glycine decarboxylase	75 ± 10	Not present	15 ± 5	Not present
Pyruvate dehydrogenase	140 ± 10	200 ± 10	30 ± 5	25 ± 5
α-Ketoglutarate dehydrogenase	ND[a]	300 ± 10	ND	30 ± 5
Isocitrate dehydrogenase	190 ± 10	195 ± 5	50 ± 5	60 ± 10

[a] Not determined because of the high instability of the α-ketoglutarate complex in the case of pea leaf mitochondria.

constants are shown in Table I. Several points of interest emerge from these studies.

First, NADH competitively inhibited all the studied dehydrogenases including NAD^+-linked malic enzyme when NAD^+ was the varied substrate. The apparent K_i values for NADH were 2 to 5 times lower than the K_m for NAD^+. These results demonstrate, therefore, that the steady state activities of these dehydrogenases are sensitive to the NADH/NAD^+ molar ratios. In other words, increasing the ratio of NADH to NAD^+ in the matrix space results in a logarithmic increase in the inhibition of the dehydrogenases. Interestingly, glycine decarboxylase, which is very sensitive to NADH, does not escape the control exerted by the pyridine nucleotides on every matrix oxidative mechanism (for review see Wiskich and Dry [22]).

Second, NAD^+-linked malic enzyme did not behave like the other matrix dehydrogenases with respect to the pyridine nucleotides (Table I). Concerning NAD^+, the different dehydrogenases exhibited various degrees of affinity for this coenzyme, the NAD^+-linked malic enzyme having the weakest one (K_m above 400 μM) (1, 9, 19). The fact that the affinity of the NAD^+-linked tricarboxylic-acid-cycle dehydrogenases for NAD^+ is much higher than that of the NAD^+-linked malic enzyme could explain why the decline in the O_2-uptake rate with malate is faster than that observed with oxoglutarate or pyruvate during mitochondrial aging (14): as the level of NAD^+ falls progressively in the mitochondria, it becomes too low to act as a substrate for NAD^+-linked malic enzyme, but it is still sufficient to engage the NAD^+-linked tricarboxylic-acid-cycle dehydrogenases. Also, this could explain numerous results showing that the concentration of NAD^+ within the mitochondrial matrix influences strongly the activity of NAD^+-linked malic enzyme, with higher concentrations stimulating and this can be altered by adding NAD^+ to isolated mitochondria (13, 15, 17) owing to the presence of a specific NAD^+ carrier on the inner mitochondrial membrane (11). In other words, NAD^+-linked malic enzyme does not compete favourably at the level of matrix NAD^+. With respect to NADH, the kinetic studies show that most of the matrix dehydrogenases, glycine decarboxylase complex from pea leaf mitochondria, pyruvate dehydrogenase complex, isocitrate dehydrogenase, and oxoglutarate complex from pea leaf and potato tuber mitochondria were strongly inhibited as soon as the NADH concentration increased in the medium because their apparent K_i values for NADH were less than 50 μM. In contrast, concerning NAD^+-linked malic enzyme, the apparent K_i value for NADH was 4 to 12 times higher (180–200 μM), displaying therefore a markedly lower sensitivity to NADH (Table I). This intrinsic property may explain why malate oxidation can occur when the NADH reoxidation by complex I is blocked by rotenone (15). Under such conditions, NAD^+-linked malic enzyme, when fully activated (19), is able to raise the matrix NADH level up to the required concentration to fully engage the rotenone-resistant internal NADH-dehydrogenase, whose affinity for NADH is weaker than complex I (16). In contrast, malate dehydrogenase with its unfavorable equilibrium constant and the other dehydrogenases with their low apparent K_i values for NADH cannot trigger the full strength of the rotenone-insensitive pathway. All these results together strongly suggest that NAD^+-linked malic enzyme and NADH oxidases (complex I and rotenone-insensitive NADH oxidase) are not linked together and interact via a soluble and nonspecific pool of NAD^+ (4, 10).

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