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Regulation of NAD- and NADP-dependent isocitrate dehydrogenases by reduction levels of pyridine nucleotides in mitochondria and cytosol of pea leaves

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Abstract

Regulation of NAD- and NADP-dependent isocitrate dehydrogenases (NAD-ICDH, EC 1.1.1.41, and NADP-ICDH, EC 1.1.1.42) by the level of reduced and oxidized pyridine nucleotides has been investigated in pea (*Pisum sativum* L.) leaves. The affinities of mitochondrial and cytosolic ICDH enzymes to substrates and inhibitors were determined on partially purified preparations in forward and reverse directions. From the kinetic data, it follows that $NADP^+$- and NAD^+-dependent isocitrate dehydrogenases in mitochondria represent a system strongly responding to the intramitochondrial NADPH and NADH levels. The NADPH, $NADP^+$, NADH and NAD^+ concentrations were determined by subcellular fractionation of pea leaf protoplasts using membrane filtration in mitochondria and cytosol in darkness and in the light under saturating and limiting CO_2 conditions. The cytosolic NADPH/NADP ratio was about 1 and almost constant both in darkness and in the light. In mitochondria, the NADPH/NADP ratio was low in darkness (0.2) and increased in the light, reaching 3 in limiting CO_2 conditions compared to 1 in saturating CO_2. At high reduction levels of NADP and NAD observed at limiting CO_2 in the light, i.e. when photorespiratory glycine is the main mitochondrial substrate, isocitrate oxidation in mitochondria will be suppressed and citrate will be transported to the cytosol ('citrate valve'), where the cytosolic NADP-ICDH supplies 2-oxoglutarate for the photorespiratory ammonia refixation.

Keywords: Ammonia refixation; Citrate valve; Isocitrate dehydrogenase; NAD(P)H/NAD(P) ratio; Photorespiration; Plant mitochondria

1. Introduction

Operation of the tricarboxylic acid (TCA) cycle in the light fulfills metabolic and energy demands of photosynthesizing cells. Mitochondrial function in the light consists in photosynthetic cells in regulating redox balance and producing metabolites for anabolic reactions and ATP as a source of energy, which is transported to the cytosol [1,2]. In ambient air, the main flux in the mitochondrial respiratory chain of C_3 plants is provided by the oxidation of NADH from photorespiratory glycine, which exceeds that from the TCA cycle [2]. When the pathways of electron transport coupled to ATP synthesis are saturated, oxidation of NADH originated from glycine may switch to the pathways of mitochondrial electron transport noncoupled to energy conservation [3].

In photosynthetic tissues, respiratory decarboxylation is usually lower in the light than in darkness [2,4]. It can be controlled at the step of the pyruvate dehydrogenase complex via inhibition by NADH, ATP and photorespiratory ammonia and reversible phosphorylation [5]. The conversion of isocitrate to 2-oxoglutarate (OG) by isocitrate dehydrogenase (ICDH) may represent another major controlling point. This step has a lower maximal capacity than other TCA cycle reactions, which is why it might be limiting for the overall rate of the cycle [6] and therefore important for the regulation of carbon flow in the TCA cycle. Thus in the light, the glycolytic product is converted

Abbreviations: GDC, glycine decarboxylase complex; cNADP-ICDH, cytosolic $NADP^+$-dependent isocitrate dehydrogenase; mNADP-ICDH, mitochondrial $NADP^+$-dependent isocitrate dehydrogenase; NAD-ICDH, NAD^+-dependent isocitrate dehydrogenase; OAA, oxaloacetate; OG, 2-oxoglutarate; TCA cycle, tricarboxylic acid cycle

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to citrate, which is transported to the cytosol. A partial TCA cycle operating in the light was proposed by Chen and Gadal [7] and further investigated by Heldt's group on the isolated mitochondria [8,9]. ^{13}C nuclear magnetic resonance studies using intact leaves confirmed that citrate is a major mitochondrial product in the light [10]. Tricarboxylate (or citrate) carriers were purified from mitochondrial membranes and characterized [11–13]. They can exchange citrate with different TCA intermediates, including OG, malate and OAA. Citrate export from mitochondria is probably more important than isocitrate export, since aconitase equilibrium is strongly displaced towards citrate [6].

How can the transition from the complete TCA cycle operating in the dark to the partial TCA cycle operating in the light be achieved? A key for understanding this mechanism is the regulation of ICDH in mitochondria. Mitochondria contain two isocitrate dehydrogenases, one NAD-dependent and the other NADP-dependent [14–16]. In the present investigation, we compare the NAD- and NADP-ICDH enzymes in plant mitochondria and cytosol by their kinetic properties, and analyze their operation in connection with regulation by reduced and oxidized pyridine nucleotide levels in the mitochondrial matrix. Particularly, we measured NAD^+, NADH, $NADP^+$ and NADPH contents in plant mitochondria and cytosol and related this to the kinetic properties of NAD- and NADP-ICDH. Our analysis leads to a conclusion that switching between the direct and reverse reactions of NADP-ICDH together with operation of NAD-ICDH represent a fine mechanism for regulation of intramitochondrial and cytosolic levels of pyridine nucleotides, OG and (iso)citrate. At high reduction levels of NAD and NADP in mitochondria in the light, caused by the flux of photorespiratory glycine, isocitrate oxidation will be suppressed resulting in citrate export to the cytosol.

2. Materials and methods

2.1. Plant material and growth conditions

Pea (*Pisum sativum* L., cv 294 040 Märgärt Kelvedon Wonder) plants were grown in a greenhouse in soil with a 17 h period of artificial light of 300 μmol m^{-2} s^{-1} (metal-halogen lamps Powerstar HQI-T 400 W/D, OSRAM, München, Germany). The temperature was 23 °C during the day and 20 °C at night.

2.2. Isolation of mitochondria and assays

Mitochondria were isolated from extracts of leaf tissue using self-generated Percoll gradients [17]. NAD^+-dependent ICDH (EC 1.1.1.41) was measured spectrophotometrically essentially as described by Cox and Davies [18] in 25 mM HEPES–KOH buffer, pH 7.5, containing 2 mM threo-D_s-isocitrate (Fluka, Switzerland), 1 mM $MnCl_2$, 1 mM NAD^+ and 0.1% Triton X-100 (only for total mitochondrial fraction). For $NADP^+$-dependent ICDH (EC 1.1.1.42), NAD^+ was substituted by $NADP^+$ (0.1 mM). At pH higher than 8.0, HEPES was substituted by Tricine (25 mM).

Reverse reaction of NADP-ICDH was spectrophotometrically measured in 25 mM Bis–Tris buffer, containing 1 mM OG, 5 mM $NaHCO_3$, 0.15 mM NADPH, and (only for total mitochondrial fraction) 0.1% Triton X-100, or radiometrically in 0.5 ml of the same medium, containing 5 mM $NaH^{14}CO_3$ (radioactivity 0.1 mBq ml^{-1}). After 15-min incubation, the reaction was stopped by addition of 0.2 ml 1 M H_2SO_4. Samples were allowed to stay overnight and radioactivity was detected by liquid scintillation counter.

Oxygen evolution by protoplasts and uptake by mitochondria was measured using a Hansatech oxygen electrode [19]. Chlorophyll concentration was measured according to Bruinsma [20]. The protein content was estimated by the method of Lowry et al. [21] after precipitation with trichloroacetic acid. BSA was used as a standard.

2.3. Purification of isocitrate dehydrogenases

For partial purification of isocitrate dehydrogenases from mitochondria, mitochondrial fractions after Percoll gradient were washed from Percoll, pelleted, resuspended in 5 mM Mops buffer, pH 7.2 and subjected to sonication five times for 5 s with 10-s interval. After centrifugation at 100,000 × *g* for 1 h, the pellet was discarded and supernatant regarded as the matrix fraction was fractionated by ammonium sulfate. NAD- and NADP-dependent isocitrate dehydrogenases were fractionated at different concentrations of ammonium sulfate (between 30–45% and 60–80% of saturation correspondingly). The pellet after ammonium sulfate fractionation containing mitochondrial NADP-ICDH (mNADP-ICDH) was desalted on a PD-10 column equilibrated with 10 mM potassium phosphate buffer, pH 7.6, containing 1 mM $MgCl_2$. Then, it was passed through a DEAE cellulose column equilibrated by the same buffer. ICDH was eluted by a step gradient of increasing concentration of the buffer between 30 and 40 mM potassium phosphate.

For purification of the cytosolic ICDH (cNADP-ICDH), the cytosolic fraction (after differential centrifugation) was fractionated by ammonium sulfate (60–80% of saturation), gel-filtrated on the PD-10 column and purified on DEAE cellulose column. ICDH was eluted by step gradient between 20 and 30 mM potassium phosphate. The partially purified cNADP-ICDH and mNADP-ICDH were quite stable and could be stored for months at −80 °C. NAD-ICDH was stable for few hours only in ammonium sulfate. It was not subjected for further purification. The activity of NAD-ICDH preparation after ammonium sulfate fractionation was 0.33 μmol NADH formed min^{-1} mg^{-1} protein, which corresponded to the threefold enrichment from the matrix fraction.

NADP-ICDH activities in the matrix and cytosolic fractions were in a range of 0.11–0.12 μmol NADPH formed mg^{-1} protein min^{-1}. After ammonium sulfate fractionation, they increased to 0.7–0.8 μmol mg^{-1} min^{-1}, and after

DEAE cellulose they were 7–9 μmol mg^{-1} min^{-1}. This corresponded to 70–80-fold purification with a recovery of 10–20%. For native electrophoresis, active fractions were concentrated by membrane filtration using Microsep Microconcentrator Pall Filtron (Pall Gelman Laboratories, Ann Arbor, MI, USA) and loaded on 7.5% polyacrylamide gel. Activity staining was performed in Tris–HCl buffer, pH 8.0, containing 5 mM threo-D_s-isocitrate, 1 mM $MgCl_2$, 0.5 mM $NADP^+$, 0.2 mM nitroblue tetrazolium and 0.1 mM phenazine methosulfate. The mitochondrial enzyme revealed higher mobility in 7.5% native PAAG (0.30 compared to 0.25) (Fig. 1).

The kinetic properties were investigated on preparations of NADP-ICDH after DEAE-cellulose and of the fraction after ammonium sulfate fractionation for NAD-ICDH. Michaelis constants were determined from double-reciprocal plots by varying concentration of one substrate at saturating concentration of the other. Inhibitory constants were obtained from Dixon plots by varying concentration of the inhibitor at fixed concentrations of a substrate [22]. The values presented in tables are average means of three to four independent measurements (in μM) with standard deviations of 25–30%.

2.4. Isolation and fractionation of protoplasts

Protoplasts were isolated from leaves of 10–12-day-old plants after stripping lower epidermis and fractionated by membrane filtration as described earlier [23]. Protoplasts (Chl concentration 70 μg ml^{-1}) were incubated in 1 ml assay medium [0.25 M sucrose, 0.25 M sorbitol, 5 mM EGTA, 0.06% (w/v) BSA, 0.2% (w/v) PVP, 10 mM HEPES, pH 8.2]. This medium was applied for separation of organelles from pea protoplasts since it prevents the adhesion of chloroplasts and mitochondria [24]. Protoplasts were irradiated for 7 min at 500 μmol m^{-2} s^{-1} (xenophot HLX slide projector lamps, OSRAM) after preincubation in the dark for 10 min. This light intensity was saturating for photosynthesis. Saturating CO_2 was provided by the addition of 10 mM $NaHCO_3$ to the medium. Limiting CO_2 (corresponding to about 50% photosynthetic O_2 evolution compared to saturating CO_2 conditions) was provided by the addition of 0.15 mM $NaHCO_3$. Protoplasts were ruptured on a selected 15-μm nylon net (Henry Simon Ltd., Stockport, UK). Chloroplasts were separated by two (12 and 8 μm) MF-Millipore membrane filters; mitochondria were separated by a 0.45-μm filter.

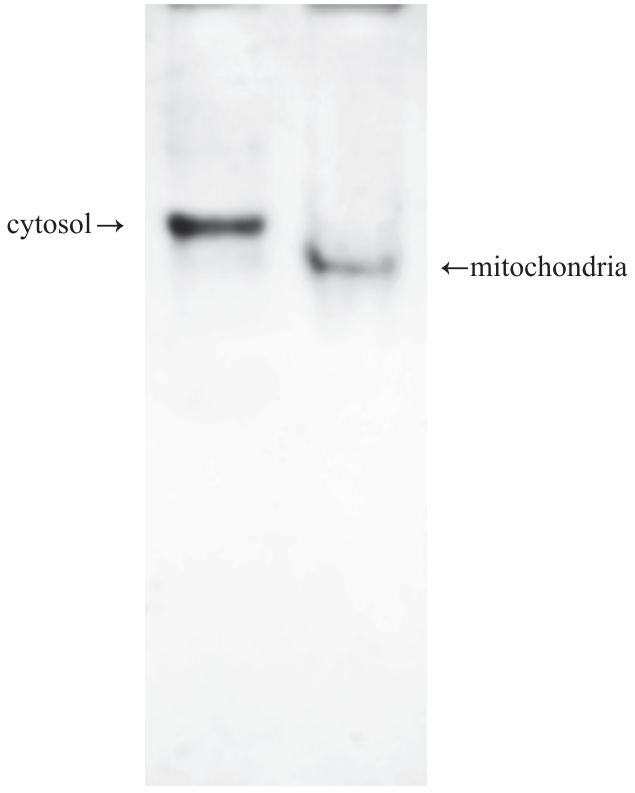

Fig. 1. Electrophoregrams of NADP-dependent isocitrate dehydrogenase from cytosol and mitochondria of pea leaves. The activity was stained with nitroblue tetrazolium as described in Materials and methods.

2.5. Determination of pyridine nucleotides

For determination of reduced and oxidized pyridine nucleotides, filtrates were mixed with 0.5 ml each of the following: (1) 0.2 M HCl + 1% (w/v) Chaps; (2) 0.2 M KOH + 0.08% (w/v) Triton X-100; and (3) 0.17 M sorbitol, 0.3 mM $MgCl_2$, 17 mM HEPES, pH 7.0 [25]. $NADP^+$ and NAD^+ were determined in acid extracts (1), NADPH and NADH in alkaline extracts (2), and marker enzymes in buffer extracts (3). Alkaline extracts were heated at 60 °C for 15 min for complete destruction of $NAD(P)^+$ and then cooled on ice [25]. The dilution of the samples after rapid fractionation was determined by comparing the refractive index of the samples to that of pure solutions. Cross-contamination between organelles was measured using the marker enzymes [23] and did not exceed 7–10%. Pyridine nucleotides were determined by enzymatic cycling [26] as described earlier [27] and quantified by fluorescence emission at 460 nm, with 365 nm as the excitation wavelength, on a FluoroMax-2 spectrofluorometer (Instruments S.A., Inc., Edison, NJ, USA). NADH/NAD and NADPH/NADP ratios were determined for the paired data of each individual experiment and standard deviations were calculated from five to six replications. Statistically significant differences according to the paired comparisons *t*-test are discussed.

3. Results

3.1. Isocitrate dehydrogenase activities in photosynthetic and nonphotosynthetic tissues

In etiolated leaves of pea and barley, the NAD-ICDH activity was similar to that in photosynthetic tissues, whereas the activity in potato tubers was slightly higher as compared to leaves (Table 1). The mitochondrial NADP-

Table 1
NADP-ICDH and NAD-ICDH activities associated with mitochondria isolated from leaves of different plants and their ratios

Plant	Organ	NADP-ICDH	NAD-ICDH	NADP-ICDH/ NAD-ICDH
Pea	green leaf	72 ± 8	84 ± 7	0.85 ± 0.11
	etiolated leaf	38 ± 3	96 ± 7	0.40 ± 0.08
Barley	green leaf	78 ± 7	95 ± 6	0.82 ± 0.10
	etiolated leaf	34 ± 5	100 ± 8	0.34 ± 0.06
Potato	green leaf	37 ± 6	88 ± 5	0.42 ± 0.07
	tuber	21 ± 5	113 ± 11	0.19 ± 0.06

Activities are given in the forward reaction at pH 7.5 and expressed in nmol mg^{-1} mitochondrial protein min^{-1}. The values are means and S.D. of three replicates based on comparisons of the paired data.

Table 3
Reverse reaction of ICDH measured as incorporation of the label from $NaH^{14}CO_3$ to acid-stable compounds

Substrate added	nmol mg^{-1} mitochondrial protein min^{-1}
No addition	5.1 ± 1.1
+OG	5.3 ± 1.4
+OG+NADH	5.4 ± 1.3
+OG+NADPH	31 ± 3
+OG+NADPH+NAD	34 ± 5
+OG+NADPH+NADP	13 ± 4

Mitochondria were incubated in the presence of OG (5 mM) and pyridine nucleotides (1 mM) in different combinations.

ICDH activity was about twice higher in green leaves than in the nonphotosynthetic tissues. The data in the Table 1 represent the values for pH 7.5. At pH 8.5, which is optimal for NADP-ICDH, the difference would be somewhat larger. For the cytosolic NADP-ICDH, the activity was similar for both nonphotosynthetic and photosynthetic tissues (data not shown). In all plants examined, the ratio of NADP- to NAD-dependent ICDH in mitochondria was higher in photosynthetic tissues as compared to nonphotosynthetic tissues.

3.2. Kinetic characteristics of ICDH enzymes in forward and reverse reactions

The kinetic parameters for NAD-ICDH (K_m for NAD^+, K_i for NADH, $K_{0.5}$ for threo-D_s-isocitrate and K_i for NADPH) in the forward direction (isocitrate + $NAD^+ \rightarrow$ OG + NADH + CO_2) are of the order of 100–250 μM (Table 2), which is close to the values previously reported, e.g. the K_m value for NAD^+ was earlier reported to be 190–200 μM [11,28]. NAD-ICDH was unable to catalyze the reverse reaction, which was shown by inability to incorporate labeled bicarbonate (Table 3) as well as to monitor oxidation of NADH in the presence of OG and $NaHCO_3$ (data not presented). NAD-ICDH revealed a pH optimum with a peak about 7.5 (Fig. 2A).

There was no activity of NADP-ICDH isoenzymes with NAD^+ at the sensitivity limit of <0.5% of the rate with $NADP^+$. The forward reaction of both mNADP-ICDH and cNADP-ICDH was about tenfold more sensitive to inhibition by NADPH than by NADH (Table 2). Also, the two forms showed similar specificity to substrates and inhibitors. Inhibition by OG was low (in the mM range) and bicarbonate had no inhibitory effect (Table 2). In the reverse reaction, the mNADP-ICDH enzyme exhibited higher affinity to its substrate, bicarbonate, NADPH and to the inhibitors $NADP^+$ and isocitrate compared to cNADP-ICDH. NADH (at 0.1 mM) and NAD^+ (at 1 mM) had no inhibitory effect. Incorporation of radioactive label from $NaH^{14}CO_3$ also confirmed inhibition by $NADP^+$ and isocitrate and no inhibition by NADH and NAD^+ (Table 3).

Both mitochondrial and cytosolic forms of NADP-ICDH showed broad optimum in forward reaction at pH 8–9 (Fig. 2B). In the reverse reaction (measured both in mitochondrial fraction by incorporation of $NaH^{14}CO_3$ and spectrophotometrically on purified enzyme fraction), the pH optimum for mNADP-ICDH was 6.8–7 (Fig. 2A,B). For the cNADP-ICDH, it was 6.3–6.5, with a low activity at pH 7 (Fig. 2B). According to the pK of bicarbonate dissociation, the K_m of 0.2 mM of the mitochondrial NADP-ICDH for bicarbonate at pH 7 corresponds to a value of about 0.05 mM for CO_2, which is the true substrate for carboxylation reaction [29]. The K_m of 1.6 mM at pH 7 for the cytosolic enzyme corresponds to a concentration of about 0.4 mM for CO_2.

3.3. Pyridine nucleotide contents in pea leaf protoplasts

Estimation of pyridine nucleotides concentrations in fractionated pea leaf protoplasts showed that the mitochondrial NADH + NAD^+ concentration was about 1.5 mM and the NADPH + $NADP^+$ concentration about 0.3 mM if we

Table 2
Kinetic constants (Michaelis constants in bold and inhibitory constants in italics, μM) of NAD-ICDH, mNADP-ICDH and cNADP-ICDH in forward and reverse reactions

	NAD^+	NADH	$NADP^+$	NADPH	Isocitrate	OG	$NaHCO_3$
NAD-ICDH (pH 7.5)	**200**	*180*	*No effect*	*260* (nc)	**120**[a]	*No effect*	*No effect*
mNADP-ICDH (pH 8.0)	*No effect*	*60*	**3**	*5*	**4**	*>1000*	*No effect*
mNADP-ICDH (reverse, pH 7.0)	*No effect*	*No effect*	*6*	**9**	*5*	**17**	**200**
cNADP-ICDH (pH 8.0)	*No effect*	*80*	**4**	*7*	**6**	*>1000*	*No effect*
cNADP-ICDH (reverse, pH 7.0)	*No effect*	*No effect*	*21*	**24**	*12*	**26**	**1600**

nc—noncompetitive inhibition; competitive type is not indicated.
[a] $S_{0.5}$ is given.

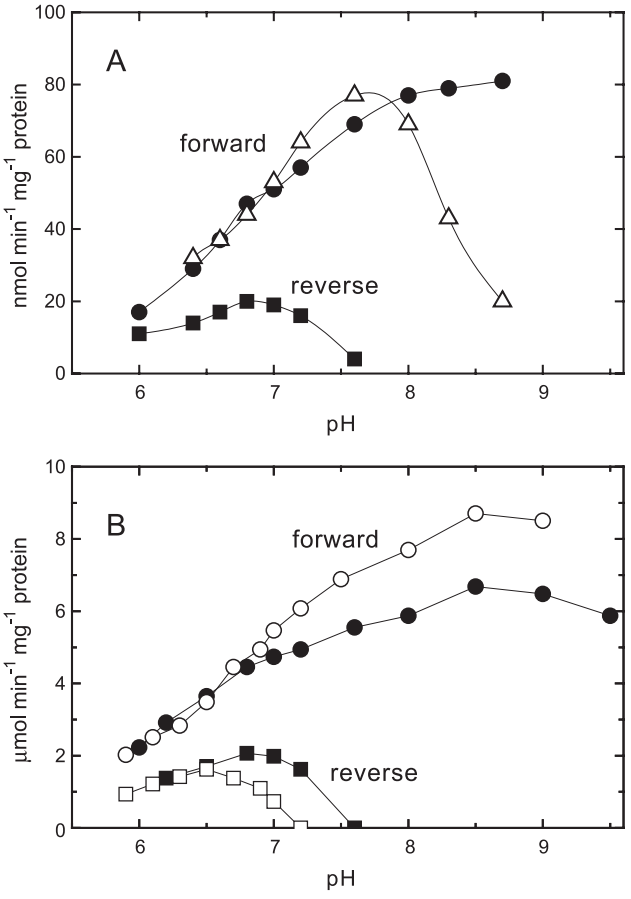

Fig. 2. The dependence of isocitrate dehydrogenase activities on pH in forward and reverse reactions. (A) Measurements on mitochondrial preparations. Open triangles—forward reaction of NAD-ICDH (spectrophotometrically measured by reduction of NAD^+); closed circles—forward reaction of mNADP-ICDH (spectrophotometrically measured by reduction of $NADP^+$); closed squares—reverse reaction of mNADP-ICDH (measured by incorporation of radioactive label from $NaH^{14}CO_3$). (B) Spectrophotometric measurements on partially purified cNADP-ICDH (open symbols) and mNADP-ICDH (closed symbols). Circles—forward reaction; squares—reverse reaction.

apply organelle volumes as determined by Heldt's group for barley [30], spinach [31] and potato [32]. In all these plants, the subcellular volumes in mesophyll protoplasts were remarkably similar, so we have used these values also for pea. The actual mitochondrial matrix content of pyridine nucleotides is somewhat higher, taking into account that the intermembrane space occupies a part of the total organelle volume. The reduction levels of NAD and NADP in cytosol and mitochondria significantly differ depending upon conditions (Table 4, Fig. 3). In darkness, CO_2 concentration did not have any effect on these ratios exceeding standard deviation of the experiments—thus, we present combined data for saturating and limiting CO_2—while in the light, CO_2 exhibited a pronounced effect.

The NAD pool was very oxidized in the cytosol with NADH concentration at the detection limit, also in mitochondria the pool was relatively oxidized (Table 4, Fig. 3) being more reduced in the light in limiting CO_2 conditions.

Table 4
Mitochondrial and cytosolic concentrations (μM) of NAD^+, NADH, $NADP^+$ and NADPH in pea leaf protoplasts

	NADH	NAD^+	NADPH	$NADP^+$
Mitochondria				
Dark	75 ± 30	1520 ± 170	45 ± 20	265 ± 45
Light, limiting CO_2	455 ± 120	1550 ± 220	235 ± 60	75 ± 10
Light, saturating CO_2	130 ± 60	1410 ± 160	115 ± 20	135 ± 20
Cytosol				
Dark	18 ± 14	570 ± 30	165 ± 25	145 ± 25
Light, limiting CO_2	55 ± 35	520 ± 15	180 ± 40	140 ± 30
Light, saturating CO_2	24 ± 12	615 ± 40	140 ± 30	140 ± 20

Protoplasts were incubated in darkness and in saturating light (500 μmol quanta m^{-2} s^{-1}) under limiting and saturating CO_2. The values (means from five to six replications and S.D.) are based on subcellular volumes estimated in Refs. [30–32].

The cytosolic NADH/NAD ratio measured in our study was however higher than that calculated by Heineke et al. [33] from concentrations of metabolites and equilibria of malate dehydrogenase and transaminase in the cytosol, since the ratio obtained in their work was the ratio of free pyridine nucleotides, while our method allowed measurement of total fractions of free and bound nucleotide species.

The NADH concentration in mitochondria in photorespiratory conditions (~ 450 μM) was more than twice higher than the K_i of NAD-ICDH (180 μM), while the K_i of mNADP-ICDH (60 μM) was lower than the concentration of NADH in the light in saturating CO_2 (~ 130 μM). The cytosolic and mitochondrial NADP pools were more reduced than the NAD pools (Table 4, Fig. 3). The cytosolic pool was about the same in darkness and in the light independently on CO_2 concentration. In darkness, the mitochondrial NADP pool was mainly oxidized; its reduction level increased in the light, much more in photorespiratory conditions approaching the same level as K_i of NAD-ICDH.

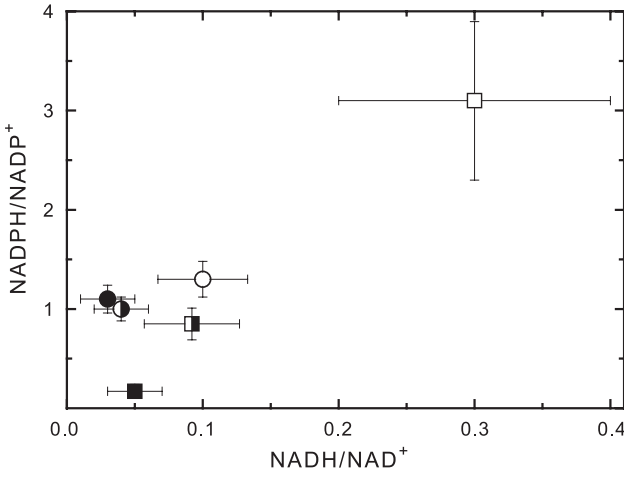

Fig. 3. NADPH/NADP ratios plotted against NADH/NAD ratios in mitochondria (squares) and cytosol (circles) rapidly fractionated from pea leaf protoplasts in darkness (black) and in the light in saturating CO_2 (half-black) and limiting CO_2 conditions (white). The S.D. are calculated from average differences between the paired data.

4. Discussion

The data obtained indicate that in vivo the ICDH enzymes are strongly regulated by the level of reduced and oxidized pyridine nucleotides. Noncompetitive inhibition of NAD-ICDH by NADPH has K_i (260 μM) similar to the total NADP + NADPH concentration in the matrix (Tables 2 and 4), which is close to the previously reported value of 300 μM [11]. Thus, NADPH will markedly inhibit the enzyme when the NADPH/NADP ratio is high (as it is in the light). NAD-ICDH operates only in the forward direction and is most active when the concentration of isocitrate becomes high, when pH is near 7.5 and the reduction level of the matrix is low. This is in line with the situation in darkness. The NAD-ICDH can be suggested to have a role in maintaining complete TCA cycle operation under conditions of sufficient isocitrate supply.

NADP-ICDH activity in mitochondria is higher in photosynthetic than in nonphotosynthetic tissues (Table 1). This may indicate an important role of this enzyme during photosynthesis. The suggestion has been made that NADP-ICDH in mitochondria can provide NADPH for redox regulation of the alternative oxidase protein via thioredoxin system [34]. Since the highest NADPH/NADP ratio in mitochondria of photosynthetic tissues is observed in the light in photorespiratory conditions (Fig. 3), an alternative is to propose that a higher NADPH/NADP ratio is a consequence of active oxidation of glycine turning on the transhydrogenation between NADH and $NADP^+$ [35,36]. As a result, NAD-ICDH will be inhibited and the mNADP-ICDH will operate in the reverse direction. This provides appreciable conditions for efflux of citrate, which may be particularly important in photorespiratory conditions for providing carbon skeletons for ammonia refixation [8], for maintaining the cytosolic NADPH/NADP ratio [37] and for expression of the alternative oxidase gene [38]. OG released from glutamate in aminotransferase reactions may be imported into mitochondria in exchange with citrate [13] and enter the reverse NADP-ICDH reaction when reduction level in mitochondria is high and the flux via OG dehydrogenase complex is low (probable situation in photorespiratory conditions). Thus, the reverse reaction of the mitochondrial NADP-ICDH may be important at higher reduction levels of NADP for recycling OG and NADPH in mitochondria. OG itself is an important signaling compound regulating pyruvate kinase and PEP carboxylase in the cytosol and citrate synthase and alternative oxidase in mitochondria, all involved in redox control between the cytosol and mitochondria [39].

Different electrophoretic mobilities of the cytosolic and mitochondrial NADP-ICDH proteins indicate that they are different isoenzymes [16,40]. The kinetic properties of both isoenzymes show their high affinity to substrates and inhibitors. The mitochondrial enzyme is more specific to its substrates and inhibitors in the reverse reaction; it possesses physiological pH optimum (6.8–7.0) as compared to the optimum of the cytosolic isoenzyme, which is displaced to the acidic range (6.3–6.5) and has higher affinity for CO_2.

Determination of NADH/NAD and NADPH/NADP ratios shows that photosynthesis particularly at limiting CO_2 concentration (in photorespiratory conditions) leads to increased reduction levels of NAD and NADP in pea mitochondria (Fig. 3). The same was observed also for the mitochondria from barley protoplasts [27]. The glycine decarboxylase complex (GDC) is strongly inhibited by NADH with the K_i of 15 μM and K_m for NAD^+ 75 μM, i.e. it has five times higher affinity for NADH than for NAD^+ [41]. The increase of NADH/NAD ratio in photorespiratory conditions will provide a restriction for GDC operation and a necessity to remove NADH. Possible pathways for this are the export of reducing equivalents by malate/OAA shuttle [42], the oxidation of NADH in the ETC, including induction of noncoupled pathways of electron transport [3] or transhydrogenation with $NADP^+$ [35,36].

In darkness, when both NADP and NAD pools in mitochondria are oxidized, both isocitrate dehydrogenases will operate in the forward direction and the partitioning of metabolic flux between them will depend on the concentration of isocitrate. During photosynthesis in nonphotorespiratory conditions, NAD-ICDH will retain some ability to oxidize isocitrate since the reduction level in mitochondria is not too high. The NADH/NAD ratio is not significantly higher in nonphotorespiratory conditions as compared to darkness. This corresponds to the oxidation of mainly the TCA intermediates both in darkness and in the light in high CO_2, while in photorespiratory conditions, also a major flux of glycine is oxidized in mitochondria. In nonphotorespiratory conditions, if concentrations of isocitrate and OG in mitochondria are in the same range, the mitochondrial NADP-ICDH will be near equilibrium between the forward and reverse reactions because of the similar levels of $NADP^+$ and NADPH and a balance between oxidation and export of (iso)citrate will be maintained. In photorespiratory conditions, when NADPH/NADP ratio is high, only the reverse reaction of NADP-ICDH may take place and NAD-ICDH is inhibited. Participation of two ICDH in mitochondria and one ICDH in cytosol in interconversions between isocitrate and OG represents a flexible mechanism in which the ICDH enzymes are involved in the maintenance of the metabolic balance between reduced and oxidized pyridine nucleotides. This mechanism also suggests a possibility of transhydrogenation between NADPH (at its high reduction level) and NAD^+ when NADP-ICDH operates in the reverse direction supplying isocitrate to NAD-ICDH.

Mass action ratio of pyridine nucleotides (NADPH/NADP)/(NADH/NAD) obtained in our study and in the previous work on barley [27] is not far from the equilibrium; the same is also observed in bacteria lacking proton-translocating and possessing a soluble non-energy-linked trans-

hydrogenase [43]. In animal mitochondria, active membrane potential dependent transhydrogenase pushes pyridine nucleotide mass action ratio to very high values, up to 500 at high potential and near 100 at low potential [44]. Sazanov and Jackson [45] proposed that NAD-ICDH in forward reaction and NADP-ICDH in reverse reaction constitute a substrate cycle via which the transmembrane proton potential gradient regulates the TCA cycle activity. Plant mitochondria lack proton-translocating transhydrogenase and possess two non-energy-linked transhydrogenase activities, one belonging to the side reaction of complex I, and the other to a soluble transhydrogenase-like enzyme [35]. In higher plant mitochondria in situ, according to our estimations, the mass action ratio is changing from lower levels (~ 1) in darkness to some higher levels (near 10) in the light (Fig. 3). Switching between the direct and reverse reactions of NADP-ICDH together with operation of the NAD-ICDH represents a fine mechanism for regulation of intramitochondrial levels of NAD^+, NADH, $NADP^+$ and NADPH, where transhydrogenase-like equilibration between the reduced and oxidized pyridine nucleotides governs the efflux/influx of citrate/OG via a specific carrier protein [13].

In stress condition, when the levels of H_2O_2 and NO increase in mitochondria, citrate efflux may also be connected with direct inhibition of aconitase by these signal compounds [46,47]. Alternatively, a flux via NAD-ICDH may provide some OG formation directly in the mitochondria, which is facilitated by induction of NAD-ICDH in the condition of increased nitrogen supply [48]. However, it was demonstrated that the citrate export is more than 10 times higher than the export of OG during oxidation of OAA by isolated pea mitochondria and nearly twice higher for spinach mitochondria [8]. High citrate levels in plant leaves present in the light decrease during light–dark transition [49]. It is very probable that the metabolic system for ammonia refixation is robust, flexible and has a redundant capacity. This is proved by the fact that the transgenic plants of potato down regulating the cICDH have very similar metabolic characteristics as wild-type plants [50]. Probably in these plants, mitochondria will supply mainly OG instead of citrate for ammonia refixation, which will decrease metabolic flexibility but will not affect their survival in nonstress conditions.

Citrate export from mitochondria may be important for maintaining the cytosolic NADPH/NADP ratio in the light. In photorespiratory conditions, a part of NADPH pool in the cytosol is used for reduction of glyoxylate and hydroxypyruvate exported from peroxisomes [37]. NADPH/NADP turnover may be provided by participation of cNADP-ICDH and NADP-dependent hydroxypyruvate reductase [51]. The kinetic properties of cNADP-ICDH favor the forward reaction. The reverse reaction has nonfavorable pH optimum and very high K_m for CO_2, shown in earlier investigations [52–54] and in the present study. Isocitrate formation can increase NADPH level also in peroxisomes via the peroxisomal NADP-ICDH [55] and in chloroplasts if some isocitrate is oxidized by the chloroplastic NADP-ICDH [56].

We propose the operation of two redox valves in photosynthetic plant cell, one driven by chloroplasts and another driven by mitochondria. The malate valve, driven by NADPH formed by photosynthetic electron transport, raises subcellular NADH/NAD ratios [57], whereas the citrate valve, driven by the increased reduction level in mitochondria, reduces subcellular NADP pools. Operation of the modified TCA cycle and the citrate valve maintains also subcellular concentrations of OG, OAA and pyruvate, important for nitrogen assimilation in the light and supporting ammonia refixation from the photorespiratory glycine. This all constitutes a major feature of bioenergetics of photosynthetic plant cells in the light.

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