The regulation of exogenous NAD(P)H oxidation in spinach (*Spinacia oleracea*) leaf mitochondria by pH and cations

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Essentially chlorophyll-free mitochondria were isolated from green leaves of spinach (*Spinacia oleracea* L. cv. Viking II). Uncoupled oxidation of exogenous NADPH (1 mM) to oxygen had an optimum at pH 6.0, and activity was relatively low at pH 7.0, even in the presence of 1 mM-CaCl₂. There was a proportional increase in the apparent K_m for NADPH with decreasing H⁺ concentrations, suggesting that NADPH protonated on the 2'-phosphate group was the true substrate. Exogenous NADH was oxidized by oxygen with an optimum at pH 6.9. Under low-cation conditions, EGTA or EDTA (both 1 mM) had no effect on the V_{max} of NADH oxidation, although the removal of bivalent cations from the membrane surface by the chelators could be observed by use of 9-aminoacridine fluorescence. In contrast, under high-cation conditions, chelators lowered the V_{max} by about 50%, probably due to a better approach of the negatively charged chelators to the negative membrane surface than under low-cation conditions. In a low-cation medium, the V_{max} of NADH oxidation was increased by about 50% by the addition of cations. This was caused by a lowering of the size of the negative surface potential through charge screening. In contrast with other cations, La³⁺ inhibited NADH oxidation, possibly through binding to lipids essential for NADH oxidation. The apparent K_m for NADH varied 6-fold in response to changes in the size of the surface potential, suggesting that the approach of the negatively charged NADH to the active site is hampered by the negative surface potential. The results demonstrate that the spinach leaf cell can regulate the mitochondrial NAD(P)H oxidation through several mechanisms: (1) the pH; (2) the cation concentration in general; and (3) the concentration of Ca²⁺ in particular. The results also emphasize the importance of electrostatic considerations when investigating the kinetic behaviour of membrane-bound enzymes.

INTRODUCTION

Mitochondria from higher plants and fungi oxidize exogenously added NADH and NADPH [see Palmer & Møller (1982) for a brief review]. NAD(P)H oxidation forms a potentially important link between the redox level of the cytoplasm and the respiratory chain. It is probably caused by two different dehydrogenases, one specific for NADH, the other specific for NADPH, both located on the outer surface of the mitochondrial inner membrane. The electrons from NAD(P)H enter the respiratory chain at ubiquinone, and their passage to oxygen is coupled to the synthesis of two ATP molecules (Koeppel & Miller, 1972; Arron & Edwards, 1979, 1980; Møller & Palmer, 1981a).

The oxidation of NAD(P)H by plant mitochondria is affected by cations in several different ways. (a) It is dependent on Ca²⁺ (Coleman & Palmer, 1971; Cowley & Palmer, 1978; Arron & Edwards, 1979; Møller & Palmer, 1981b; Møller *et al.*, 1981; Moore & Åkerman, 1982), which is often found bound to the mitochondrial membranes in sufficient amounts to ensure maximal activity (Møller *et al.*, 1981). (b) In the presence of sufficient Ca²⁺, it is stimulated unspecifically by cations in the order of efficiency C³⁺ > C²⁺ > C⁺ (Hackett, 1961; Johnston *et al.*, 1979; Møller & Palmer, 1981c; Møller *et al.*, 1984). This is due to electrostatic screening of the

negatively charged membrane, i.e. to a reduction in the size of the surface potential which enhances rate-limiting step(s) in electron transport between ubiquinone and oxygen (Møller & Palmer, 1981c; Møller *et al.*, 1984). (c) It is inhibited by La³⁺ binding in mitochondria from some tissues (Møller *et al.*, 1982). (d) Its pH optimum is 7.0–7.2 for NADH (Arron & Edwards, 1980; Møller & Palmer, 1981a, b; Møller *et al.*, 1983) and lower, pH 5.5–6.7 depending on the tissue, for NADPH (Møller & Palmer, 1981a, b). These properties provide the plant cell with several different ways of regulating the rate of NAD(P)H oxidation *in vivo*.

The investigation of mitochondria from green tissues was long hampered by the lack of suitable methods for removing thylakoid membranes in the mitochondrial preparation. However, we now have available purified mitochondria from spinach leaves with a minimum of contamination (Bergman *et al.*, 1980). It has thus been possible to show that mitochondria from photosynthetic tissue differ from mitochondria from non-photosynthetic tissue of the same plant in several important aspects, such as the ability to oxidize glycine (Gardeström *et al.*, 1980). This oxidation of glycine by leaf mitochondria has precedence over the oxidation of other substrates like external NADH, malate and succinate (Bergman & Ericson, 1983; Dry *et al.*, 1983). To understand this preference for glycine, we need to know more about the

Abbreviations used: (DM)Br₉, NNNN'N'N'-hexamethyldecane-1,10-diamine bromide; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenyl-hydrazine; (TEC)Cl₃, tris(ethylenediamine)cobalt(III) chloride.

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basic properties of substrate oxidation. The purpose of the present investigation was to characterize the effect of cations and pH on the oxidation of external NADH and NADPH by spinach leaf mitochondria.

MATERIALS AND METHODS

Plant material

Spinach (*Spinacia oleracea* L. cv. Viking II) was grown at 18 °C under artificial light ($80 \mu\text{E} \cdot \text{s}^{-1} \cdot \text{m}^{-2}$) with a light period of 12 h in a nutrient solution as described by Siegenthaler & Depéry (1976), except that the Fe^{2+} concentration was doubled. Fully expanded leaves (6–8 weeks old), with mid-veins removed, were used for the preparation of mitochondria.

Isolation of mitochondria

Essentially chlorophyll-free mitochondria were isolated by a preparation procedure using differential centrifugation, phase partition and a discontinuous Percoll gradient (Bergman *et al.*, 1980). The mitochondria were freed from Percoll by centrifugation twice at 20000 *g* for 10 min after dilution with at least 10 vol. of 0.3 M-sucrose/2 mM- K^+ -Mops, pH 7.00. The resulting pellet was resuspended in the same medium.

NAD(P)H oxidation

The NAD(P)H oxidation was measured either as oxygen consumption or as NAD(P)H consumption. The oxygen uptake was measured in a Hansatech (King's Lynn, Norfolk, U.K.) oxygen electrode at 25 °C in a total volume of 0.5 ml. The electrode was calibrated against air-saturated water, assuming an O_2 concentration of 250 μM . Measurements of the NAD(P)H consumption were made in a Beckman Acta III spectrophotometer at 340 nm at 20–21 °C. The medium employed and the additions made are specified for each experiment in the legends to the Figures and Tables. In all cases the mitochondria were uncoupled by the presence of 0.4 μM -FCCP.

9-Aminoacridine fluorescence

The fluorescence of 9-aminoacridine was measured as described by Johnston *et al.* (1979) in a Perkin-Elmer 512 fluorescence spectrophotometer. Excitation wavelength was 398 nm (slit width 10 nm) and emission was measured at 456 nm (slit width 10 nm). The medium employed and the additions made are as specified in the legend to Fig. 2.

Protein determination

Protein content was measured by the method of Lowry *et al.* (1951), with bovine serum albumin (Sigma) as standard.

RESULTS AND DISCUSSION

pH-dependence

Uncoupled NADH oxidation by chlorophyll-free mitochondria from spinach leaves was optimal at pH 6.7–7.0 (Fig. 1), in good agreement with previous results on mitochondria from potato (*Solanum tuberosum*) (Arron & Edwards, 1980) and Jerusalem-artichoke (*Helianthus tuberosus*) tubers, *Arum maculatum* (cuckoo-pint) spadices (Møller & Palmer, 1981a; Møller *et al.*,

Fig. 1. NAD(P)H oxidation by spinach leaf mitochondria as a function of pH

The rate of O_2 consumption was measured in 0.3 M-sucrose/0.1 M- K^+ -Mes (○, Δ) [or 0.1 M- K^+ -Mops (●, ▲)]/0.4 μM -FCCP/2 mM-(DM)Br₂, 60 μg of mitochondrial protein/ml being used. The reactions were started by adding 1 mM-NADH (○, ●) or 1 mM-NADPH (Δ, ▲).

1983) and the fungus *Neurospora crassa* (Møller *et al.*, 1982).

Uncoupled NADPH oxidation by spinach leaf mitochondria showed a pH optimum at pH 6.0 (Fig. 1), as did NADPH oxidation by mitochondria from Jerusalem-artichoke tubers, but lower than for *Arum* (pH 6.6) (Møller & Palmer, 1981a, b) and *Neurospora* (pH 7.8) (Møller *et al.*, 1982) mitochondria.

In Jerusalem-artichoke and *Arum* mitochondria the rates of NADH and NADPH oxidation were identical at pH 4.0–5.5, indicating that a common rate-limiting step was shared (Møller & Palmer, 1981a). In contrast, spinach leaf mitochondria oxidized NADPH significantly faster than NADH at pH 5.0–5.5 (Fig. 1). This is consistent with the conclusion that two different dehydrogenases are responsible for the oxidation of NADH and NADPH in plant mitochondria (Arron & Edwards, 1979, 1980; Møller & Palmer, 1981a; Nash & Wiskich, 1983; Petit, 1984).

At pH 7.0–7.4, NADPH oxidation was low, and, in contrast with what has been reported for mitochondria from maize (*Zea mays*) leaves (Petit, 1984) and pea (*Pisum sativum*) leaves (Nash & Wiskich, 1983), 1 mM- CaCl_2 did not cause a significant stimulation of NADPH oxidation under high-cation conditions (results not shown). The reason for this difference is not clear, but it may be of importance for the understanding of the function of leaf mitochondria. On the other hand, the difference is probably not of direct physiological interest, since the concentration of free Ca^{2+} in the cytoplasm of plant cells probably lies in the micromolar range (Williamson, 1981).

Cation-membrane interactions

The interaction between the mitochondrial membranes and cations was investigated by use of 9-aminoacridine fluorescence. In solution, 9-aminoacridine is a fluorescent univalent cation, the fluorescence of which decreases when negatively charged particles (e.g. carboxymethyl-cellulose particles, liposomes, biological membranes) are

Fig. 2. Effect of EGTA, EDTA and KCl on 9-aminoacridine fluorescence

The fluorescence were measured as described in the Materials and methods section by using 0.18 mg of mitochondrial protein/ml in 0.3 M-sucrose/2 mM-K⁺-Mops (pH 7.2)/20 μM-9-aminoacridine. EGTA, EDTA and KCl were added consecutively to the final concentrations indicated. At the end of the experiment, 16 mM-MgCl₂ was added to obtain the highest release of fluorescence quenching (Searle *et al.*, 1977).

added (Chow & Barber, 1980). This is due to concentration quenching as 9-aminoacridine is concentrated into the diffuse layer associated with the negatively charged surface of the particles (Searle *et al.*, 1977; Chow & Barber, 1980). This effect has been seen with plant mitochondria from various sources and was also seen for spinach leaf mitochondria (Fig. 2). The addition of EGTA decreased the fluorescence further, indicating an increased concentration of 9-aminoacridine in the vicinity of the membrane surfaces brought about by an increase in the negative surface potential due to the

removal of Ca²⁺ by the chelator (Møller *et al.*, 1981). The addition of EDTA after EGTA caused the removal of Mg²⁺ and thus a further decrease in fluorescence (Fig. 2). The relative effect of EGTA and EDTA has been suggested to be a measure of the relative amount of Ca²⁺ and Mg²⁺ bound to the membranes (Møller *et al.*, 1981; Møller, 1983). For spinach leaf mitochondria the ratio of the decrease in fluorescence caused by EGTA and EDTA was 0.68 ± 0.02 (mean ± s.e.m. for three preparations) (Fig. 2), indicating more Ca²⁺ than Mg²⁺ bound to the spinach mitochondria. After the fluorescence was brought down to its minimal value by the chelators, the initial fluorescence could be restored by the addition of cations, causing a decrease in the size of the surface potential through electrostatic screening (Fig. 2).

NADH oxidation and cations

At neutral pH, NADH oxidation was strongly stimulated by the addition of salt to the basic low-salt medium (Table 1). There appeared to be no specificity, as Mg²⁺ and DM²⁺ stimulated as much as Ca²⁺ at the same concentration. K⁺ stimulated at 100 mM, whereas the tervalent cation, tris(ethylenediamine)Co³⁺, stimulated at only 200 μM. The anion was unimportant (Table 1). These results are similar to what has been observed with other types of plant (Johnston *et al.*, 1979; Møller & Palmer, 1981c; Møller *et al.*, 1981, 1984) and fungal mitochondria (Møller *et al.*, 1982). They indicate that the stimulation of NADH oxidation by cations is due to electrostatic screening, which decreases the negative surface potential of the mitochondrial inner membrane as shown in Fig. 2. This will be treated in more detail below.

In the absence of FCCP, Ca²⁺ appeared to uncouple NADH oxidation at pH 7.0–7.4 (results not shown). The reason for this effect is not known. Ca²⁺ in combination with chlorotetracycline inhibited NADH oxidation strongly (Table 1). In Jerusalem-artichoke mitochondria such an effect was interpreted as being due to a Ca²⁺ requirement by the NADH dehydrogenase (Møller *et al.*, 1983), but the relatively small effect of the chelators

Table 1. Effect of cations and chelators on NADH oxidation in spinach leaf mitochondria

Rates, given as nmol of O₂/min per mg of protein, are means ± s.d. (number of independent preparations). The measurements were done as described in the Materials and methods section, in 0.3 M-sucrose/2 mM-K⁺-Mops (pH 7.2)/0.4 μM-FCCP/mitochondrial protein (0.18–0.25 mg/ml). Cations and chelators were added to the final concentration indicated before the reaction was started by addition of 1 mM-NADH. Abbreviation used: CTC, chlorotetracycline

Salt	Chelator...	Rate (nmol/min per mg)		
		None	EGTA (1 mM)	EDTA (1 mM)
None		131 ± 12 (4)	120 ± 18 (3)	121 ± 15 (3)
Preincubation				
11 min		—	—	114 (1)
5 min		—	—	112 (1)
10 min		—	—	119 (1)
KCl (100 mM)		226 ± 67 (3)	93 ± 23 (2)	86 ± 6 (2)
(DM)Br ₂ (2 mM)		210 ± 37 (3)	149 ± 14 (2)	140 ± 1 (2)
MgCl ₂ (2 mM)		195 ± 32 (3)	126 ± 9 (2)	—
CaCl ₂ (2 mM)		213 ± 26 (2)	—	—
CaCl ₂ (2 mM) + CTC (50 μM)		53 ± 18 (2)	—	—
(TEC)Cl ₃ (200 μM)		210 ± 38 (2)	—	—
LaCl ₃ (200 μM)		74 ± 21 (4)	—	—

Fig. 3. The effect of La^{3+} on the fluorescence of 9-aminoacridine and on the oxidation of exogenous NADH by spinach leaf mitochondria

The oxidation of NADH (Δ) by spinach mitochondria (0.27 mg of protein/ml) was measured in 0.3 M-sucrose/2 mM-(DM)Br₂/5 mM-K⁺-Mops (pH 7.2)/0.4 μM -FCCP with 1 mM-NADH as substrate. The fluorescence of 9-aminoacridine ($\circ$) was measured in 0.3 M-sucrose/2 mM-K⁺-Mops (pH 7.2)/20 μM -9-aminoacridine/mitochondrial protein (0.27 mg/ml). LaCl_3 was added to the final concentration indicated.

EDTA and EGTA on NADH oxidation by spinach leaf mitochondria (Table 1) makes such a conclusion less secure (see, however, below). La^{3+} , another relatively specific inhibitor of the NADH dehydrogenase in Jerusalem-artichoke mitochondria (Møller *et al.*, 1983), also inhibited NADH oxidation by spinach leaf mitochondria strongly (Table 1). This inhibition by La^{3+} was closely correlated with the decrease in the size of the surface potential of the membranes as monitored by 9-aminoacridine fluorescence (Fig. 3). This is the fourth type of plant and fungal mitochondrion on which the effect of La^{3+} on NADH oxidation and 9-aminoacridine fluorescence has been investigated (Figs. 5 and 6 in Møller *et al.*, 1982), and in all cases different patterns have been obtained: (1) stimulation of NADH oxidation and monophasic increase in 9-aminoacridine fluorescence with increasing La^{3+} concentration (*Arum maculatum*); (2) inhibition of NADH oxidation and a concentration lag in the effect on 9-aminoacridine fluorescence (Jerusalem artichoke); (3) inhibition of NADH oxidation after a concentration lag and a two-phase effect on 9-aminoacridine fluorescence (*Neurospora crassa*); (4) inhibition and a monophasic increase in 9-aminoacridine fluorescence (Fig. 3). It would be interesting to know what causes these differences, which may be connected to differences in the amount of charged lipids to which La^{3+} has been suggested to bind (Møller *et al.*, 1982).

Although the experiments with 9-aminoacridine clearly showed that Ca^{2+} (and Mg^{2+}) was removed from the membrane surface by EGTA and EDTA at pH 7.0 under low-cation condition (Fig. 2), the chelators had no effect on NADH oxidation under the same conditions. Preincubation for 10 min did not cause any additional effect under low-cation conditions (Table 1). In the presence of 2 mM-(DM)Br₂, about a one-third inhibition was observed and about 60% in the presence of 100 mM-KCl (Table 1). An inhibition by EGTA was observed even in the presence of an excess of Mg^{2+} (Table

1), pointing at Ca^{2+} as the ion involved. An attempt to induce a Ca^{2+} requirement by extensive washing with EGTA/ MgCl_2 or EGTA/(DM)Br₂ was not successful. It was not possible to remove more than about two-thirds of the original Ca^{2+} content as measured with atomic-absorption spectroscopy. NADH oxidation by these mitochondria still showed no specific requirement for Ca^{2+} (results not shown).

In Jerusalem-artichoke mitochondria the effect of chelators on NADH oxidation was also dependent on the cation concentration (Møller *et al.*, 1981): the chelators were very ineffective when added during NADH oxidation under low-cation conditions. When added before NADH, chelators inhibited less under low- than under high-cation conditions. These effects were interpreted as being due to problems of approach of the negatively charged chelators (EGTA and EDTA have two and three negative charges respectively, at pH 7; Sillén & Martell, 1964, 1971) to the highly negative surface of the mitochondrial inner membrane under low-cation conditions (Møller *et al.*, 1981). However, the situation was more extreme with spinach leaf mitochondria, since chelators had no effect on NADH oxidation under low-cation conditions.

From the results mentioned above the surface charge of spinach leaf mitochondria would be expected to be more negative than in Jerusalem-artichoke mitochondria. This hypothesis will be further investigated by comparative studies on membrane properties like isoelectric points, charge densities and lipid composition. Some results of such studies have been reported (Edman & Ericson, 1984).

Kinetics of NADH oxidation

In a low-cation medium the size of the negative surface potential of a biological membrane is maximal (i.e. most negative). Addition of cations causes a decrease in the size of the surface potential through electrostatic screening, as illustrated in Fig. 2 for spinach leaf mitochondria. A reduction of the surface potential affects the kinetics of membrane-bound enzymes: the apparent K_m for charged substrates is increased (positive substrate) or decreased (negative substrate) (Wojtczak & Nalecz, 1979; Nalecz *et al.*, 1980; Møller & Palmer, 1981c; Wojtczak *et al.*, 1982; Møller *et al.*, 1984; Thibaud *et al.*, 1984), and the apparent pH optimum shifted to lower values (Douzou & Maurel, 1977). In the case of multi-step electron transfer, electrostatic screening can affect the V_{max} of the process (Møller & Palmer, 1981c; Gibrat & Grignon, 1982; Møller *et al.*, 1984). The lack of inhibition of NADH oxidation by chelators in spinach leaf mitochondria under low-cation conditions (Table 2) allowed us to investigate the effect of three different surface potentials on the kinetics of NADH oxidation: (a) high cation concentration, where the surface potential is near zero; (b) low-cation conditions, where the surface potential is low; and (c) low-cation conditions + 1 mM-EDTA, where the surface potential has been made even more negative through the removal of bound bivalent cations (as observed by a decrease in 9-aminoacridine fluorescence in Fig. 2).

The apparent K_m for NADH varied in response to the surface potential (Table 2). It was highest when the surface potential was most negative (low cation + EDTA) and lowest when the surface potential was near zero (high cation). When the ratios of the apparent K_m , obtained in

Table 2. Effect of salt on the kinetic parameters of NADH oxidation of spinach leaf mitochondria

The oxidation of NADH was measured at 340 nm as described in the Materials and methods section by using 33–50 μg of mitochondrial protein/ml. The low-salt medium contained 0.3 M-sucrose, 5 mM- K^+ -Mops, pH 7.0, and 0.4 μM -FCCP. Low salt + EDTA was low salt plus 1 mM EDTA, and high-salt conditions were obtained by adding 2 mM-(DM) Br_2 to the low-salt medium. V_{max} values (in nmol of NADH/min per mg of protein) and K_m values (in μM) were calculated by linear-regression analysis of v (nmol of NADH/min per mg of protein) against v/s (nmol of NADH/min per mg of protein and μM). Data are given as means $\pm$ S.D. (number of preparations). Values for *Arum* spadix and Jerusalem-artichoke tubers are from Møller & Palmer (1981c).

Conditions	Parameter	Mitochondria from...	Spinach leaf	<i>Arum</i> spadix	Jerusalem-artichoke tubers
Low salt	V_{max}		200 $\pm$ 51 (3)	1260 $\pm$ 50 (2)	390 (1)
	K_m		26 $\pm$ 7 (3)	31 $\pm$ 4 (2)	91 (1)
Low salt + EDTA	V_{max}		190 $\pm$ 41 (5)	—	—
	K_m		52 $\pm$ 18 (5)	—	—
High salt	V_{max}		250 $\pm$ 57 (8)	1490 $\pm$ 160 (2)	555 (1)
	K_m		14 $\pm$ 4 (8)	19 $\pm$ 1 (2)	54 (1)

media (a)–(c) above, were calculated from experiments made on the same preparation, the mean proportion $\pm$ S.D. (number of preparations) was found to be 3.0 ± 0.38 (3):1:0.42 $\pm$ 0.03 (4). Since NADH is negatively charged at pH 7.0, this is consistent with the suggestion that the NADH concentration near the active site of the external NADH dehydrogenase in the inner membrane of plant mitochondria is regulated by the repulsion of NADH from the membrane surface (Johnston *et al.*, 1979). The relative size of the observed change in the apparent K_m with changes in the surface potential (by a factor 3–6) is comparable with what has been observed on other membrane-bound enzymes (for references, see Johnston *et al.*, 1979). The apparent K_m for NADH in spinach leaf mitochondria is very similar to that in *Arum* mitochondria, but 3–4 times lower than in Jerusalem-artichoke mitochondria (Table 2).

The V_{max} values determined for NADH oxidation in the spectrophotometer (Table 2) were about 30% lower than expected from the rates observed with the oxygen electrode (Table 1). At least part of this discrepancy may be due to different temperatures used for the two assays, i.e. 25 °C for O_2 consumption and 20 °C for NADH oxidation. The trend in the changes in V_{max} were the same, however (cf. Tables 1 and 2), which was the main point of the V_{max} determinations (Table 2).

The observed increase in V_{max} for the multistep reaction $\text{NADH} \rightarrow \text{O}_2$ in spinach leaf mitochondria as the surface potential is made less negative by electrostatic screening (Tables 1 and 2) is consistent with that observed for other types of plant mitochondria (Table 2). We have previously suggested that decreased repulsion between respiratory complexes and/or between complexes and charged lipids caused by a lowering in the size of the surface potential might result in an increased lateral mobility of the respiratory complexes and thus in an increased collision frequency and higher rates of oxidation (Møller & Palmer, 1981c).

Kinetics of NADPH oxidation

The V_{max} for NADPH oxidation at pH 6.0 (optimum, see Fig. 1) was unaffected by chelators, but increased by about 50% under high-cation conditions (Table 3). The latter effect is comparable with what was observed on NADPH oxidation by *Arum* mitochondria (Møller &

Table 3. Effect of salt on the kinetic parameters of NADPH oxidation of spinach leaf mitochondria

The oxidation of NADPH was measured at 340 nm as described in the Materials and methods section by using 60–100 μg of mitochondrial protein/ml. The low-salt medium contained 0.3 M-sucrose, 5 mM- K^+ -Mes, pH 6.0 or 6.3, or 5 mM- K^+ -Mops, pH 7.0, and 0.4 μM -FCCP. Low salt + EDTA was low salt plus 1 mM-EDTA and high-salt conditions were obtained by adding 2 mM-(DM) Br_2 to the low-salt medium. V_{max} (in nmol values of NADPH/min per mg of protein) and K_m values (in μM) were calculated by linear-regression analysis of v (nmol of NADPH/min per mg of protein) against v/s (nmol of NADPH/min per mg of protein and μM). Data are given as means $\pm$ S.D. (number of preparations). For *Arum* spadix, V_{max} and K_m values in high-salt medium were 760 nmol/min per mg of protein and 370 μM respectively (Møller & Palmer 1981a).

pH	Conditions	V_{max}	K_m
6.0	Low salt	110 $\pm$ 25 (3)	440 $\pm$ 80 (3)
	Low salt + EDTA	90 $\pm$ 36 (3)	340 $\pm$ 72 (3)
	High salt	160 $\pm$ 39 (6)	170 $\pm$ 40 (6)
6.3	High salt	176 (1)	226 (1)
7.0	High salt	41 $\pm$ 18 (3)	1040 $\pm$ 370 (3)

Palmer, 1981c). High-cation conditions also caused a 50% decrease in the apparent K_m for NADPH at pH 6.0 as compared with low-cation conditions (Table 3), just as was the case with NADH oxidation at pH 7.0 (Table 2). The K_m for NADPH was higher than for NADH (cf. Tables 2 and 3), which is consistent with the findings of Koeppe & Miller (1972) and Arron & Edwards (1979).

We have previously considered the possibility that the pH-dependence of NADPH oxidation is linked to the protonation of the 2'-phosphate group (Møller & Palmer, 1981a), which has a pK of 6.2–6.3 (Theorell, 1935). Such an effect has been observed with lactate dehydrogenase (Navazio *et al.*, 1957). The apparent K_m for NADPH should have relative sizes of 0.5:1.0:5.0 at pH 6.0, 6.3 (pK) and 7.0 respectively if the protonated form of NADPH were the true substrate of NADPH oxidation. The ratio determined was 0.7:1.0:4.6 (Fig. 4, Table 3), or fairly close to that predicted, particularly considering

Fig. 4. Kinetics of NADPH oxidation by spinach leaf mitochondria at pH 6.0 (○), 6.3 (□) and 7.0 (△)

The measurements were done at 340 nm as described in the Materials and methods section. The medium used contained 0.3 M-sucrose, 5 mM-K⁺-Mes, pH 6.0 or 6.3, or 5 mM K⁺-Mops, pH 7.0, 0.4 μM-FCCP, 2 mM-(DM)Br₂ and 75–100 μg of mitochondrial protein/ml. The reactions were started by addition of NADPH to the final concentrations indicated. $V_{\max}$ (in nmol of NADPH/min per mg of protein) and K_m (in μM) values were calculated by linear-regression analysis of v (nmol of NADPH/min per mg of protein) against v/s (nmol of NADPH/min per mg of protein and μM).

the low activity and high K_m at pH 7.0 compared to pH 6.0 and 6.3 (Fig. 4), and the resulting relatively large errors (Table 3). It therefore appears reasonable to conclude that NADPH oxidation by spinach leaf mitochondria, and possibly also by other types of plant mitochondria, is relatively inactive at neutral pH unless the concentration of NADPH is high in the cytoplasm (Fig. 4, Table 3).

Conclusions

(1) The oxidation of NADH and NADPH by spinach leaf mitochondria has properties which are in general similar to what has been observed on other plant mitochondria: pH optimum (Fig. 1), unspecific stimulation by cations (Table 3) and dependence on bound Ca²⁺ (Table 1). Some differences are found, however, which include: (i) the low NADPH oxidation at neutral pH, even in the presence of 1 mM-Ca²⁺; (ii) the absence of any effect of chelators on NADH oxidation under low-cation conditions (Table 1) and only 50% inhibition by chelators on NADH oxidation under high-cation conditions (Table 1); (iii) a more rapid NADPH oxidation than NADH oxidation at low pH (Fig. 1); (iv) a special pattern of the effect of La³⁺ on NADH oxidation and 9-aminoacridine fluorescence (Fig. 3). From a physiological point of view these differences are probably less interesting, with the possible exception of the partial Ca²⁺-dependence. NAD(P)H oxidation by spinach leaf mitochondria *in vivo* would therefore appear to have similar properties as in other types of tissues and species, including non-photosynthetic tissue. This is interesting, since the reduction level of NAD(P)H changes with dark–light transitions of photosynthetic cells (Hampp *et al.*, 1985).

(2) The responses of NADH and NADPH oxidation to cations and chelators can be rationalized in terms of changes in the surface potential. This is true for the increase in $V_{\max}$ under high-cation conditions (Tables 1–3, Fig. 2), the changes in apparent K_m for NADH and NADPH under low-cation, low-cation + EDTA and high-cation conditions (Tables 2 and 3), as well as the different sensitivity of NADH oxidation to chelators under low- and high-cation conditions (Table 1). Electrostatic considerations should always be applied when working with membrane-bound enzymes to ensure against misinterpretations of results.

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