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Dissecting the Diphenylene Iodonium-Sensitive NAD(P)H: Quinone Oxidoreductase of Zucchini Plasma Membrane[1]

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Quinone oxidoreductase activities dependent on pyridine nucleotides are associated with the plasma membrane (PM) in zucchini (*Cucurbita pepo* L.) hypocotyls. In the presence of NADPH, lipophilic ubiquinone homologs with up to three isoprenoid units were reduced by intact PM vesicles with a K_m of 2 to 7 μM. Affinities for both NADPH and NADH were similar (K_m of 62 and 51 μM, respectively). Two NAD(P)H:quinone oxidoreductase forms were identified. The first, labeled as peak I in gel-filtration experiments, behaves as an intrinsic membrane complex of about 300 kD, it slightly prefers NADH over NADPH, it is markedly sensitive to the inhibitor diphenylene iodonium, and it is active with lipophilic quinones. The second form (peak II) is an NADPH-preferring oxidoreductase of about 90 kD, weakly bound to the PM. Peak II is diphenylene iodonium-insensitive and resembles, in many properties, the soluble NAD(P)H:quinone oxidoreductase that is also present in the same tissue. Following purification of peak I, however, the latter gave rise to a quinone oxidoreductase of the soluble type (peak II), based on substrate and inhibitor specificities and chromatographic and electrophoretic evidence. It is proposed that a redox protein of the same class as the soluble NAD(P)H:quinone oxidoreductase (F. Sparla, G. Tedeschi, and P. Trost [1996] Plant Physiol. 112:249–258) is a component of the diphenylene iodonium-sensitive PM complex capable of reducing lipophilic quinones.

Plant cells appear to contain several types of NAD(P)H-dependent quinone oxidoreductases in addition to those of energy-conserving reactions of plastids and mitochondria. The purification of some of these plant proteins has been accomplished, but their biochemical characterization is still at an initial stage. Recently, NAD(P)H-QR from plant tissues was purified and characterized (Rescigno et al., 1995; Trost et al., 1995; Sparla et al., 1996). This plant oxidoreductase represents a functional equivalent of animal DT-diaphorase, since it reduces short-chain quinones to quinols by two-electron donation without semiquinone intermediates, thereby resulting in enhanced quinone conjugation and low probability of formation of active oxygen species (Trost et al., 1995). However, a number of properties are different from animal-type DT-diaphorase, i.e. the NAD(P)H-QR contains FMN, it has a mass of about 90 kD with subunits of 21.4 kD (by MS), and the hydride transfer from NAD(P)H to the flavin is B-stereospecific (Sparla et al., 1996), whereas animal DT-diaphorase is an FAD-containing, A-stereospecific, dimeric dehydrogenase with 26- to 31-kD subunits (Ernster, 1987; Jaiswal et al., 1990). The basidiomycete *Phanaerochete chrysosporium* has also been shown to contain an FMN-binding oxidoreductase of the NAD(P)H-QR type involved in the detoxification of quinones naturally produced by the fungal degradation of lignin (Brock et al., 1995; Brock and Gold, 1996).

The NAD(P)H-QR bears some structural and kinetic resemblance to some enzymes purified from plant PM (Luster and Buckhout, 1989; Serrano et al., 1994) and mitochondria, in the latter as rotenone-insensitive NAD(P)H dehydrogenases (Luethy et al., 1991; Rasmusson et al., 1993). They are relatively unspecific toward pyridine nucleotides and use hydrophilic quinones efficiently, whereas Cyt *c*, oxygen, and ferri-chelates are not reduced to a significant extent. With the exception of the 43-kD NAD(P)H dehydrogenase purified from sugar beet mitochondria by Luethy et al. (1991), these dehydrogenases are composed of subunits of 25 to 27 kD (Luster and Buckhout, 1989; Rasmusson et al., 1993; Serrano et al., 1994), similar to the apparent molecular mass of 22 to 24 kD of the NAD(P)H-QR, as calculated by SDS-PAGE (Brock et al., 1995; Rescigno et al., 1995; Trost et al., 1995; Sparla et al., 1996). Moreover, detergents are not required to extract the mitochondrial dehydrogenases from the membrane (Luethy et al., 1991; Rasmusson et al., 1993). Therefore, these proteins seem to be rather hydrophilic, like NAD(P)H-QR.

Another type of particulate pyridine nucleotide-dependent quinone oxidoreductase named NADH-DQ reductase (Pupillo et al., 1986) is widespread in plant microsomal membranes, although its purification has never been achieved. Unlike soluble NAD(P)H-QR, the NADH-DQ reductase is strongly bound to the membrane (Valenti et al., 1990) and the molecular mass is larger (340 kD, according to Guerrini et al. [1994]). Whereas this form prefers NADH in sugar beet microsomes (Guerrini et al., 1994), NADPH is also a good electron donor in zucchini (*Cucurbita pepo* L.) PM, and we shall refer to this enzyme as the intrinsic NAD(P)H:quinone oxidoreductase.

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Abbreviations: DPI, diphenylene iodonium; DQ, duroquinone (2,3,5,6-tetramethyl-benzoquinone); LPC, lysophosphatidylcholine; NAD(P)H-QR, soluble NAD(P)H:quinone oxidoreductase (EC 1.6.99.2); PM, plasma membrane; SN167,000*g*, supernatant 167,000*g* from hypocotyl tissue; TEA, triethanolamine; TX-100R, Triton X-100, reduced; UQ-*n*, ubiquinone homolog with *n* isoprenoid units.

Other NADH-specific oxidoreductases are also found in plant microsomes and PM (Serrano et al., 1994; Bagnaresi and Pupillo, 1995; Bérczi et al., 1995; Bagnaresi et al., 1997). Most of these preparations consist of proteins having prominent ferricyanide reductase activity with some capacity to reduce ferri-chelates, but they are inactive with DQ, an artificial quinone commonly used in redox enzyme assays. These oxidoreductases are therefore distinct from both the intrinsic NAD(P)H:quinone oxidoreductase and the soluble NAD(P)H-QR. In the present work we have addressed the purification of the intrinsic NAD(P)H:quinone oxidoreductase from the PM of etiolated zucchini hypocotyls. The findings are unexpected, since solubilized preparations tend to lose certain properties of the intrinsic complex in the course of purification, to reveal those of a soluble NAD(P)H-QR-like flavoprotein.

MATERIALS AND METHODS

Chemicals

Ubiquinone homologs (UQ-1, UQ-2, and UQ-3) were a gift from Eisai (Tokyo, Japan). The inhibitor 7-iodo-acridone-4-carboxylic acid was from Dr. W. Oettmeier (Ruhr University, Bochum, Germany; Oettmeier et al., 1993). All other compounds were of the highest purity grade available. Chromatographic resins were from Pharmacia.

Plant Material and Membrane Purification

Zucchini (*Cucurbita pepo* L. hybrid Storr's Green, Asgrow, Milan, Italy) seedlings were grown for 5 d in the dark at 25°C on moistened vermiculite. For preparation of membrane fractions, hypocotyls were processed, as described by Valenti et al. (1990), with some modifications. Briefly, the hypocotyls were homogenized on ice in 2 volumes of buffer A (50 mM Tris-Cl, 2 mM EDTA, 10 mM 2-mercaptoethanol, 0.5 mM PMSF, and 250 mM Suc, pH 7.5) in a blender (20 s, full speed). The homogenate was filtered on a 30-μm nylon net and centrifuged at 9,000*g* for 20 min. The resulting supernatant was then centrifuged at 167,000*g* for 1 h and the pellet (microsomes) was resuspended in buffer B (5 mM potassium phosphate, 3 mM KCl, and 330 mM Suc, pH 7.8). Microsomes were partitioned in an aqueous two-phase system according to the method of Larsson et al. (1994), with final concentrations in the phase system being 6.3% (w/w) dextran, 6.3% (w/w) PEG, 5 mM potassium phosphate, 3 mM KCl, and 330 mM Suc, pH 7.8. Phase partitioning was performed at 5°C by centrifugation for 5 min at 5,000*g* in a swinging bucket rotor. Right-side-out PM vesicles, recovered in the upper phase following two washing steps with fresh lower phase, were diluted 10-fold with buffer B, spun down at 167,000*g* for 1 h, and resuspended in buffer A in a Teflon/glass homogenizer. Membranes were stored at −65°C until further use.

To separate PM proteins from contaminating soluble proteins (either trapped inside the vesicles or unspecifically adsorbed), PM vesicles were diluted 4-fold in buffer A also containing 0.15 M KCl, frozen (−20°C) and thawed (room temperature) three times, and centrifuged at 100,000g for 1 h. Both the supernatant and the sediment, resuspended in buffer A, were collected for further analysis.

Enzyme Assays

Spectrophotometric assays were carried out at 25°C with a spectrophotometer (Uvikon 941 Plus, Kontron, Milan, Italy). NAD(P)H:DQ oxidoreductase activity was measured, if not otherwise stated, in 40 mM Mops-Na, pH 7.0, 1 mM EDTA, 0.5 mM KCN, 0.2 mM DQ, and 0.2 mM NAD(P)H following the decrease in A_{340} with an extinction coefficient of 6.23 mM^{-1} for pyridine nucleotides. With quinone acceptors other than DQ, corrected extinction coefficients were used to take into account the interfering A_{340} of the quinone/quinol couple. Assays of membrane activities were conducted in 3-mL cuvettes with stirring. The reaction was started by the addition of acceptor. Kinetic data at variable concentrations of electron donors and acceptors were interpolated by nonlinear regression analysis as described by Sparla et al. (1996). The results shown are representative of at least three experiments with different PM preparations.

NADPH-Cyt *c* reductase and Cyt *c* oxidase were assayed as described by Valenti et al. (1989), and NAD(P)H-ferricyanide reductase was as described by Guerrini et al. (1994). Glucan synthase II was tested according to the method of Ray (1977) with some modifications. In a total volume of 150 μL, the assay mixture consisted of 50 mM Tris-HCl, pH 8.0, 0.5 mM DTT, 0.2 mM spermine, 0.05 mM $CaCl_2$, 20 mM cellobiose, 0.5 mM [U-^{14}C]UDP-Glc (0.67 Ci mol^{-1}), and about 10 μg of protein. The reaction (45 min at 25°C) was stopped by the addition of 1 mL of ethanol (70%, v/v). Fibrous cellulose (7.5 mg/test tube) was added to assist in the precipitation of the newly synthesized polymer. Following three ethanol-washing steps, the ethanol-insoluble material was counted in a liquid scintillator. Blanks with no protein were subtracted. Activity is expressed as nanomoles of UDP-Glc incorporated per minute per milligram of protein.

Membrane Solubilization

Solubilization of membrane proteins was obtained by gently stirring the membrane preparation (30 min, room temperature) in the presence of 2 to 5 mg of LPC to 1 mg of protein (1–3%, w/v, LPC), as indicated. Solubilized proteins were recovered following sedimentation of the insoluble material at 100,000*g* for 1 h.

Chromatographic Techniques

All chromatographic steps were performed by fast protein liquid chromatography or the SMART system (Pharmacia). For gel-filtration chromatographies a Superdex 200 column (HL 16/60) was used. The equilibration buffer was 20 mM TEA-Cl, pH 7.8, 1 mM EDTA, and 150 mM KCl, with or without 0.01% (w/w) TX-100R as indicated. Sample volume was 2 to 3 mL and flow rate was 1.0 mL min^{-1}. Molecular masses were estimated on the basis of calibration runs, as reported by Trost et al. (1995).

For the purification of the intrinsic NAD(P)H:quinone oxidoreductase of intact PM vesicles, the sample recovered after LPC solubilization was subjected to a Superdex 200 step, and the active fractions were pooled and equilibrated to 20 mM TEA-Cl, pH 7.8, 0.01% (w/w) TX-100R by means of Sephadex G-25 Hi Trap Fast Desalting columns. The enzyme was then loaded on a Mono-Q column (HR 5/5, Pharmacia) equilibrated with the same buffer at an elution rate of 0.7 mL min^{-1}. Following 5-mL column washing, 20 mL of a 0 to 0.5 M KCl gradient was applied and 0.5-mL fractions were collected. Active fractions were finally purified by means of a further Superdex 200 step under the same conditions as above, apart from the absence of detergent in the elution buffer.

The soluble NAD(P)H-QR was purified from the SN167,000*g* following the method of Trost et al. (1995), which consists of an ammonium sulfate precipitation followed by anion-exchange (Q-Sepharose and KCl-gradient), gel-filtration (Superdex 200), and affinity chromatographies (Blue Sepharose CL 6B and NADPH-gradient).

Other Analytical Techniques

Protein contents were measured by a modification of the Lowry method, as described by Bensadoun and Weinstein (1976), with BSA as a standard.

For SDS-PAGE (Trost et al., 1995), samples were equilibrated to 0.1% (w/v) ammonium bicarbonate by column desalting (SMART Phast Desalting PC 3.2/10), lyophilized, and resuspended in a sample buffer. Homogeneous gels (12.5% polyacrylamide) were silver-stained according to the instructions of the manufacturer (Sigma-Aldrich S.r.l., Milan, Italy).

RESULTS

NAD(P)H:Quinone Oxidoreductase Activities of Purified PM Vesicles

PM vesicles were isolated from zucchini hypocotyls by aqueous two-phase partitioning (Table I). Compared with microsomes, the PM marker glucan synthase II was 3-fold enriched in the PM, whereas NADPH-Cyt *c* reductase and the mitochondrial marker Cyt *c* oxidase decreased by a factor of 2 and 6, respectively. The specific activities of NADH- and NADPH-dependent DQ oxidoreductases in both PM and microsomes were similar. Therefore, NAD(P)H:DQ oxidoreductase activities are suggested to be associated with both the PM and other membranes present in the microsomal fraction (9,000–167,000*g* precipitate).

Table II. *NAD(P)H-dependent PM redox activity with different acceptors*

Pyridine nucleotide concentration was 0.2 mM and each quinone was 0.02 mM. Ferricyanide was 0.5 mM. Assays performed at 25°C under stirring with 0.1 to 0.2 mg of protein in 2.0 mL total volume. Assay medium contained 40 mM Mops-Na, 2 mM EDTA, and 0.5 mM KCN, pH 7.0. NAD(P)H oxidation rate in the absence of acceptors (10–20 nmol NAD(P)H min^{-1} mg^{-1}) was subtracted. Mean data (±SE) are from three independent PM preparations.

Acceptor	NADH	NADPH
	nmol NAD(P)H min^{-1} mg^{-1}	
UQ-0	65 ± 6	75 ± 14
UQ-1	24 ± 5	44 ± 9
UQ-2	53 ± 5	58 ± 5
UQ-3	15 ± 1	15 ± 6
DQ	28 ± 2	43 ± 1
Juglone	62 ± 2	47 ± 3
Menadione	40 ± 4	55 ± 7
Ferricyanide	534 ± 44	215 ± 29

Several quinones were tested as electron acceptors of the redox activities of purified PM, as shown in Table II. With most quinones the activities measured with either NADPH or NADH were similar. However, activities measured with DQ and UQ-1 were chiefly NADPH-dependent, whereas juglone and ferricyanide were better reduced in NADH-dependent reactions.

The NAD(P)H:UQ-2 oxidoreductase activity of intact PM vesicles was partially inhibited by DPI (Fig. 1), a compound best known as an inhibitor of mammalian NADPH-oxidase (Doussiere and Vignais, 1992). Although DPI was strongly inhibitory in the low micromolar range, a considerable residual activity was retained up to DPI concentrations as high as 200 μM. Fifty-percent inhibition of the DPI-sensitive reaction with either NADH or NADPH was observed at about 0.7 μM DPI (Fig. 1).

By varying the concentration of ubiquinone homologs in the presence of NADPH (0.2 mM), the responses exhibited saturation kinetics with K_m values between 2 and 7 μM (Table III). Hyperbolic saturation patterns were also obtained when pyridine nucleotide concentrations were varied in the presence of 20 μM UQ-2 (Fig. 2A), with similar K_m and V_{max} values for both NADH and NADPH (Table III). In the presence of equimolar and saturating levels of both pyridine nucleotides, the UQ-2 oxidoreductase activity was higher than with either NADH or NADPH alone

Table I. *Occurrence of NAD(P)H-dependent DQ oxidoreductase activity in PM of zucchini hypocotyls*

DQ reductase activity was assayed in the presence of 0.01% (w/w) TX-100R. Results are means ± SE of four preparations.

Membrane Fraction	CCO[a]	NADPH-CCR[b]	GSII[c]	NADH:DQ Oxidoreductase	NADPH:DQ Oxidoreductase
	nmol Cyt c min^{-1} mg^{-1}	*nmol NAD(P)H min^{-1} mg^{-1}*	*nmol UDP-Glc min^{-1} mg^{-1}*	*nmol NAD(P)H min^{-1} mg^{-1}*	
Microsomes	14 ± 6	57 ± 1	4.3 ± 0.6	62 ± 4	95 ± 9
PM	2.3 ± 0.9	24 ± 6	12 ± 1	66 ± 8	123 ± 8

[a] CCO, Cyt *c* oxidase. [b] NADPH-CCR, NADPH-dependent Cyt *c* reductase (antimycin A insensitive). [c] GSII, Glucan synthase II.

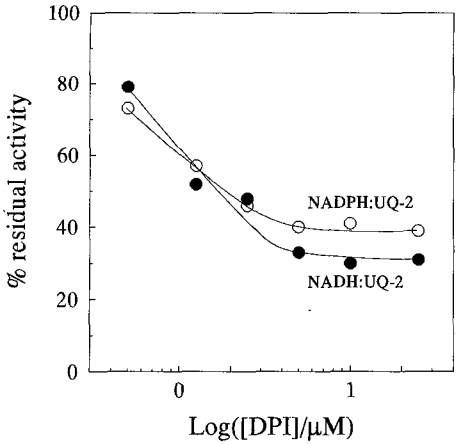

Figure 1. DPI inhibition of NAD(P)H:UQ-2 oxidoreductase activity of PM vesicles. Assays were conducted with 0.2 mM NAD(P)H and 0.02 mM UQ-2 in 40 mM Mops-Na, 1 mM EDTA, and 0.5 mM KCN, pH 7.0, under stirring. PM vesicles (0.1 mg of protein) were incubated with inhibitor for 5 min in the assay mixture before NAD(P)H and UQ-2 were added to start the reaction.

(Fig. 2A) but lower than the theoretical sum of the NADH- plus the NADPH-dependent activities (as expected in the case of two distinct enzymes). The theoretical curve for a single NAD(P)H-dependent quinone oxidoreductase in the presence of both electron donors would be intermediate between the NADH- and NADPH-dependent ones. The UQ-2 oxidoreductase activity of the PM did not fit either pattern (Fig. 2A). This result suggests that more than one UQ-2 oxidoreductase, with different specificities for pyridine nucleotides, was active in PM preparations.

We also examined the pyridine nucleotide dependence of the residual NAD(P)H:UQ-2 oxidoreductase activity in the presence of DPI (Fig. 2B). Contrary to the experiments in the absence of inhibitor, the substrate saturation kinetics matched the typical response of a single DPI-insensitive oxidoreductase, with higher NADPH- than NADH-dependent activity and an intermediate response with both nucleotides. Such a response is typical for the NAD(P)H-QR (results not shown). It seems that DPI selectively inhibited an oxidoreductase with prevailing NADH-dependent activity.

Identification of Two NAD(P)H:Quinone Oxidoreductase Forms of the PM

The PM-bound quinone oxidoreductase activity was partially solubilized by LPC (3%, w/v, 5 mg LPC/mg protein). The recovery of activity assayed with several quinones (DQ, UQ-2, and UQ-3) was in the 30 to 60% range. About 20% of the activity was usually measured in the insoluble fraction, but higher LPC concentrations were not effective in increasing the yield. Following solubilization, two main enzyme peaks of quinone oxidoreductase activity were resolved by gel-filtration fast-protein liquid chromatography (Fig. 3). A high-molecular-mass peak (peak I, about 300 kD) accounted for most of the UQ-3 reductase activity but for only a minor part of the total activity with DQ. On the other hand, the main NAD(P)H:DQ oxidoreductase (peak II) eluted as a 80- to 100-kD protein and it showed lower activity with UQ-3. The activity of peak I was strongly inhibited by DPI, which had little effect on peak II. The effect was independent of the electron donor and quinone used in the assay (Table IV).

The NAD(P)H:quinone oxidoreductases corresponding to peaks I and II were shown to have different types of interaction with the PM. A fraction containing hydrophilic proteins (either trapped inside the vesicles or unspecifically bound to the membranes) was obtained by repeatedly freezing and thawing the PM preparation in the presence of 0.15 M KCl. Following high-speed centrifugation the supernatant was analyzed by gel-filtration chromatography and peak II, but not peak I, was detected in the eluate (Fig. 4A). On the other hand, LPC-solubilized proteins from the pellet following this treatment contained both peaks I and II (Fig. 4B). Also, peak I was not released by PM vesicles following such treatments as sonication, osmotic shock, and salt (1 M KCl) or detergent washing (0.01% TX-100R, w/v; not shown). The DPI-sensitive peak I is therefore suggested to be intrinsically bound to the PM, in agreement with the high affinity of peak I for hydrophobic quinones (Fig. 3).

Peak II, contrary to peak I, clearly represents a hydrophilic, DPI-insensitive quinone oxidoreductase form. Furthermore, peak II is definitely more active with NADPH than NADH, whereas peak I shows some preference for NADH when UQ-2 is the acceptor (Fig. 4; Table IV). Since

Table III. *Kinetic parameters of NAD(P)H:quinone oxidoreductase activity of PM vesicles*

Kinetic parameters were calculated by nonlinear regression analysis of data obtained either by varying the concentration of quinones at 0.2 mM NAD(P)H or by varying the concentration of NAD(P)H at 0.02 mM UQ-2, in 40 mM Mops-Na, 2 mM EDTA, and 0.5 mM KCN, pH 7.0. Mean values ± SE are of three PM preparations.

Electron Donor	Quinoid Electron Acceptor	$K_{m(quinone)}$	$K_{m(NAD(P)H)}$	V_{max}
		μM		nmol min^{-1} mg^{-1}
NADPH	UQ-0	6 ± 1	nt[a]	107 ± 18
NADPH	UQ-1	6 ± 3	nt	67 ± 4
NADPH	UQ-2	7 ± 1	62 ± 11	61 ± 4
NADPH	UQ-3	2 ± 1	nt	19 ± 0
NADH	UQ-2	nt	51 ± 5	51 ± 8

[a] nt, Not tested.

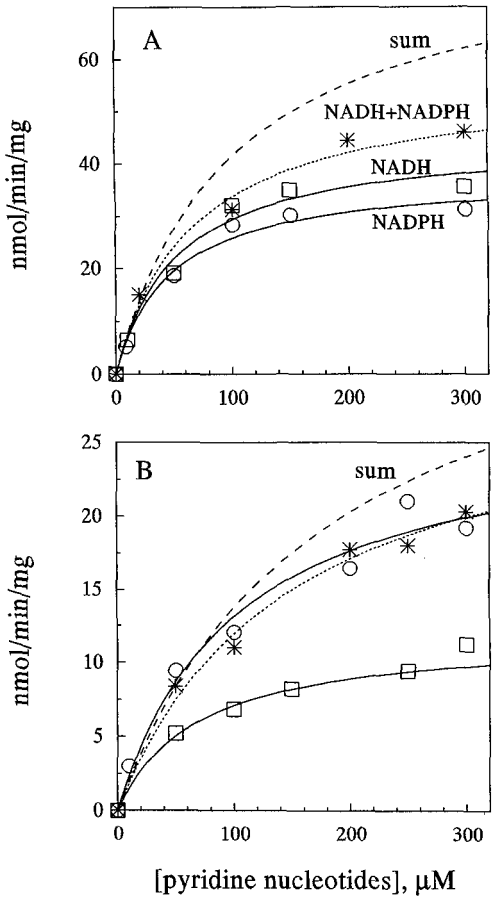

Figure 2. Pyridine nucleotide specificity of redox activities of PM vesicles with UQ-2 as an electron acceptor, with or without DPI. A, Assay solution (2.0 mL) contained 40 mM Mops-Na, pH 7.0, 1 mM EDTA, 0.5 mM KCN, 20 μM UQ-2, 0.1 mg of PM protein, and variable amounts of NADH (□) or NADPH (○) or an equimolar mixture of NADH plus NADPH (*). The curve indicated as "sum" refers to the equation

$$v = V_{max(NADH)}*[NADH]/(K_{m(NADH)} + [NADH]) + V_{max(NADPH)}*[NADPH]/(K_{m(NADPH)} + [NADPH])$$

and represents the expected kinetic response in the case of two distinct dehydrogenases, each specific for either NADH or NADPH. The numerical values of the parameters V_{max} and K_m are obtained by nonlinear regression analysis of experimental data with either NADH or NADPH alone. B, The same as A, except that 50 μM DPI was present in the medium; 5 min of preincubation.

the NAD(P)H:UQ-2 oxidoreductase activity of the PM is mainly NADPH-dependent in the presence of DPI (Fig. 2B), the DPI-insensitive NAD(P)H:UQ-2 oxidoreductase is suggested to depend entirely on peak II. DPI thus appears to discriminate between peak I and peak II oxidoreductases in unfractionated PM vesicles.

Purification of the Intrinsic NAD(P)H:Quinone Oxidoreductase from Zucchini PM Vesicles

Peak I, obtained by gel-filtration experiments as in Figure 3, was collected for further purification. Following anion-exchange chromatography on Mono-Q, the enzyme was quite active as NAD(P)H:DQ oxidoreductase (Fig. 5A), but the activity with UQ-3 was now vestigial (not shown). Surprisingly, a second gel filtration on Superdex 200 showed that the activity was now associated with a quinone oxidoreductase, eluting at the same position as peak II (Fig. 5B). In this final step, identical results were obtained with or without detergent in the column buffer (not shown). The purification scheme of the intrinsic NAD(P)H:quinone oxidoreductase from zucchini PM vesicles is reported in Table V.

A very active NAD(P)H-QR was recovered in the soluble fraction (SN167,000*g*) of zucchini hypocotyls, co-eluting with peak II in Superdex 200 gel-filtration columns (not shown), and it has been purified to homogeneity (Fig. 6), as detailed in "Materials and Methods." A comparison, based on electron acceptor specificity and the effect of inhibitors, clearly showed that the NAD(P)H:quinone oxidoreductase of the PM, obtained upon purification of peak I, was quite similar to the NAD(P)H:QR purified from the SN167,000*g* (Table VI). They both preferred NADPH over NADH as electron donors to reduce hydrophilic quinones or ferricyanide but not Cyt *c* or oxygen. The isoprenylated quinone UQ-3 was totally ineffective as an acceptor for the NAD(P)H-QR (Trost et al., 1995) and a weak acceptor for the purified PM oxidoreductase. DPI had no effect on either enzyme, whereas Cibacron blue, 7-iodo-acridone-4-carboxylic acid, and dicumarol were partially inhibitory as for tobacco NAD(P)H-QR (Sparla et al., 1996).

A comparison between NAD(P)H-QR and PM-derived NAD(P)H:quinone oxidoreductases was also performed by SDS-PAGE (Fig. 6). Three bands of 18, 24, and 27 kD were detectable in the final NAD(P)H:quinone oxidoreductase

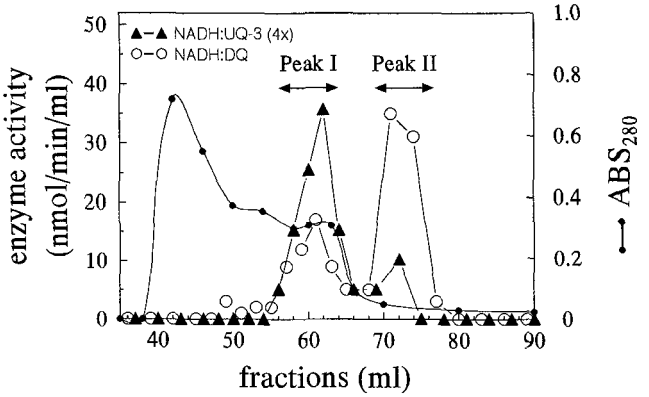

Figure 3. Gel-filtration chromatography of PM proteins following solubilization with LPC. PM vesicles were solubilized in the presence of 5 mg/LPC to 1 mg protein (3%, w/v, LPC) for 30 min at room temperature and centrifuged (100,000*g*, 1 h). A 3-mL sample was loaded on a Superdex 200 column (fast-protein liquid chromatography), previously equilibrated with 20 mM TEA-Cl, pH 7.8, 1 mM EDTA, 150 mM KCl, and 0.01% (w/w) TX-100R. The flow rate was 1 mL min^{-1} and 1-mL fractions were collected. NADH:UQ-3 and NADH:DQ oxidoreductase activities were measured as described in "Materials and Methods," with 0.2 mM NADH and either 0.2 mM DQ or 0.02 mM UQ-3. For the sake of clarity, the NADH:UQ-3 oxidoreductase activity was multiplied by a factor of 4. ABS, Absorbance.

Table IV. *DPI inhibition of LPC-solubilized quinone reductase activities of PM*

Peaks I and II were separated by Superdex 200 gel-filtration chromatography as in Figure 3. Assays were conducted with 0.2 mM NAD(P)H and 0.2 mM DQ or 0.02 mM UQ-2. DPI concentration was 50 μM. Enzyme was incubated with the inhibitor for 5 min before starting the reaction by adding the substrates.

Donor:Acceptor	Peak I		Peak II	
	Activity	+DPI	Activity	+DPI
	nmol min^{-1} mL^{-1}	*% inhibition*	*nmol min^{-1} mL^{-1}*	*% inhibition*
NADH:UQ-2	10.4	(98)	6.7	(1)
NADPH:UQ-2	6.8	(95)	22.1	(6)
NADH:DQ	6.0	(95)	10.7	(7)
NADPH:DQ	6.7	(92)	43.8	(0)

preparation obtained from the PM following purification of peak I (Figs. 3 and 5). The 24-kD band was the size of the subunit of the NAD(P)H-QR purified from the SN167,000*g* (Fig. 6), as well as subunits of tobacco and sugar beet NAD(P)H-QR (Trost et al., 1995; Sparla et al., 1996).

DISCUSSION

In this paper we describe some biochemical properties and the partial purification of the intrinsic NAD(P)H:quinone oxidoreductase of the PM of zucchini hypocotyls. The enzyme, formerly defined as NADH-DQ reductase, was initially characterized by its sensitivity to inhibition by detergents, Triton X-100 in particular (De Luca et al., 1984; Asard et al., 1987). Following infiltration of tobacco leaves with bacterial protein-lipopolysaccharide complexes, the protection observed against hypersensitive necrosis caused by challenge bacteria was associated with an increase of NADH:DQ reductase activity of the PM. This enzyme was suggested to play a role in the prevention of superoxide formation (Valenti et al., 1989). An activity increase was also apparent in particulate fractions of sugar beet suspension cells subjected to calcium limitation (Guerrini et al., 1994).

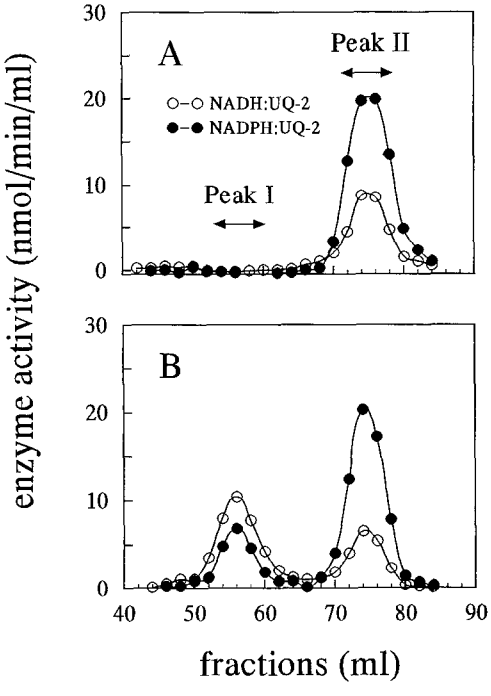

Figure 4. Gel-filtration chromatography of PM NAD(P)H:UQ-2 oxidoreductases following freeze/thaw treatment and centrifugation. A Superdex 200 column (fast-protein liquid chromatography) was run under the same conditions as in Figure 3. PM vesicles were subjected to freeze/thawing and centrifuged at high speed (see "Materials and Methods"). Assays were conducted as in "Materials and Methods" with 0.2 mM NAD(P)H and 0.05 mM UQ-2. A, Supernatant (3 mL) containing hydrophilic proteins. B, The pellet, enriched in intrinsic PM proteins, was resuspended and solubilized with 2 mg LPC mg^{-1} protein (1%, w/w, LPC). Unsolubilized material was sedimented by centrifugation and the supernatant (3 mL) was applied to the column.

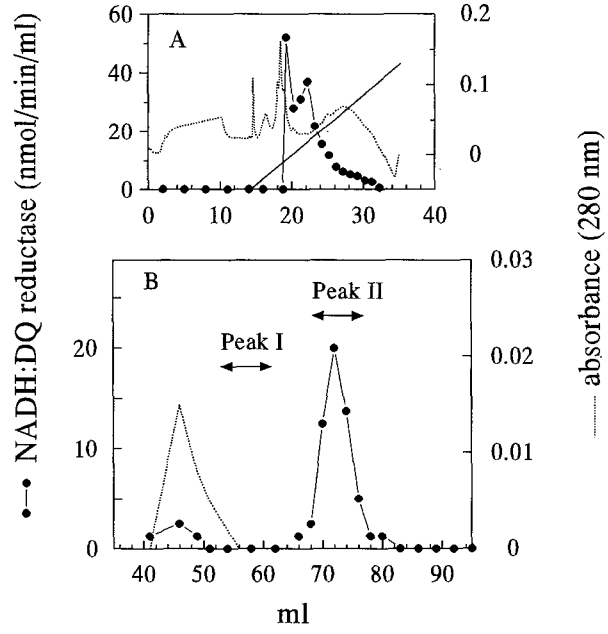

Figure 5. Purification of the intrinsic NAD(P)H:quinone oxidoreductase of the PM. A, SMART-Mono-Q (HR 5/5) anion-exchange chromatography. The sample loaded on the column was peak I from a Superdex 200 separation of PM proteins following LPC solubilization. After the sample was loaded, the column was washed (5 mL) and a 25-mL salt gradient (0–0.5 M KCl) was applied. The fraction volume was 0.5 mL and the flow rate was 0.7 mL min^{-1}. B, Superdex 200 column run under identical conditions as in Figure 3, but without detergent in the column buffer. A sample of 2 mL from pooled fractions 19 to 23 of the Mono-Q run shown in A was loaded on the column.

Table V. *Purification of the intrinsic NAD(P)H:quinone oxidoreductase of PM vesicles*

Activity measured with 0.2 mM NADH and 0.2 mM DQ in 40 mM Mops-Na, 1 mM EDTA, and 0.5 mM KCN, pH 7.0 LPC (0.01%, w/v) was present in assays of PM vesicles and solubilized proteins.

Purification Step	Activity (NADH:DQ)	Protein	Specific Activity	Purification	Yield
	μmol min^{-1}	*mg*	*μmol min^{-1} mg^{-1}*	*-fold*	*%*
PM vesicles	1.177	9.6	0.123	1	100
Solubilized (LPC)	0.328	5.6	0.059	0.5	28
Superdex 200 (first)	0.278	1.9	0.146	1.2	24
Mono-Q	0.118	0.21	0.561	4.6	10
Superdex 200 (second)	0.117	nd[a]	nd	nd	10

[a] nd, Not detectable (using conventional methods of protein quantification).

NAD(P)H:quinone oxidoreductase activities of the PM have previously been characterized by using artificial electron acceptors, including relatively hydrophilic quinones, mostly DQ (Rubinstein and Luster, 1993). Although DQ is efficiently reduced by the PM redox system, it is sparingly soluble in membranes (Braun et al., 1986) and, therefore, it is not expected to interact with enzyme sites deeply embedded in lipid bilayers. PM dehydrogenases, on the other hand, may interact with lipophilic quinones. Whereas the occurrence of ubiquinone has been demonstrated for the PM of animal cells (Kalén et al., 1987), vitamin K_1 (phylloquinone) was recently identified in the PM of maize roots (Döring and Lüthje, 1996).

To reveal PM redox activities potentially involved in the reduction of membrane-soluble quinones, we have used ubiquinone homologs with up to three isoprenoid units, which are readily incorporated into lipid bilayers (Degli Esposti et al., 1981). These UQ homologs seem to feature appropriate kinetic parameters for putative physiological functions at the PM of zucchini hypocotyls. In the presence of 0.2 mM NADPH, a concentration approaching cytosolic NADPH levels (Heineke et al., 1991), the affinity of the PM redox system for isoprenylated UQ homologs was comparable to the affinity of mitochondrial complex I for the same molecules (Fato et al., 1995), although the activities of PM vesicles were about 1 order of magnitude lower than observed in plant mitochondria (Rugolo and Zannoni, 1992).

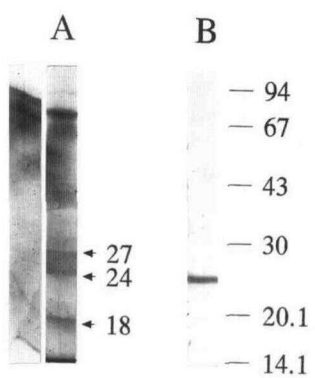

Figure 6. SDS-PAGE of NAD(P)H:quinone oxidoreductases purified from PM and the soluble fraction of zucchini hypocotyls. A, right lane, NAD(P)H:quinone oxidoreductase (70 nmol min^{-1}, NADH:DQ activity) purified from PM vesicles by a first gel filtration (Fig. 3, peak I), an anion-exchange step (Fig. 5A), and a second gel filtration (Fig. 5B, peak II). The gel was silver-stained. Left lane, Sample buffer was used only for background comparison. B, NAD(P)H-QR from SN167,000*g* of zucchini hypocotyls (770 nmol min^{-1}) was purified according to the method of Trost et al. (1995). Right lane, The migration of molecular mass standards (in kD) are indicated.

We have found evidence for at least two NAD(P)H:UQ-2 oxidoreductase activities of PM vesicles of zucchini hypocotyls, in agreement with results obtained with DQ (Pupillo et al., 1986). In fact, the inhibition exerted by DPI was strong but incomplete, indicating that both DPI-sensitive and DPI-insensitive NAD(P)H:quinone oxidoreductases were involved in the reaction with UQ-2. At variance with the total activity measured in PM vesicles, the DPI-insensitive oxidoreductase showed complete competition between NADH and NADPH and clearly represents a single protein accepting both pyridine nucleotides.

Following LPC-solubilization of zucchini PM vesicles, two NAD(P)H:quinone oxidoreductases were separated by gel-filtration chromatography. Peak I, having an apparent molecular mass of about 300 kD, represents a DPI-sensitive oxidoreductase and may be operationally defined as an intrinsic membrane protein (or protein complex) on the basis of its detergent requirement for solubilization and a relative preference for lipophilic quinones (e.g. UQ-3, Fig. 3). Peak II, on the other hand, is weakly interacting with the PM and can be largely released following a freeze-thaw treatment. Peak II has low sensitivity to DPI, prefers NADPH to NADH, and elutes as an 80- to 100-kD protein. Native molecular weight and other basic features of peak II are also similar to the NAD(P)H-QR of the soluble fraction (Sparla et al., 1996), including the hydrophilic nature of the protein and the NADPH/NADH activity ratio (Table VI).

By attempting to purify peak I, however, we obtained a peak II-like quinone oxidoreductase. The interpretation of this result is not straightforward. A reasonable possibility is that oxidoreductases of the NAD(P)H-QR type may be bound in vivo to other PM proteins, to constitute an intrinsic membrane complex catalyzing the NAD(P)H-dependent, DPI-sensitive reduction of membrane quinones. Following LPC solubilization and gel-filtration chromatography, the membrane complex was found to be associated with peak I, on the basis of DPI sensitivity and higher activity toward membrane quinones. At this stage, the peak I electrophoretic pattern (SDS-PAGE) was too complex to provide any reliable information about its subunit composition (not shown). However, the stability of peak I was

Table VI. *Electron donors, acceptors, and inhibitors of NAD(P)H:quinone oxidoreductases purified from PM vesicles and SN167,000g*

Assays in 40 mM Mops, pH 7.0, 1 mM EDTA, and 0.2 mM NAD(P)H. Activities are expressed as percentages with respect to the NADH:DQ oxidoreductase activity.

Donor	Acceptor	Inhibitor	NAD(P)H:quinone oxidoreductase from:	
			PM	SN167,000*g*
	mM	mM	%	
NADH	DQ (0.2)	–[a]	100	100
NADPH	DQ (0.2)	–	162	139
NADH	Juglone (0.2)	–	163	193
NADH	UQ-0 (0.02)	–	134	156
NADH	UQ-3 (0.02)	–	16	0.4
NADH	Ferricyanide (0.5)	–	129	113
NADH	Cyt *c* (0.05)	–	3.0	4.5
NADH	O_2 (0.25)	–	nd[b]	nd
NADH	DQ (0.2)	Ciba Blue (0.01)	50	78
NADH	DQ (0.2)	7-Iodo-acridone-4-carboxylic acid (0.1)	24	26
NADH	DQ (0.2)	Dicumarol (0.3)	51	78
NADH	DQ (0.2)	DPI (0.025)	92	110

[a] –, No inhibitor present. [b] nd, Not detectable.

clearly affected by the conditions, and ionic strength changes during a subsequent ion-exchange step may have caused the separation of a putative DPI-binding component from the NAD(P)H:quinone oxidoreductase moiety. Following this step, the NAD(P)H:quinone oxidoreductase was stable in the absence of detergents and behaved as a soluble protein with little activity with lipophilic quinones, probably due to the loss of bound lipids or hydrophobic protein(s). Accordingly, the UQ-2 reductase activity of peak I was strongly inhibited by high detergent concentrations (e.g. 0.1% [w/w] TX-100R; not shown). Membrane interactions may also be invoked to explain the shift in NAD(P)H dependence shown by the purified oxidoreductase with respect to peak I, which (contrary to peak II) slightly preferred NADH over NADPH. Alternatively, a NADH-specific enzyme might be lost following purification of peak I. Since, however, DPI showed a strong inhibitory effect on membrane activity, and a complete one against peak I (Table IV), the results are consistent with a single NAD(P)H-dependent oxidoreductase in the DPI-sensitive redox complex.

In spite of the fact that DPI, interacting with the heme-binding component of Cyt b_{558} (Doussiere and Vignais, 1992), is often claimed to be a specific inhibitor of the mammalian NAD(P)H-oxidase, this compound also inhibits other redox proteins, including NAD(P)H-dehydrogenases of plant mitochondria (Roberts et al., 1995). The existence of an NADPH-oxidase complex of the mammalian type has recently been suggested for plants as well (Groom et al., 1996). Superoxide production in plant defense responses is sensitive to DPI (Levine et al., 1994), as is the NAD(P)H-dependent O_2^- synthesis detected in purified PM vesicles (Murphy and Auh, 1996). In our experiments the DPI effect was only evident in PM vesicles and partially purified protein fractions but not for the purified oxidoreductase. At present we also do not have any evidence for the participation of NAD(P)H:quinone oxidoreductase in a superoxide-generating system. We observe that DPI is less specific as an inhibitor than is usually thought.

On the basis of the peculiar mechanism of quinone reduction producing fully reduced hydroquinones, we have proposed that plant NAD(P)H-QR is a functional analog of animal DT-diaphorase (Trost et al., 1995). DT-diaphorase is now thought to interact with biological membranes, and it has been proposed that the two-electron reduction of ubiquinone effected by DT-diaphorase may be a general mechanism of protection of membrane components from free radical damage (Beyer et al., 1996). A similar antioxidant function of NAD(P)H:quinone oxidoreductase in the PM seems likely.

This is not to say that these membrane flavoproteins may not also participate in further redox reactions, e.g. transmembrane electron transport (Rubinstein and Luster, 1993). NAD(P)H:quinone oxidoreductases measured with impermeable electron acceptors are in *cis* configuration, i.e. their donor- and acceptor-binding sites face the cytosol (Askerlund et al., 1988; Cordoba et al., 1996). However, the capability of the PM redox system of reducing lipophilic quinones, by means of the intrinsic NAD(P)H:quinone oxidoreductase complex, supports a possible transmembrane electron transport via quinones shuttling through the membrane and perhaps interacting with other redox components, for instance, *b*-type cytochromes, also present in the plant PM (Asard et al., 1989; Askerlund et al., 1989).

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