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NAD(P)H:(Quinone-Acceptor) Oxidoreductase of Tobacco Leaves Is a Flavin Mononucleotide-Containing Flavoenzyme

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The soluble NAD(P)H:(quinone-acceptor) oxidoreductase [NAD(P)H-QR, EC 1.6.99.2] of *Nicotiana tabacum* L. leaves and roots has been purified. NAD(P)H-QR contains noncovalently bound flavin mononucleotide. Pairs of subunits of 21.4 kD are linked together by disulfide bridges, but the active enzyme is a homotetramer of 94 to 100 kD showing an isoelectric point of 5.1. NAD(P)H-QR is a B-stereospecific dehydrogenase. NADH and NADPH are electron donors of similar efficiency with K_{cat}:K_m ratios (with duroquinone) of 6.2×10^7 and 8.0×10^7 M^{-1} s^{-1}, respectively. Hydrophilic quinones are good electron acceptors, although ferricyanide and dichlorophenolindophenol are also reduced. The quinones are converted to hydroquinones by an obligatory two-electron transfer. No spectral evidence for a flavin semiquinone was detected following anaerobic photoreduction. Cibacron blue and 7-iodo-acridone-4-carboxylic acid are inhibitory. Tobacco NAD(P)H-QR resembles animal DT-diaphorase in some respects (identical reaction mechanism with a two-electron transfer to quinones, unusually high catalytic capability, and donor and acceptor substrate specificity), but it differs from DT-diaphorase in molecular structure, flavin cofactor, stereospecificity, and sensitivity to inhibitors. As in the case with DT-diaphorase in animals, the main NAD(P)H-QR function in plant cells may be the reduction of quinones to quinols, which prevents the production of semiquinones and oxygen radicals. The enzyme appears to belong to a widespread group of plant and fungal flavoproteins found in different cell compartments that are able to reduce quinones.

Plant NAD(P)H-QR (EC 1.6.99.2) has long been thought to be a peculiar type of DT-diaphorase (Pupillo et al., 1986; Valenti et al., 1989). Like the DT-diaphorase of animal tissues (Ernster, 1987), which does not seem to be present in higher plant cells (Trost et al., 1995), this plant enzyme can use either NADPH or NADH as an electron donor and hydrophilic quinones as acceptors. However, NAD(P)H-QR differs from animal DT-diaphorase in molecular weight and other properties, e.g. dicumarol is relatively ineffective as an inhibitor (Guerrini et al., 1987). NAD(P)H-QR occurs mainly in the soluble cell fraction, although NAD(P)H-dependent duroquinone reductase activities are also found in the mitochondria, microsomes, and plasma membrane (Asard et al., 1987; Luster and Buckhout, 1989; Valenti et al., 1990; Serrano et al., 1994; Rescigno et al., 1995).

Plant mitochondria contain rotenone-insensitive NAD(P)H dehydrogenases that interact with the ubiquinone pool. These proteins are thought to be peripherally bound to the inner mitochondrial membrane, both on the matrix and the cytosolic side (Møller et al., 1993). Several preparations of "external" or "internal" NAD(P)H dehydrogenases show a distinct preference for short-chain quinones as NAD(P)H-QR does. However, their subunits appear to vary in size depending on the species (Chauveau and Lance, 1991; Luethy et al., 1991, 1995; Rasmusson et al., 1993). Plant plasma membrane also contains an NAD(P)H-dependent quinone oxidoreductase of 27-kD subunits (Luster and Buckhout, 1989; Serrano et al., 1994), the function of which has not yet been elucidated.

We have recently characterized an NAD(P)H-QR from sugar beet suspension cells (Guerrini et al., 1994). The active enzyme, which has a mass of 85 to 94 kD, is composed of four identical subunits, each with a molecular mass of approximately 24 kD. NAD(P)H-QR shows a huge catalytic activity with short-chain acceptor quinones (Trost et al., 1995). In competition with other flavoproteins catalyzing a one-electron reduction of quinones, e.g. Cyt P450 reductase (Iyanagi, 1987), NAD(P)H-QR may be the only soluble protein of plant cells that produces fully reduced quinols without semiquinone intermediates; therefore, it is expected to prevent the buildup of active oxygen species resulting from semiquinone autooxidation (Trost et al., 1995). Similar views were originally developed to explain the DT-diaphorase function in animal cells (Prochaska et al., 1985; Ernster, 1987). Quinols are relatively stable compounds and can be detoxified in plant cells to vacuole-stored conjugates (Harborne, 1980).

In the present paper we describe the purification and properties of the soluble NAD(P)H-QR of tobacco (*Nicotiana tabacum* L.) leaves, which has been biochemically characterized for the first time. The active enzyme is an FMN-containing, tetrameric flavoprotein that consists of two pairs of subunits (21.4 kD each) linked by disulfide bridges. If the relationship between soluble NAD(P)H-QR and membrane-bound dehydrogenases is confirmed, rapid advances may be expected to occur in the knowledge of the structure and function of particulate NAD(P)H:quinone oxidoreductases.

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Abbreviations: Bis-Tris, bis(2-hydroxyethyl)imino-Tris-(hydroxymethyl)methane; DQ, 2,3,5,6-tetramethyl-1,4-benzoquinone (duroquinone); FNR, Fd:NADP$^+$ oxidoreductase; FPLC, fast protein liquid chromatography; I_{50}, concentration of inhibitor resulting in 50% inhibition; IAC, 7-iodo-acridone-4-carboxylic acid; NAD(P)H-QR, NAD(P)H:(quinone-acceptor) oxidoreductase; TEA, triethanolamine.

MATERIALS AND METHODS

Chemicals

Chromatographic media and systems (FPLC, SMART) were from Pharmacia. The compound IAC was synthesized and kindly provided by Dr. W. Oettmeier (Rhur University, Bochum; Oettmeier et al., 1992). Stigmatellin, myxothiazol, ubiquinone-1, and undecyl-ubiquinone were the generous gifts of Dr. Anna Ghelli of this university. Spinach Fd was kindly offered by Dr. Giorgio Forti (University of Milan).

Plant Material

Nicotiana tabacum L. cv Samsun plants were grown on soil for 6 to 8 weeks at 21°C. Light was supplied in a 12-h light/12-h dark cycle at a constant PPFD of 250 μmol m^{-2} s^{-1}.

Purification of NAD(P)H-QR from Tobacco Leaves

NAD(P)H-QR preparations were obtained from tobacco leaves using a recently described procedure (Trost et al., 1995) with major modifications. In a typical preparation, 1 kg of leaves was homogenized on ice with 1.75 volumes of 50 mM Tris-Cl, pH 7.5, 300 mM Suc, 2 mM EDTA, 14 mM 2-mercaptoethanol, and 0.5 mM PMSF. The homogenate was filtered through a nylon net (80 μm) and spun at 30,000*g* for 60 min. The resulting supernatant was brought to 40% $(NH_4)_2SO_4$ saturation, equilibrated in the cold for 30 min, and spun at 12,400*g* for 30 min. The pellet was discarded and the supernatant was adjusted to 65% saturated $(NH_4)_2SO_4$. After 30 min of equilibration, the NAD(P)H-QR was pelleted by centrifugation (12,400*g* for 30 min). The sediment, resuspended in 20 mM TEA-Cl, pH 7.8, 2 mM EDTA, and 0.5 mM PMSF, was adjusted to 1 M $(NH_4)_2SO_4$ and mixed by gentle stirring with a batch of Phenyl-Sepharose CL 4B (Pharmacia) (about 10 mg of protein per 1 mL of drained gel) previously equilibrated with 20 mM TEA-Cl, pH 7.8, 2 mM EDTA, and 1 M $(NH_4)_2SO_4$. The resin was washed with 2 volumes of the same buffer, and the NAD(P)H-QR activity was then recovered in 2 volumes of 20 mM TEA-Cl, 2 mM EDTA, pH 7.8. The enzyme preparation was concentrated by ultrafiltration (8400 cell, YM30 membrane, Amicon, Beverly, MA) and equilibrated to 20 mM TEA-Cl, pH 7.8, by desalting (Sephadex G-25 HiTrap Fast Desalting, Pharmacia). Aliquots of 500 mg of protein were loaded at 1.5 mL/min on a Q-Sepharose HP 16/10 anion-exchange column (Pharmacia) equilibrated with the same TEA-Cl buffer. After washing with a one-column volume of equilibration buffer, a 100-mL linear gradient of 0 to 0.5 M KCl in TEA-Cl buffer was applied. Active fractions eluting around 0.2 M KCl were pooled and concentrated by ultrafiltration (52 cell, PM10 membrane, Amicon), and 3-mL aliquots (containing about 40 mg of protein each) were loaded on a Superdex 200 FPLC 16/60 gel-filtration column (Pharmacia) equilibrated with 20 mM TEA-Cl, pH 7.8, 1 mM EDTA, and 150 mM KCl, and run at 1 mL/min. Fractions containing NAD(P)H-QR activity were concentrated and equilibrated as above with 20 mM Bis-Tris, pH 6.4. The protein preparation was then loaded on a anion-exchange column (Mono Q HR 5/5 [Pharmacia]) connected to an HPLC system (SMART, Pharmacia), equilibrated with the same buffer, and eluted at 0.2 mL/min by 20 mL of 0 to 0.5 M KCl gradient. The active fractions eluting at about 0.2 M KCl were pooled again and desalted (Sephadex G-25 HiTrap Fast Desalting, equilibrated with 5 mM Mes-Na, pH 6.4). A Blue Sepharose CL 6B column (Pharmacia) (3 mL) connected to the HPLC system (SMART, Pharmacia) and equilibrated with the same buffer was loaded with the sample at 0.25 mL/min. After exhaustive washing with equilibration buffer, NAD(P)H-QR was recovered by a linear NADPH gradient (10 mL, 0–20 μM NADPH). The pure protein could be stored at −20°C for months without appreciable loss of activity. In some experiments, minor contaminants were still detectable at this stage and a second anion-exchange column (MonoQ PC 1.6/5, SMART, Pharmacia) run at pH 6.4 was used to reach total purification. The same purification procedure, except for the Phenyl Sepharose step and the Mono Q chromatography (pH 6.4), has been applied to tobacco roots collected from the same plants grown for harvesting the leaves.

Determination of the Flavin Cofactor

Absorbance spectra were recorded on a spectrophotometer (model 7850, Jasco, Easton, MD). Fluorescence spectroscopy was performed on a fluorometer (model FP770, Jasco). Excitation and emission wavelengths were 468 and 525 nm, respectively. The NAD(P)H-QR flavin cofactor was separated by ultrafiltration (LGC, 10-kD cut-off, Millipore) following heat denaturation of the protein (100°C, 3 min). The ultrafiltrate was first brought to pH 7.6 and then to pH 2.0 by NaOH/HCl additions, and both excitation and emission spectra were recorded (Siegel, 1978). Standard FAD and FMN were treated under the same conditions before fluorometric analysis.

HPLC analysis was performed by loading 0.5-mL samples on a Sephasil C_{18} SC 2.1/10 column (SMART, Pharmacia). A_{260} and A_{450} were monitored, and flavins were resolved by isocratic elution at 1 mL/min in 20% (v/v) methanol, 5 mM ammonium acetate, pH 6.0 (Light et al., 1980).

Photochemical Reduction

Photoreduction of NAD(P)H-QR was achieved by irradiating the protein under anaerobic conditions in the presence of 15 mM EDTA, 1 mM 5-deazaflavin-3-sulfonate as catalyst and 5 mM benzylviologen at 25°C in 50 mM Hepes, pH 8.0, according to Massey and Hemmerich (1978).

Determination of the Extinction Coefficient

The spectrum of NAD(P)H-QR was recorded in 50 mM Hepes, pH 8.0, at 25°C and compared with the spectrum of free FMN released upon heat denaturation of the protein. The extinction coefficient at 448 nm for the holoenzyme was calculated from the ratio of absorbance before and after denaturation using an extinction coefficient of 11,300 M^{-1} for free FMN at this wavelength. A stoichiometry of 1 FMN per 21.4-kD subunit was assumed.

MS

The molecular weight was determined by flight-MS (MALDI/TOF-MS; Vestec Lasertec, Perseptive, Frieburg, Germany) operating in a linear mode. Ions formed by a pulse UV laser beam (nitrogen laser, wavelength = 337 nm) were accelerated through 28 kV. The instrument was calibrated with bovine carbonic anhydrase II (M_r 28,980). The calibration protein and the sample were dissolved in a solution of ferulic acid in 30% acetonitrile:70% water with trifluoroacetic acid 0.1%, to a final concentration of 10 pmol/μL.

Other Analytical Methods

Chromatofocusing, SDS-PAGE, amino acid analysis, and protein measurements were performed as described (Trost et al., 1995). Holoprotein concentration in pure NAD(P)H-QR preparations was estimated by both A_{448} (11,554 M^{-1} for 21.4-kD subunits, see "Results") and amino acid analysis, with similar results.

Steady-State Kinetic Analysis

Initial velocity data were analyzed by nonlinear regression (Trost and Pupillo, 1993). Data points, obtained by varying the concentrations of one substrate at a fixed concentration of the second substrate, were interpolated by the following equation:

$$v = (V_{max(app)} \times S) / (K_{m(app)} + S),$$

where S is the concentration of the varying substrate and $V_{max(app)}$ and $K_{m(app)}$ are apparent kinetic constants. The limiting kinetic constants V_{max} and K_m were then obtained by analogous nonlinear regression of the apparent kinetic constants. K_{cat} values refer to a theoretical molecular mass of the active protein equal to 85.6 kD (4 × 21.4-kD subunits).

The inhibition parameters (I_{50}) were obtained by titrating with inhibitor the NAD(P)H-QR reaction assayed with 0.2 mM NADH, 0.2 mM DQ in 40 mM Mops, pH 7.0. The inhibitor concentrations were increased until 90% inhibition was attained.

Enzyme Assays

In early purification steps NAD(P)H-QR activity was measured in the presence of 40 mM Mops, 2 mM EDTA, 0.5 mM KCN, pH 7.0, 0.2 mM NADH, and 0.2 mM DQ. Potassium cyanide was omitted in assays with pure NAD(P)H-QR. The decrease in A_{340} was followed with a spectrophotometer (Uvikon 941 plus, Kontron, Zurich, Switzerland) thermostatted at 25°C. An extinction coefficient at 340 nm of 6.23 mM^{-1} for NAD(P)H was used for activity calculations. With other quinones, activity was measured under the same conditions, but corrected extinction coefficients were used to take into account the A_{340} given by the interfering quinone. Activity in the presence of the following electron acceptors was measured as reported: Cyt *c*, nitrate, oxygen, riboflavine (Trost et al., 1995); ferricyanide, Fe^{III}-EDTA, Fe^{III}-citrate (Bagnaresi and Pupillo, 1995); ascorbate free radical (Luster and Buckout, 1989); lipoamide (Rasmusson et al., 1993); Fd (Zanetti and Curti, 1980); oxidized glutathione (Bergmeyer, 1984); and oxaloacetate (Valenti et al., 1990). Dichlorophenolindophenol reduction was followed with an extinction coefficient at 600 nm of 20.6 mM^{-1}.

Stereospecificity of the Hydride Transfer

The hydride transfer stereospecificity has been determined by means of the ^1H NMR technique essentially as described by Arnold et al. (1976) using a 300-MHz spectrometer (Gemini, Varian, Palo Alto, CA). Purified 4*R*-[^2H]NADH was prepared according to Michels et al. (1994) with deuterated acetaldehyde and yeast aldehyde dehydrogenase (Sigma). Complete deuteration at the C-4 position was checked by ^1H NMR by comparison with standard NADH.

The reaction mixture for the determination of NAD(P)H-QR stereospecificity (100 mL) contained 50 mM NH_4HCO_3, pH 8.5, 0.2 mM 4*R*-[^2H]NADH, 0.2 mM DQ, and 0.5 μmol min^{-1} purified tobacco NAD(P)H-QR. The mixture was incubated at 25°C and the reaction was stopped by ultrafiltration when more than 90% of NADH had been converted to NAD^+ as judged by A_{340}. NAD^+ was then purified as described (Michels et al., 1994). Samples of 5 to 10 mg of pyridine nucleotide were repeatedly lyophilized and resuspended in 99.8% ^2H_2O prior to ^1H NMR analysis.

RESULTS

Purification and Biochemical Properties of NAD(P)H-QR from Tobacco Leaves

Nearly 90% of the total NAD(P)H-dependent duroquinone reductase activity of tobacco leaves was not sedimented after 1 h of centrifugation at 100,000*g*. The enzyme remaining in the supernatant [NAD(P)H-QR] was purified to homogeneity. Table I summarizes the purification procedure.

Native NAD(P)H-QR of tobacco leaves was estimated to have a molecular mass of 94 to 100 kD by gel-filtration chromatography (Superdex 200 and Superose 12 columns) (Fig. 1). It is formed by 21.4-kD polypeptides as determined by MS (Fig. 2). Interestingly enough, the protein migrated as an apparent 43-kD polypeptide on denaturating SDS-polyacrylamide gels in the absence of reductants, but as a 24-kD subunit upon reduction with 25 mM or higher concentrations of 2-mercaptoethanol (Fig. 3). Thus, tobacco NAD(P)H-QR appears to be a tetramer of 21.4-kD subunits, which are paired to 43-kD dimers by means of disulfide bridges. Two 43-kD dimers seem to form the catalytically active enzyme, since we have never observed free 43-kD dimers in nondenaturating gel-filtration experiments either in the absence (Fig. 1) or in the presence of 25 mM 2-mercaptoethanol. The final NAD(P)H-QR preparation yielded a single form of pI 5.1 ± 0.1 determined by chromatofocusing. The NAD(P)H-QR has also been purified from tobacco roots by using a simplified procedure (see "Materials and Methods"). No significant differences in specific activity, properties, or molecular weight were de-

tected between enzymes of photosynthetic and nonphotosynthetic tissues.

The absorbance spectrum of NAD(P)H-QR recalls the typical spectra of flavoproteins (Fig. 4). Peaks are visible at 375 and 448 nm, plus a shoulder at approximately 475 nm. A 274- to 448-nm absorbance ratio around 7 was normally observed with the oxidized pure protein. The absorption in the visible region was abolished by treating the protein with an excess of dithionite or NADPH. The presence of a flavin cofactor was confirmed by fluorometric and absorption spectra of both the native enzyme and the cofactor released from heat-denatured apoprotein. Native NAD(P)H-QR showed an emission maximum at 525 nm upon excitation at 468 nm, and excitation peaks at 373 and 447 nm (emission recorded at 525 nm). Identical wavelength values but much higher fluorescent yields were observed when the isolated flavin was analyzed (Fig. 5). In both cases, fluorescence intensities dropped dramatically upon dithionite reduction.

The flavin was demonstrated to be noncovalently bound FMN by two independent approaches. First, the emission of the flavin cofactor of NAD(P)H-QR was found to decrease following acidification in a manner similar to FMN, but contrary to FAD (Fig. 5) (Siegel 1978). Second, the retention time of the NAD(P)H-QR flavin cofactor on the HPLC reverse-phase column was coincident with standard FMN; no peaks were detected in the correspondence of standard FAD and riboflavin elutions (Table II). An extinction coefficient of 11,544 M^{-1} at 448 nm was calculated for the 21.4-kD subunit of the holoenzyme, with the assumption of a 1:1 stoichiometry with FMN.

When absorption spectra of NAD(P)H-QR were observed following anaerobic photoreduction, no evidence of a flavin radical intermediate was found. Increasing the reduction of NAD(P)H-QR led to a general absorption decrease at wavelengths beyond the isosbestic point (345 nm, Fig. 4). Since half-reduced flavoproteins are known to show typical absorption increments at visible wavelengths (Massey and Hemmerich, 1978), the spectral data of Figure 4 indicate that NAD(P)H-QR semiquinones had no significant thermodynamic and kinetic stability under the present conditions. By allowing the reduced NAD(P)H-QR to reoxidize in the air, the typical spectrum of the oxidized form was soon recovered, again with no evidence of semiquinoid intermediate forms. These results are consistent with the two-electron reduction of quinoid acceptors by NAD(P)H-QR that is discussed below.

Kinetic Properties

The electron transfer to quinones catalyzed by NAD(P)H-QR in comparison with another flavoenzyme, FNR, was investigated. The latter enzyme is known to react with quinones by forming radical semiquinones (Iyanagi, 1987; Trost et al., 1995). Table III reports the initial rates of NADPH oxidation and Cyt *c* reduction triggered by the addition of benzoquinone in the presence of NAD(P)H-QR or FNR. A theoretical velocity ratio of 2 (Cyt *c* reduced/NADPH oxidized) would show that two semiquinones are formed for each NADPH molecule oxidized; a ratio of 0 indicates that one quinol is directly produced by the oxidation of one NADPH. Ratios of 2.0 and 0.074 were found for FNR and NAD(P)H-QR, respectively (Table III). Hydrobenzoquinone (quinol) proved to be quite stable and not reactive with Cyt *c* under the current experimental conditions (pH 7.0). Semibenzoquinone ($E'_m[Q/Q^{\cdot-}] = +100$ mV) does not react with molecular oxygen ($E'_m[O_2/O_2^{\cdot-}] = -155$ mV), but it readily reduces Cyt *c* ($E'_m = +260$ mV). Therefore, the experiment shows that semiquinone production from NAD(P)H-QR is negligibly low.

The reaction mechanism of tobacco leaf NAD(P)H-QR has been investigated by steady-state kinetic analysis. With any pyridine nucleotide and acceptor tested (see Table IV), parallel kinetics in double-reciprocal plots were obtained, strongly suggesting a ping-pong reaction mechanism (not shown; see Trost et al., 1995). A survey of NAD(P)H-QR kinetic parameters in the presence of duroquinone is given in Table V. Table V also shows that NADPH and NADH are both suitable electron donors, as indicated by their similar V_{max} values and not vastly different K_{cat}:K_m ratios.

On the basis of the K_{cat}:K_m ratios (Fersht, 1985), we have examined the specificity of the tobacco enzyme for various electron acceptors (Table IV) and found ubiquinone-0 to be the best acceptor. The effectiveness of other quinones as acceptors was negatively correlated with hydrophobicity, as previously shown for the sugar beet enzyme (Trost et al., 1995). Relatively lipophilic quinones such as undecylubiquinone, phylloquinone, pyrroquinoline quinone, and lapachol were not reduced at all. No activity (i.e. less than 1% compared with duroquinone) was detected when test-

Table I. *Purification scheme of tobacco leaf NAD(P)H-QR*

Data from a typical preparation of 1 kg of leaves. Activity was measured with the standard assay (NADH:DQ).

Purification Step	Activity	Protein	Specific Activity	Yield	Purification
	μmol min⁻¹	*mg*	*μmol min⁻¹ mg⁻¹*	*%*	*-fold*
Supernatant 30,000*g*	2228	2912	0.77	100	1
$(NH_4)_2SO_4$ 40–65%	1184	nd[a]	nd	53	nd
Phenyl-Sepharose	989	1470	0.67	44	<1
Q-Sepharose (pH 7.8)	760	57	13	34	17
Superdex 200	485	13	37	22	48
MonoQ (pH 6.4)	264	0.83	316	12	410
Blue-Sepharose	181	0.06	3017	8	3918

[a] nd, Not determined.

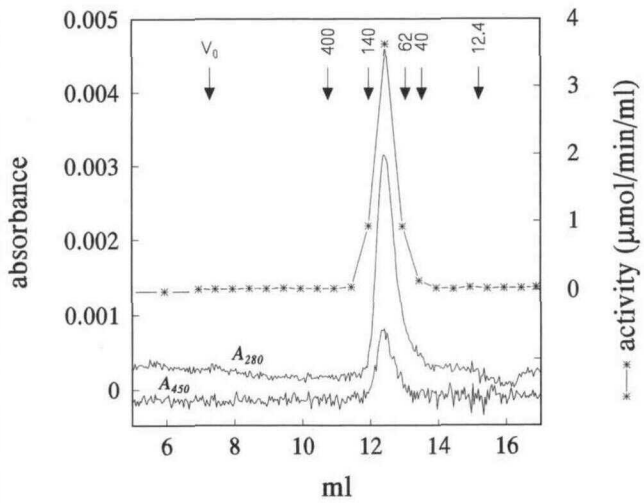

Figure 1. Gel-filtration chromatography of purified tobacco NAD(P)H-QR. A Superose 200 column (HR 10/30) equilibrated with 20 mM TEA-Cl, pH 7.8, 150 mM KCl, and connected to the Smart System was loaded with 0.2 mL of sample (2 μg protein). Flow rate was 300 μL/min. Fractions of 0.5 mL were collected for measure of NADH:DQ reductase activity. A_{280} and A_{450} were simultaneously monitored. Elution volumes of standard protein were determined in independent runs under identical conditions. Molecular mass standards (kD) are indicated (400, ferritin; 140, lactate dehydrogenase; 62, malate dehydrogenase; 40, phosphoglycerate kinase; 12.4, Cyt *c*); V_o, Void volume.

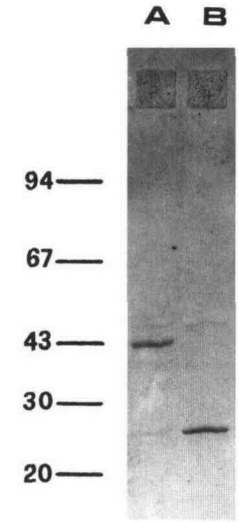

Figure 3. SDS-PAGE of purified tobacco NAD(P)H-QR. Denaturating 12.5% PAGE. Samples of 0.5 μg of purified NAD(P)H-QR were boiled for 3 min in sample buffer: 10 mM PBS, pH 7.1, 1% (w/v) SDS, 0.25% (w/v) bromphenol blue without (lane A) or with (lane B) 23 mM 2-mercaptoethanol. The M_r of the NAD(P)H-QR dimer and monomer were estimated on the basis of the markers indicated on the left of the gel (kD).

ing a number of possible physiological electron acceptors other than quinones (Cyt *c*, ascorbate free radical, oxidized glutathione, Fe^{III}-citrate, Fe^{III}-EDTA, riboflavin, nitrate, oxaloacetate, oxygen, lipoamide, and Fd).

The effects of several potential inhibitors of the NAD(P)H-QR reaction are summarized in Table VI. Compounds thought to interact with quinone sites of the mitochondrial respiratory chain (rotenone, stigmatellin, and myxothiazol) had no effect, and other respiratory inhibitors such as flavone, dinitrophenol, KCN, and salicylhydroxamic acid were also not particularly active. The enzyme activity was slightly affected by 4-hydroxymercuribenzoate. Among inhibitors of animal NAD(P)H:QR (DT-diaphorase), dicumarol and warfarin were effective only at substantial concentrations (I_{50} of 2×10^{-4} M), but Cibacron blue was active in the low micromolar range (I_{50} of 6×10^{-6} M). The enzyme was effectively inhibited by IAC (I_{50} of 2×10^{-5} M), a compound active at similar concentrations against the rotenone-insensitive NAD(P)H dehydrogenases of plant mitochondria (Roberts et al., 1996). The inhibition was

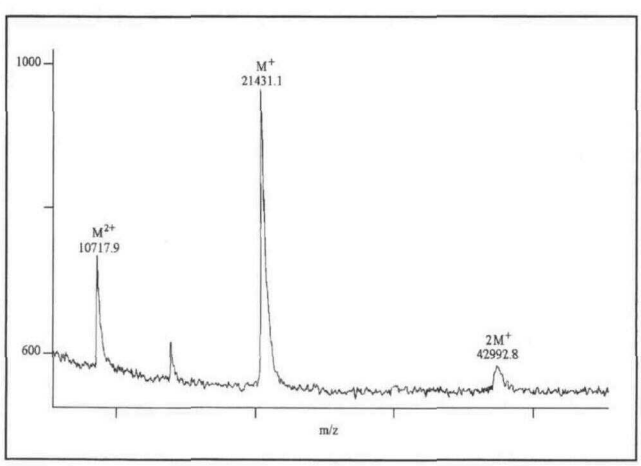

Figure 2. MS of purified tobacco NAD(P)H-QR. Mass spectrum was obtained using carbonic anhydrase II (M_r = 28,980) as a calibration protein. The number of charges on each ion species is given by the superscript *n*+.

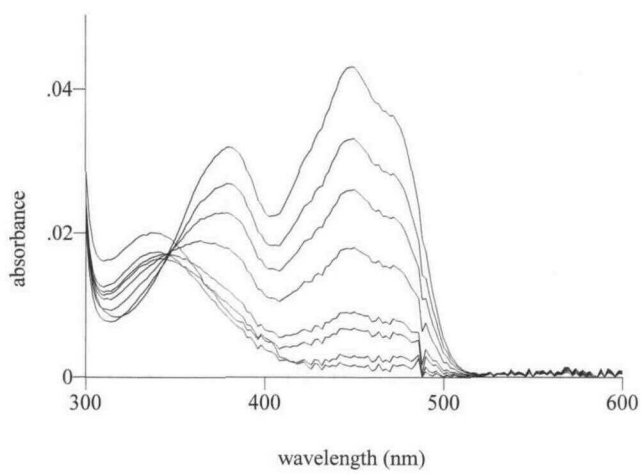

Figure 4. Absorption spectra of NAD(P)H-QR following photoreduction under anaerobiosis. NAD(P)H-QR in 50 mM Hepes, pH 8.0, 1 mM 5-deazaflavin-3-sulfonate as catalyst, and 5 mM benzylviologen was irradiated under anaerobic conditions at 25°C. Selected spectra are shown for different times of irradiation (top to bottom): oxidized enzyme, 8, 13, 18, 23, 25, 27, and 29 s of irradiation.

mixed, although it tended toward pure competition with pyridine nucleotides (Fig. 6) with K_i versus NADH of 2×10^{-5} M.

Hydride Transfer Stereospecificity

Purified NAD(P)H-QR has been used to determine the stereospecificity of the hydride transfer from the C-4 position of the nicotinamide of NADH to the flavin. A sample of 4*R*-[^{2}H]NADH obtained by the reaction of aldehyde dehydrogenase, an A-specific enzyme (You et al., 1978), was oxidized by NAD(P)H-QR in the presence of duroquinone. The resulting NAD^+ proved to be still deuterated at carbon 4 of nicotinamide (4-[^{2}H]NAD^+), as shown by the lack of the corresponding doublet (Fig. 7, PC_4H) in the ^{1}H NMR spectra. Also, the triplet-to-doublet conversion of the signal associated with the proton at carbon 5 (Fig. 7, PC_5H) confirms the presence of deuterium at carbon 4. Thus, NAD(P)H-QR has opposite stereospecificity with respect to aldehyde dehydrogenase, and so it belongs to the B-stereospecific class of dehydrogenases.

DISCUSSION

We studied the biochemical characterization of the NAD(P)H-QR obtained from tobacco leaves. In this tissue, as in nonphotosynthetic tissues, a single dominant enzyme present in the soluble cell fraction is responsible for most of the NAD(P)H:duroquinone reductase activity, although minor activities are found to be associated with membranes (Valenti et al., 1990; Trost et al., 1995). Of many compounds tested on purified NAD(P)H-QR, hydrophilic quinones proved to be highly effective acceptors, whereas hydrophobic quinones such as phylloquinone (vitamin K_1) and undecyl-ubiquinone were not accepted. Artificial electron acceptors such as ferricyanide and dichlorophenolindophenol were also reduced; molecular oxygen and Cyt *c* were not. Similar results were previously obtained with the NAD(P)H-QR of heterotrophic sugar beet cells (Trost et al., 1995). Quinones may be the electron acceptors of NAD(P)H-QR in vivo. By contrast, most other flavoenzymes, e.g. Fd:$NADP^+$ oxidoreductase or Cyt P_{450} reductase, perform definite physiological reactions in addition to

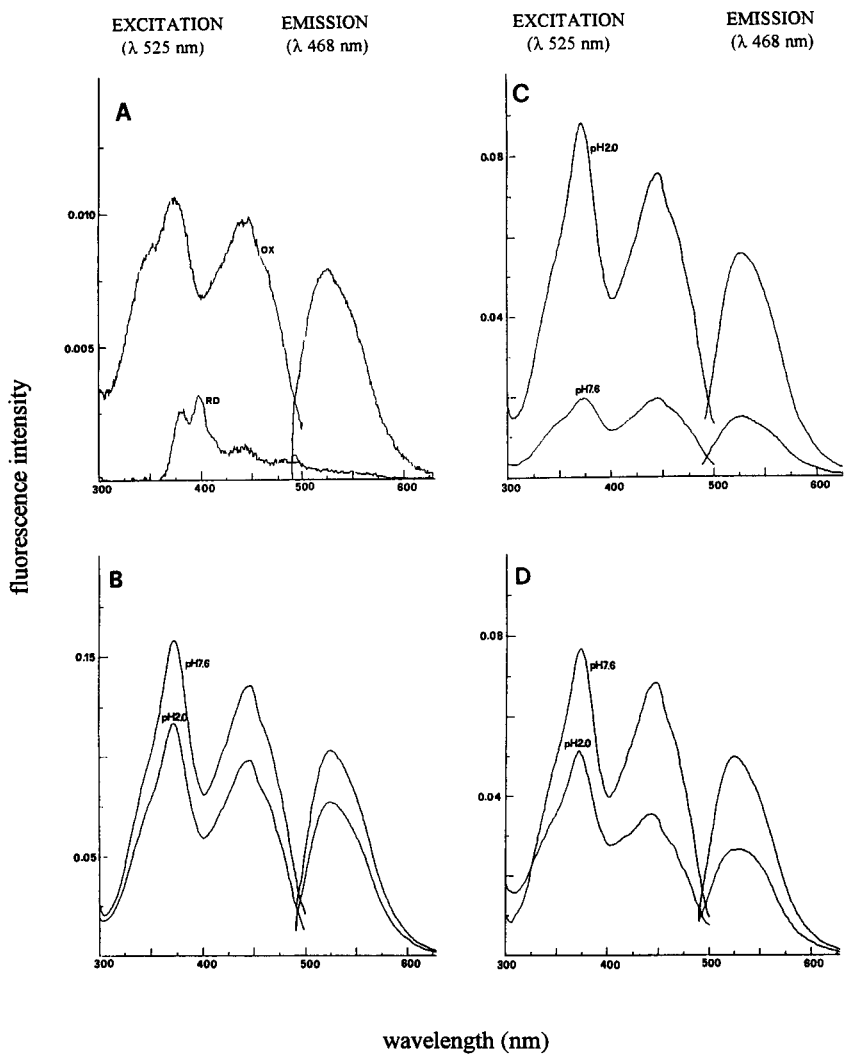

Figure 5. Fluorometric spectra of NAD(P)H-QR holoprotein and NAD(P)H-QR prosthetic group. Excitation spectra recorded with an emission wavelength of 468 nm and by excitating at 525 nm. A, NAD(P)H-QR holoprotein (6 μg/mL). OX, Oxidized enzyme; RD, dithionite-reduced enzyme. B, NAD(P)H-QR prosthetic group obtained after heat denaturation and ultrafiltration of the sample shown in A. C, FAD (0.16 μM), and D, FMN (0.16 μM) spectra recorded at pH 7.6 and 2.0, respectively.

the unspecific reduction of quinones of suitable redox potential (Iyanagi, 1987).

Most flavo-oxidoreductases can use quinones as substrates and reduce them to semiquinone radicals in one-electron reactions. Animal NAD(P)H:(quinone-acceptor) oxidoreductase, better known as DT-diaphorase, is a notable exception in that it produces only fully reduced hydroquinones by a compulsory two-electron transfer (Iyanagi, 1987). Plant NAD(P)H-QR appears to react with quinones in the same way that DT-diaphorase does, and shares with this animal flavoprotein an unusually high catalytic power and the specificity toward donor and acceptor substrates.

That hydroquinones are the products of the NAD(P)H-QR reaction is shown here by the use of Cyt *c* as a radical trap, in an experimental comparison between NAD(P)H-QR and FNR reactions with benzoquinone. Furthermore, spectral measurements of NAD(P)H-QR following anaerobic photoreduction rule out the existence of stable flavin semiquinonic intermediates. This is an uncommon feature among flavo-oxidoreductases (Massey et al., 1969). The finding that NAD(P)H-QR can also reduce artificial one-electron acceptors such as ferricyanide is not in contrast with the present interpretation. Although DT-diaphorase is also active with ferricyanide, Tedeschi et al. (1995) were unable to detect flavoprotein semiquinones in rapid reaction studies with DT-diaphorase and concluded that the reduction of the second ferricyanide molecule was much faster than that of the first. Therefore, the simultaneous two-electron transfer model seems suitable for both DT-diaphorase and plant NAD(P)H-QR, and we assume that in general NAD(P)H-QR and DT-diaphorase share a common and peculiar reaction mechanism.

The catalytic potential of tobacco NAD(P)H-QR with hydrophilic quinones is elevated. Hydrophilic quinoid species are widespread in plants mostly as quinols, but there is little information about their actual concentrations in vivo. Quinols can be conjugated with sugars by the action of glycosyltransferases and accumulated into vacuoles (Harborne, 1980). On the other hand, oxidized quinones may have toxic effects, since they can be reduced to semiquinones by a number of enzymes, with the consequent formation of noxious active oxygen species (Hassan and Fridovich, 1979). By reducing hydrophilic quinones directly to quinols through an irreversible reaction (Trost et al., 1995), NAD(P)H-QR could partially prevent oxygen radical production in plant cells (Guerrini et al., 1994). Although the K_m for quinones is relatively high (that of duroquinone is 0.3–0.4 mM) and the K_m values for NADH and NADPH (0.4 mM) are higher than the expected concentrations of both pyridine nucleotides in plant cells (Heineke et al., 1990), the enzyme is potentially able to scavenge any soluble quinones, since the apparent affinity for the substrates in a ping-pong reaction mechanism tends to increase with decreasing acceptor concentrations. Also, the calculated K_{cat}:K_m ratios for NAD(P)H-QR are close to the diffusion rate of soluble molecules in water, indicating high kinetic proficiency (Fersht, 1985). Therefore, the available evidence supports an important role of this enzyme in maintaining a low level of oxidized quinones in the plant cell.

Tobacco leaf NAD(P)H-QR is a soluble flavoprotein consisting of 21.4-kD subunits arranged in dimers by sulfur bonds. The catalytically competent enzyme form is a tetramer of 94 to 100 kD resulting from the noncovalent interaction of two 43-kD dimers. FMN is the prosthetic group, noncovalently bound to the apoprotein. Our results confirm that plant NAD(P)H-QR has a structure that is different from that of the DT-diaphorase of mammals, which is in the form of 30-kD dimers, each

Table II. *Identification of the NAD(P)H-QR prosthetic group by HPLC-reverse-phase chromatography*

Isocratic runs with an HPLC column (Sephasil C18 SC 2.1/10 SMART System) in 20% (v/v) methanol, 5 mM ammonium acetate, pH 6.0. Flow rate was 1 mL/min and 0.5-mL samples were loaded. Flavins were detected by A_{260} and A_{450}. The NAD(P)H-QR flavin was previously separated from the apoprotein by heat treatment and 10-kD cut-off ultrafiltration.

Type of Flavin	Retention Time
	min
FAD	4.04
FMN	5.27
Riboflavin	21.74
NAD(P)H-QR flavin	5.28

Table III. *Enzymatic production of semibenzoquinone by NAD(P)H-QR and FNR*

NADPH oxidation was followed at 340 nm in 40 mM Mops, pH 7.0, 0.2 mM NADPH, and purified tobacco NAD(P)H-QR or FNR in the absence of Cyt *c*. The reaction was started by adding 20 μM benzoquinone to the cuvette while stirring. Initial velocity was taken during the first 10 s of the reaction. Blanks without enzyme were subtracted. Cyt *c* reduction was measured at 550 nm in the same reaction mixture with 30 μM Cyt *c*. Neither NAD(P)H-QR nor FNR are able to use Cyt *c* as a direct electron acceptor at a significant rate. Reaction was started with 20 μM benzoquinone and initial velocity was taken during the first 10 s. Data represent mean values ± SD of three assays for each type of measure.

Type of Reaction	NAD(P)H-QR	FNR
NADPH oxidation (nmol NADPH/min^{-1})	4.3 ± 0.3	4.8 ± 0.0
Cyt *c* reduction (nmol Cyt *c*/min^{-1})	0.32 ± 0.05	9.6 ± 0.2
(Cyt *c* reduction):(NADPH oxidation) ratio	0.074	2.0

Table IV. *NAD(P)H-QR specificity for electron acceptors*

The electron donor was NADPH. A single kinetic analysis was performed for every electron acceptor.

Electron Acceptor	$K_{cat}/K_{m(acceptor)}$
	$M^{-1}\ s^{-1}$
Quinones	
Ubiquinone-0	2.2×10^8
Benzoquinone	1.5×10^8
Juglone	1.4×10^8
Duroquinone	8.4×10^7
Menadione	2.8×10^7
Ubiquinone-1	2.2×10^7
General acceptors	
Dichlorophenolindophenol	9.8×10^7
Ferricyanide	5.8×10^6

Table V. *Limiting kinetic constants of NAD(P)H-QR as estimated by steady-state analysis [NAD(P)H:DQ reaction]*

Data are mean values ± SD of three experiments for each electron donor.

Donor	V_{max}	$K_{m(donor)}$	$K_{m(DQ)}$	$K_{cat}/K_{m(donor)}$	$K_{cat}/K_{m(DQ)}$
	$\mu mol\ min^{-1}\ mg^{-1}$	μM	μM	$M^{-1}\ s^{-1}$	$M^{-1}\ s^{-1}$
NADH	$(16.5 \pm 3.0) \times 10^3$	384 ± 33	436 ± 70	$(6.2 \pm 1.0) \times 10^7$	$(5.2 \pm 0.2) \times 10^7$
NAD(P)H	$(16.7 \pm 5.8) \times 10^3$	377 ± 68	280 ± 183	$(8.0 \pm 2.6) \times 10^7$	$(8.4 \pm 3.2) \times 10^7$

binding one FAD molecule (Li et al., 1995). Moreover, potent inhibitors of DT-diaphorase (dicumarol, warfarin, and Cibacron blue; Ernster, 1987) affect plant NAD(P)H-QR activity only at concentrations that are orders of magnitude higher than that necessary for DT-diaphorase to be affected.

The stereospecificity of the hydride transfer is a strongly conserved feature of dehydrogenases. DT-diaphorase is generally considered to be A-stereospecific (Lee et al., 1965), although this conclusion has been challenged by recent crystallographic studies (Li et al., 1995). On the other hand, we have shown in the present study that tobacco NAD(P)H-QR is B-stereospecific. Thus, NAD(P)H-QR and DT-diaphorase are unlikely to share much structural homology.

The NAD(P)H-QR from heterotrophic sugar beet cells has been described as having many structural and kinetic properties in common with tobacco NAD(P)H-QR, e.g. size of the subunits and of the active enzyme, catalytic competence, reaction mechanism, two-electron donation, substrate specificity, and inhibitors (Guerrini et al., 1994; Trost et al., 1995). Etiolated soybean hypocotyls also contain a similar NAD(P)H-diaphorase with 22-kD subunits showing flavoprotein spectra (Rescigno et al., 1995). Another NAD(P)H-diaphorase has been purified from the white-rot basidiomycete *Phanaerochete chrysosporium* as a dimer of 22-kD subunits with one shared FMN molecule (Brock et al., 1995). The fungal degradation of lignin yields products that include toxic quinones (e.g. 2,6-dimethoxy-1,4-benzoquinone). The NAD(P)H-diaphorase in *P. chrysosporium* was severalfold enhanced during growth in the presence of wood degradation products. A specific function for NAD(P)H-QR was thus apparent in this organism (Brock et al., 1995).

According to some reports (Luster and Buckhout, 1989; Serrano et al., 1994), the plant root plasma membrane contains dehydrogenases with 27-kD subunits with properties of the same general protein type as NAD(P)H-QR, e.g. they show high NAD(P)H-dependent activity with hydrophilic quinones but low activity with Cyt *c*. The NAD(P)H-QR of corn root plasma membrane was stimulated by added FMN, so this flavin was thought to be a weakly bound enzyme cofactor (Luster and Buckhout, 1989). An FMN-containing diaphorase has recently been found in bean hook plasma membranes (Van Gestelen et al., 1996), and proteins similar to NAD(P)H-QR are present in microsomal membranes (Guerrini et al., 1994; P. Trost, S. Foscarini, V. Preger, P. Bonora, L. Vitale, and P. Pupillo, unpublished results). Another plasmalemma flavoprotein with some activity toward quinones was recently purified from spinach leaves (Bérczi et al., 1995); however, it is mostly active as NADH:ferricyanide reductase, it is formed by 45-kD subunits, and it contains FAD, indicating that it is unrelated to NAD(P)H-QR.

Table VI. *Effect of several compounds on NAD(P)H-QR activity*

Data are mean values ± SD of two experiments with three replicates each. Reaction mixture was 40 mM Mops, pH 7.0, 0.2 mM NADH, 0.2 mM DQ ± inhibitor as indicated.

Inhibitor	Concentration	NADPH-QR	Residual Activity	NADH-QR	Residual Activity
	mM	$mmol\ min^{-1}\ mg^{-1}$	%	$mmol\ min^{-1}\ mg^{-1}$	%
Control		2.5 ± 0.1	100	2.5 ± 0.2	100
Stigmatellin	0.01	2.6 ± 0.2	104	n.t.[a]	
Myxothiazol	0.01	2.7 ± 0.5	108	n.t.	
KCN	1.0	2.2 ± 0.1	88	1.9 ± 0.1	76
Rotenone	0.02	n.t.		2.2 ± 0.1	88
	0.05	2.3 ± 0.7	92	n.t.	
Flavone	0.1	2.2 ± 0.1	88	2.2 ± 0.1	88
Salicylhydroxamic acid	1.0	3.1 ± 0.2	124	2.4 ± 0.1	96
Quinacrine	0.5	2.0 ± 0.1	80	2.2 ± 0.2	88
4-Hydroxymercuribenzoate	0.1	2.0 ± 0.2	80	1.5 ± 0.3	60
Dinitrophenol	0.05	2.3 ± 0.2	92	2.3 ± 0.2	92
Cibacron blue	0.02	0.70 ± 0.05	28	0.75 ± 0.04	30
Warfarin	0.1	1.7 ± 0.2	68	1.7 ± 0.1	68
Dicumarol	0.1	1.7 ± 0.1	68	1.8 ± 0.1	72
7-iodo-acridone-4-carboxylic acid	0.1	0.89 ± 0.03	36	0.37 ± 0.02	15

[a] n.t., Not tested.

Similar proteins present in other cell compartments have been the object of numerous investigations. An NAD(P)H-diaphorase with 26-kD subunits isolated from beet mitochondria was identified as the internal, rotenone-insensitive NAD(P)H-dehydrogenase of the inner membrane (Rasmusson et al., 1993). The latter diaphorase, as well as the external NADH-dehydrogenase of the inner membrane (Douce et al., 1973), are B-stereospecific like NAD(P)H-QR. Moreover, the NAD(P)H dehydrogenase isolated from beet mitochondria (Rasmusson et al., 1993) shows a very high specific activity, also a typical feature of NAD(P)H-QR (Trost et al., 1995). Mitochondria of the same species treated by a different procedure yielded a diaphorase with 42-kD polypeptides, active with both NADH and NADPH (Luethy et al., 1991). These contrasting findings could be reconciled if the 42-kD protein could be split up into smaller subunits, as we have done for NAD(P)H-QR.

Roberts et al. (1996) reported that IAC is an efficient inhibitor of mitochondrial NAD(P)H-dehydrogenases. We have observed a similar effect of IAC on sugar beet (Trost et al., 1995) and tobacco NAD(P)H-QR, which may suggest further complex relationships between NAD(P)H-QR and rotenone-insensitive NAD(P)H dehydrogenases. It can be speculated that the soluble NAD(P)H-QR or similar proteins may interact in vivo with lipid bilayers either by posttranslational modification (e.g. with fatty acids) or by forming a redox complex with other integral membrane proteins. In summary, we suggest that the NAD(P)H-QR described in the current study may be a member of a widespread family of flavoproteins having a role in quinone detoxification, possibly fulfilling other transmembrane redox reactions between reduced pyridine nucleotides and quinones at cytosol-membrane interfaces of plant and fungal cells.

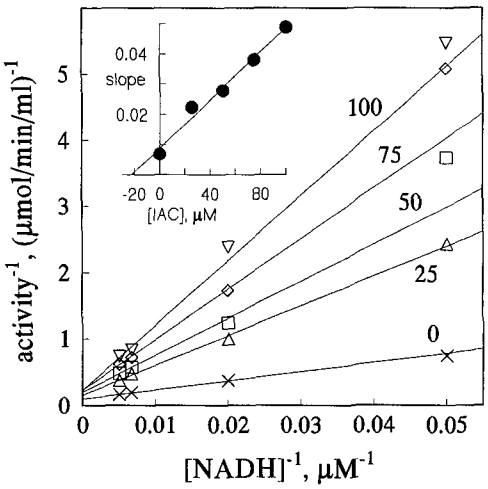

Figure 6. Inhibition of IAC on NADH-DQ reductase activity. Double-reciprocal plot with NADH as the variable substrate, DQ fixed at 0.2 mM, and increasing concentrations of IAC (μM) as indicated. Every set of data obtained at each inhibitor concentration was interpolated by nonlinear regression analysis. The inset shows the secondary plot of slope versus the IAC concentration; the K_i versus the NADH site is calculated from the intercept with the [IAC] axis.

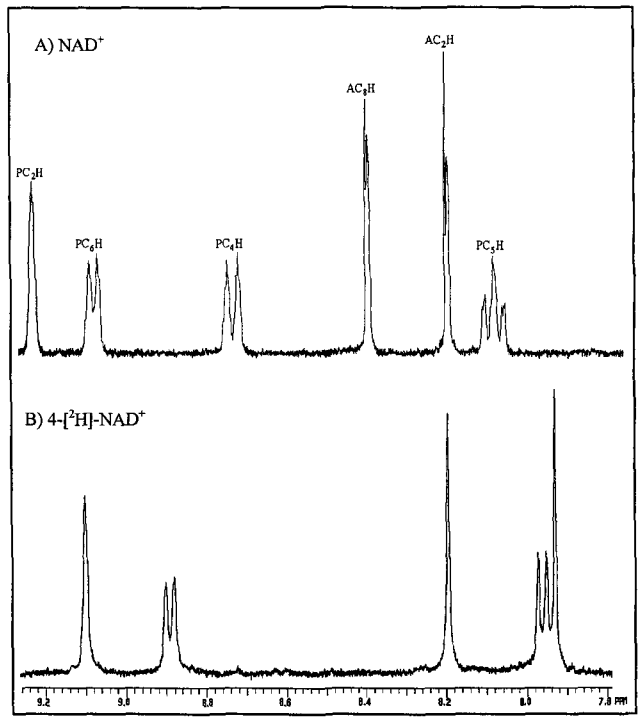

Figure 7. Hydride transfer stereospecificity of NAD(P)H-QR determined by ^{1}H NMR analysis. A, ^{1}H NMR spectrum of 10 mg of NAD$^+$ dissolved in 1 mL of ^2H_2O. Only the aromatic region is shown. By comparison with Arnold et al. (1976), the peaks were identified as indicated (PC_xH, proton at the carbon in position *x* of the pyridinium ring; AC_xH, proton at the carbon in position *x* of the adenine moiety). B, ^{1}H NMR spectrum of 5 mg of 4-[^{2}H]NAD$^+$ produced by the reaction of purified NAD(P)H-QR with 4*R*-[^{2}H]NADH and duroquinone. By comparison with spectrum A, the signal of the proton PC_4H is absent and the PC_5H triplet is converted to a doublet, demonstrating the presence of deuterium at the PC_4 position. The relative shift between the two spectra is due to the presence of impurities in the sample.

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