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Comparison of the Stereospecificity and Immunoreactivity of NADH-Ferricyanide Reductases in Plant Membranes[1]

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The substrate stereospecificity of NADH-ferricyanide reductase activities in the inner mitochondrial membrane and peroxisomal membrane of potato (*Solanum tuberosum* L.) tubers, spinach (*Spinacea oleracea* L.) leaf plasma membrane, and red beetroot (*Beta vulgaris* L.) tonoplast were all specific for the β-hydrogen of NADH, whereas the reductases in wheat root (*Triticum aestivum* L.) endoplasmic reticulum and potato tuber outer mitochondrial membrane were both α-hydrogen specific. In all isolated membrane fractions one or several polypeptides with an apparent size of 45 to 55 kD cross-reacted with antibodies raised against a microsomal NADH-ferricyanide reductase on western blots.

Most, if not all, plant membranes contain a redox chain (Møller and Lin, 1986). The presence of a redox chain in the inner mitochondrial membrane, the respiratory chain, and in the thylakoid membrane, the photosynthetic electron transport chain, is well established. The presence of a shorter redox chain consisting of an NAD(P)H-Cyt *b* reductase and a *b*-type Cyt has been established for the ER, the outer mitochondrial membrane, the PM, the glyoxysomal membrane, the peroxisomal membrane, and the tonoplast (Møller and Lin, 1986; Struglics et al., 1993, and refs. therein).

In the present study we compared the stereospecificity and immunoreactivity of the NADH-ferricyanide reductases in the mitochondrial membranes, PM, tonoplast, ER, and peroxisomal membrane.

MATERIALS AND METHODS

All of the membranes were isolated as described in the literature: inside-out SMP (the inner mitochondrial membrane) (Rasmusson and Møller, 1991) and the outer mitochondrial membrane (Pical et al., 1993) from potato (*Solanum tuberosum* L. cv Bintje) tuber mitochondria, the peroxisomal membrane from potato tuber peroxisomes (Struglics et al., 1993), PM from spinach (*Spinacia oleracea* L.) leaves (Larsson et al., 1987), ER from wheat (*Triticum aestivum* L.) roots (Widell and Sommarin, 1991), and tonoplasts from vacuoles isolated from red beetroots (*Beta vulgaris* L.) (Bennett et al., 1983).

To determine the substrate stereospecificity of the NADH-ferricyanide reductases for the α- or the β-hydrogen of NADH in the different membrane fractions, [4-^{3}H]NAD$^+$ (Amersham, England) was reduced with alcohol dehydrogenase (EC 1.1.1.1) or Glc-6-P dehydrogenase (EC 1.1.1.49) to produce [β-^{3}H]- and [α-^{3}H]NADH, respectively. When the reduction was completed, as judged by an increase in A_{340}, the sample was boiled, the enzyme removed by ultrafiltration through a 10-kD cutoff filter (YM 10, Amicon, Beverly, MA), and the filtrate stored at −20°C until further use.

A membrane aliquot, equivalent to a total NADH-ferricyanide reductase activity of 10 nmol NADH oxidized min^{-1} (2–100 μg of protein), was incubated for 10 min in a total volume of 1.0 mL containing 0.18 μM [β-^{3}H]- or [α-^{3}H]NADH and 100 μM ferricyanide in 50 mM Mops-KOH, pH 7.2. NAD$^+$ and NADH were separated from H_2O by adding 200 μL of the assay mixture to a 9-mL Sephadex G-10 (Pharmacia, Uppsala, Sweden) size-exclusion chromatography column (separation range 100–1000 D), 1-mL fractions were collected, and the radioactivity was detected by liquid scintillation.

SDS-PAGE was performed according to the method of Laemmli (1970) using a gradient gel (total monomer 12–18%, cross-linker 2.7%). Samples were solubilized in 2% (w/v) SDS, 5% (v/v) glycerol, 4% (v/v) 2-mercaptoethanol, and 62.5 mM Tris-HCl, pH 6.8, at 95°C for 3 min, except the PM, which was solubilized at room temperature for 30 min. Protein bands were stained with Coomassie brilliant blue. For western blotting a polyacrylamide minigel (total monomer 12%, cross-linker 2.7%) was used, which was blotted and immunoreacted as described by Struglics et al. (1993) except that the primary and secondary antibodies were diluted 7500- and 5000-fold, respectively. The blots were developed by the enhanced chemiluminescence method according to the manufacturer's instructions (Amersham).

Protein was determined by the method of Lowry et al. (1951) using BSA as the standard.

RESULTS AND DISCUSSION

Stereospecificity of NADH-Ferricyanide Reductases

NADH contains two nonequivalent hydrogens, one on either side of the nicotinamide ring. NAD(P)H dehydrogen-

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Abbreviations: PM, plasma membrane; SMP, submitochondrial particles.

ases are specific for removal of one or the other hydrogen (α and β specific, respectively; You et al., 1978). The stereospecificity of NAD(H)-binding dehydrogenases is determined by whether the conformation of the active site allows NAD(H) binding with anti or syn orientation of its nicotinamide ring (You et al., 1978, and refs. therein). It is possible to determine which of the two hydrogens the enzyme removes, i.e. the stereospecificity of the enzyme can be determined. When an α-specific enzyme is incubated with [4α-^{3}H]NADH and ferricyanide, the ^{3}H will be released into water, whereas if [4β-^{3}H]NADH is used, the ^{3}H remains in the NAD$^+$ after the reaction. A gel filtration column will delay the smaller water molecules much more than NAD$^+$ (and unreacted NADH) (Fig. 1, cf. G with A–F). We used the above method to determine the stereospecificity of the NADH-ferricyanide reductases in the six membranes isolated (Fig. 1).

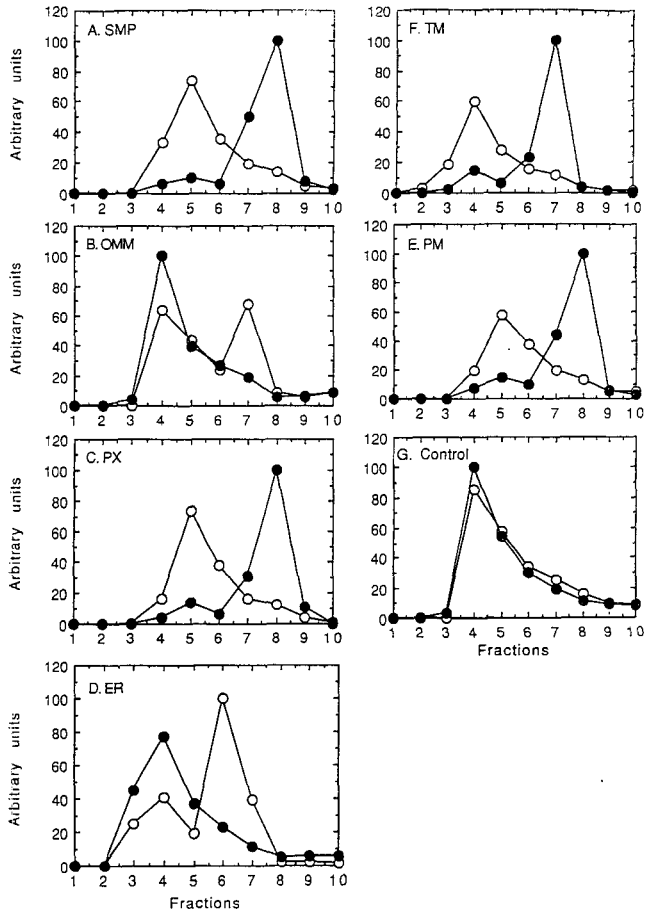

Figure 1. Stereospecificity of NADH-ferricyanide reductases in plant membranes. A, Inside-out SMP from potato tubers; B, outer mitochondrial membrane (OMM) from potato tubers; C, peroxisomal membrane (PX) from potato tubers; D, ER from wheat roots; E, PM from spinach leaves; F, tonoplast membrane (TM) from red beetroots; and G, control (no membranes in the incubation). The membranes (in each case a total activity equivalent to 10 nmol NADH oxidized min^{-1}) were incubated with 0.18 μM [α-^{3}H]NADH (open symbols) or [β-^{3}H]NADH (closed symbols) for 10 min. After boiling, 200 μL of assay mixture were passed through a 9-mL Sephadex G-10 gel filtration column, and 1-mL fractions were collected and counted. NAD(H) elutes in fractions 4 and 5 (see G) and water in fractions 6 to 8. The results shown are from a single experiment for each membrane, but the same results have been obtained on at least two independent preparations of each membrane.

The activity catalyzed by inside-out SMP is β specific (Fig. 1A). This activity is catalyzed by two enzymes, complex I and the rotenone-insensitive NAD(P)H dehydrogenase, both linked to the electron transport chain (Møller and Lin, 1986; Møller et al., 1993). The rotenone-insensitive enzyme is β specific in beetroot mitochondria (Rasmusson and Møller, 1991), but in assays with potato tuber SMP, complex I activity (NADH-ferricyanide) is totally dominant (Rasmusson et al., 1993). Thus, our assays measured β-specific complex I activity. This is in agreement with data for beef heart SMP (Lee et al., 1965), which lack the internal rotenone-insensitive dehydrogenase.

The NADH-ferricyanide reductase in the outer membrane of potato tuber mitochondria is α specific (Fig. 1B), thereby confirming the observation of Douce et al. (1973) for mung beans. The activity in the peroxisomal membrane is β specific (Fig. 1C) as reported by Struglics et al. (1993).

The NADH-Cyt b_5 reductase in animal ER is α specific (Drysdale et al., 1961), which we confirm here for plants (Fig. 1D).

The activity in the PM and the tonoplast is β specific (Fig. 1, E and F), which has not been reported previously. Since the ER activity is α specific, this shows clearly that the redox activity in the PM (and in the tonoplast) is not due to contamination by ER, a point that has hitherto been difficult to prove unequivocally (Askerlund, 1990; Widell and Larsson, 1990). Thus, since the specific activity of NADH-ferricyanide and NADH-Cyt *c* reductase (Widell and Sommarin, 1991; Møller et al., 1994) in the ER is very high, the stereospecificity provides a simple and sensitive test for ER contamination in preparations of PM or tonoplasts. In assays in which ER is added to purified PM, it has been possible to detect less than 1% ER contamination on a protein basis (K.M. Fredlund, S. Widell, and I.M. Møller, unpublished observation).

Comparison of Polypeptide Pattern and Immunoreactivity

When the membrane fractions were analyzed by SDS-PAGE, very different polypeptide patterns were observed (Fig. 2). In particular, the tonoplast was quite different from the ER and the PM. These membranes were isolated from different species, but that should not affect their polypeptide pattern much, e.g. PM from different species and organs show essentially the same pattern when analyzed by SDS-PAGE (Larsson et al., 1990).

One or several polypeptides of 45 to 55 kD cross-reacted in all membranes (Fig. 3) when a western blot of a 12% gel loaded with PM, tonoplast, ER, peroxisomes, SMP, and outer mitochondrial membranes was probed with antibodies raised against a potato tuber microsomal NADH-ferricyanide reductase (Galle et al., 1984; Askerlund et al., 1991). Askerlund et al. (1991) partially purified an NADH-ferricyanide reductase from spinach leaf PMs, and we have recently further purified that 45-kD enzyme to homogeneity. It cross-reacts

with the anti-NADH-ferricyanide reductase antibodies on western blots and exhibits β-specific NADH oxidation (A. Bérczi, K.M. Fredlund, and I.M. Møller, unpublished data).

CONCLUSIONS AND PERSPECTIVES

The cross-reactivity of membrane proteins with antibodies raised against a microsomal NADH-ferricyanide reductase suggests that an NADH-ferricyanide reductase(s) of similar size and comparable antigenicity might exist in all of the membranes studied. Stereospecificity is considered to be one of the most conserved characteristics of a given dehydrogenase (You et al., 1978), i.e. it is independent of the source of the enzyme. Therefore, it has been speculated that dehydrogenases with the same stereospecificity share common structural features around the active site (Branden and Tooze, 1991). Yet, the reductases fall into two distinct groups based on substrate stereospecificity. This raises two questions about the origin and function of the enzymes.

First, how do the the proteins recognize their respective membrane after synthesis in the cytoplasm? In this connection it is interesting that the two α-specific enzymes are located in the ER and the outer mitochondrial membrane since these two membranes have been reported to be physically connected (Gasnier et al., 1993). The Golgi in rat liver cells contains an NADH-Cyt b_5 reductase immunologically similar to that of the ER and the outer mitochondrial membrane (Meldolesi et al., 1980). A 45-kD polypeptide cross-reacts when western blots of rat liver Golgi are probed with antibodies raised against the potato tuber microsomal NADH-ferricyanide reductase. The rat liver Golgi shows only α-specific NADH-ferricyanide reductase activity (Møller et al., 1994).

Second, what is the function of these enzymes in the membranes? The function of the NADH-Cyt b_5 reductase in the ER is in fatty acid desaturation and Cyt P450 reactions. A possible function of the NADH-ferricyanide reductase in the PM is iron reduction (Møller and Crane, 1990), whereas the role of the enzyme in the outer mitochondrial membrane, the peroxisomal membrane, and the tonoplast is, as yet, unknown.

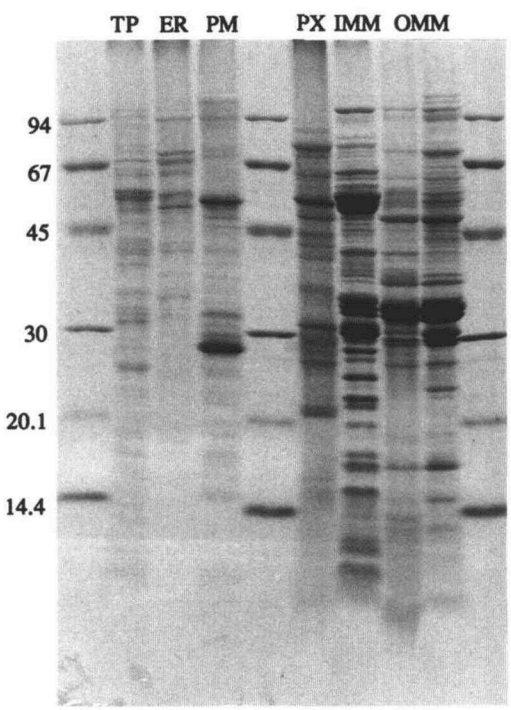

Figure 2. SDS-PAGE of plant membranes visualized by Coomassie staining. Lanes 1, 5, and 10, Standards; lane 2, tonoplast (TP, 30 μg of protein); lane 3, ER (30 μg); lane 4, PM (30 μg); lane 5, standards; lane 6, peroxisomal membrane (PX, 50 μg); lane 7, inner mitochondrial membrane (IMM, 50 μg); lanes 8 and 9, outer mitochondrial membrane (OMM, 25 and 50 μg, respectively). The sizes (in kD) of the molecular mass markers in lane 5 are given on the left.

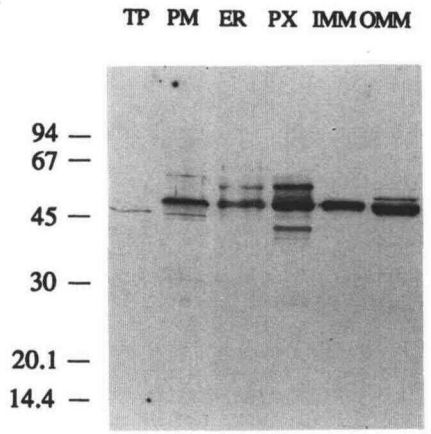

Figure 3. Western blot of an SDS-PAGE gel using antibodies raised against a microsomal NADH-ferricyanide reductase. Lane 1, Tonoplast (TP, 10 μg of protein); lane 2, PM (20 μg); lane 3, ER (10 μg); lane 4, peroxisomal membrane (PX, 5 μg); lane 5, inner mitochondrial membane (IMM, 50 μg); lane 6, outer mitochondrial membrane (OMM, 5 μg). The sizes (in kD) of the molecular mass markers are given on the left.

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