A 43-kDa NAD(P)H dehydrogenase was purified from red beetroot mitochondria. An antibody against this dehydrogenase was used in conjunction with the membrane-impermeable protein cross-linker 3,3'-dithiobis(sulfo succinimidylpropionate) to localize the dehydrogenase on the matrix side of the inner membrane. Immunoblotting showed that the dehydrogenase was found in mitochondria isolated from several plant species but not from rat livers. Antibodies against the purified dehydrogenase partially inhibited rotenone-insensitive internal NADH oxidation by inside-out sub-mitochondrial particles. The level of rotenone-insensitive respiration with NADH-linked substrates correlated with the amount of 43-kDa NAD(P)H dehydrogenase present in mitochondria isolated from different soybean tissues. Based on these results, we conclude that the 43-kDa NAD(P)H dehydrogenase is responsible for rotenone-insensitive internal NADH oxidation in plant mitochondria.

It has been known for some time that plant mitochondria, unlike their mammalian counterparts, can oxidize NAD-linked substrates in the presence of the complex I inhibitor rotenone (1). However, rotenone lowered the ADP/O ratio for NADH-linked substrates by one third (1), suggesting the presence of an internal NADH dehydrogenase that did not translocate protons and which was insensitive to rotenone. This was supported by the finding (2) that inside-out sub-mitochondrial particles (SMP) from Jerusalem artichoke exhibited two different $K_m$ values for NADH, depending on whether rotenone was present or not. These results suggested that the rotenone-insensitive bypass was mediated by a distinct NADH dehydrogenase, rather than by direct evidence, that a 26-kDa NAD(P)H dehydrogenase is responsible for oxidation of cytosolic NAD(P)H. In contrast, Rasmusson et al. (16) concluded by a process of elimination, rather than by direct evidence, that a 26-kDa NAD(P)H dehydrogenase from red beetroot mitochondria was responsible for rotenone-insensitive internal NADH oxidation.

In this paper, we report on the purification of a 43-kDa NAD(P)H dehydrogenase from red beetroot mitochondria and provide strong evidence that this enzyme is responsible for rotenone-insensitive internal NADH oxidation.

**EXPERIMENTAL PROCEDURES**

*Materials—Red beetroot (Beta vulgaris L.) and Potatoes (Solanum tuberosum L.) were purchased from local markets. Soybean (Glycine max L. cv. Stevens) plants were glass house-grown and cotyledons and roots harvested at 7 days, nitrogen-fixing nodules were harvested at 8 weeks.*

*Preparation and Fractionation of Mitochondria—Beetroot mitochondria were prepared by the method of Menz et al. (17), rat liver mitochondria by the method of James et al. (18), potato tuber mitochondria by the method of Liden and Akerland (19), and soybean mitochondria by the method of Day et al. (20). The mitochondrial "soluble fraction" was prepared by resuspending the mitochondrial pellet in 20 mM Tris/HCl (pH 8.0) containing 1 mM phenylmethylsulfonyl fluoride and 5 mM EDTA, protease inhibitor and sonicating for 5 x 5-s bursts using an MSE (Sussex, United Kingdom) ultrasonic disintegrator at maximum power. The sonicate was centrifuged at 300,000 x g for 45 min, and the resulting supernatant represented the soluble fraction. Sub-mitochondrial particles were prepared as detailed previously (17).**

*Chromatographic Techniques—All chromatographic procedures were carried out using a Pharmacia (Sydney, Australia) fast protein liquid chromatography system. Anion exchange chromatography was performed on a Pharmacia RESOURCE Q (1 ml) column, while blue affinity chromatography was performed on a Pharmacia Hi-Trap blue column.*
Plant Mitochondrial 43-kDa NAD(P)H Dehydrogenase

RESULTS

Purification of a 43-kDa NAD(P)H Dehydrogenase—We (26) and others (14, 16) have shown that rotenone-insensitive NADH dehydrogenase activity is released from mitochondrial membranes by sonication and can be recovered in the soluble fraction (see “Experimental Procedures”). Use of this fraction has the advantage of avoiding complications due to complex I, which excludes DTSSP from proteins located on the inside of the inner membrane, and sonicated mitochondria, with a disrupted and exposed inner membrane that could not exclude DTSSP, were incubated with the cross-linker. Following cross-linking, the mitochondria were subjected to SDS-PAGE and Western blot analysis. In the absence of β-mercaptoethanol, the 43-kDa protein was only detected in the intact mitochondria and not in the sonicated mitochondria (Fig. 4). However, when SDS-PAGE was performed in the presence of β-mercaptoethanol, which cleaves the cross-linker, Western analysis detected the 43-kDa protein in both the intact and sonicated mitochondria (Fig. 4). These results demonstrate that the 43-kDa protein was protected from the cross-linker in intact mitochondria and therefore must be located within the mitochondrial inner membrane (DTSSP can pass through the outer membrane; Ref. 13). These results also suggest that DTSSP cross-links the 43-kDa protein to other proteins forming a supermolecular weight complex which, because of its large size, is not transferred to the blotting membrane.

Correlation between 43-kDa Protein and Matrix Rotenone-Insensitive NADH Oxidation—Mitochondria from several different plant species as well as from rat livers were subjected to Western analysis with the antibodies raised against the 43-kDa red beet protein. The antibodies were found to cross-react with a protein of approximately 43 kDa in mitochondria from potato tuber and soybean cotyledons but not in rat liver mitochondria (Fig. 5). The presence of the 43-kDa protein in a variety of plant species, but not in completely rotenone-sensitive mammalian mitochondria, is consistent with the 43-kDa protein being one of the unique NADH dehydrogenases found in plant mitochondria.
It has been shown that mitochondria isolated from different tissues of soybean plants exhibit different levels of the internal rotenone-insensitive bypass activity (17). Therefore, mitochondria were isolated from soybean cotyledons, roots, and nodules, and the level of rotenone-insensitive internal NADH oxidation measured. The level of this activity was found to vary, with that in cotyledons being greater than in roots which in turn were greater than in nodules (Fig. 5). These mitochondria were also subjected to Western analysis with the antibody against the 43-kDa NAD(P)H dehydrogenase. A good correlation between the level of this protein and the amount rotenone-insensitive internal NADH oxidation was found (Fig. 5). This correlation provides further evidence that the 43-kDa protein mediates rotenone-insensitive internal NADH oxidation.

Antibodies against the 43-kDa protein were tested for their ability to inhibit rotenone-insensitive internal NADH and NADPH oxidation, which are likely to be mediated by different proteins (27). Rotenone-insensitive internal NADH oxidation was measured in inside-out beetroot SMP that were incubated with either the 43-kDa NAD(P)H dehydrogenase antiserum or preimmune serum, for 2 h on ice. The 43-kDa protein antiserum inhibited rotenone-insensitive internal NADH oxidation by approximately 20% (not shown). In contrast, there was no significant inhibition of NADPH oxidation by SMP with the 43-kDa NAD(P)H dehydrogenase antiserum. This result supports the hypothesis that there are separate enzymes for NADH and NADPH oxidation (27) and that NADH oxidation is mediated by the 43-kDa NAD(P)H dehydrogenase.

Characterization of the 43-kDa NAD(P)H Dehydrogenase—
The relative effects of different substrates and acceptors on the dehydrogenase activity are shown in Table I. The enzyme was capable of oxidizing both NADH and NADPH at an equal rate with Q$_0$ as acceptor. In this regard the purified enzyme appears similar to both the 42-kDa dehydrogenase purified by Luethy et al. (14) and the 26-kDa protein purified by Rasmusson et al. (16). The rate of oxidation by the enzyme with FeCN as acceptor was measured using 10 mM malate, 10 mM glutamate, 0.5 mM ADP, and 25 mM rotenone as detailed under “Experimental Procedures.” The same mitochondria were subjected to Western analysis using the 43-kDa NAD(P)H dehydrogenase antiserau.

The effect of different substrates, acceptors, and various compounds on 43-kDa NAD(P)H dehydrogenase activity

Reductase activities were assayed as detailed under “Experimental Procedures.” Various compounds (additions) were tested for their effect on NADH:Q$_0$ reductase activity. All rates are means (n = 3) and are expressed relative to the NADH:Q$_0$ reductase rate. pCMB, p-chloromercuribenzoic acid.

| Substrate:acceptor or addition | Relative activity |
|-------------------------------|------------------|
| NADH:Q$_0$                     | 100              |
| NADPH:Q$_0$                    | 100              |
| Deamino-NADH:Q$_0$             | 45               |
| NADH:FeCN                     | 60               |
| 5 mM EGTA                     | 95               |
| 1 mM CaCl$_2$                  | 99               |
| 200 mM NAD                    | 96               |
| 200 mM NADP                   | 95               |
| 200 mM ADP                    | 86               |
| 200 mM ATP                    | 91               |
| 150 mM pCMB                   | 74               |
| 150 mM mersalyl                | 68               |
| 150 mM dicumarol              | 12               |
| 150 mM platanetin             | 99               |
| 150 mM Bavone                  | 100              |
We have purified a 43-kDa NAD(P)H dehydrogenase from red beet mitochondria. The enzyme was located on the matrix side of the inner membrane. It was present in mitochondria from several plant species, but not rat liver, and there was good correlation between the abundance of the 43-kDa NAD(P)H dehydrogenase and rotenone-insensitive oxidation by soybean mitochondria. Taken together, these results provide the strongest evidence to date that the 43-kDa protein is the internal rotenone-insensitive bypass of complex I found in most plant mitochondria. The antibody inhibition of rotenone-insensitive internal NADH oxidation, but not NADPH oxidation, further supports this idea and suggests that a separate enzyme is responsible for rotenone-insensitive internal NADPH oxidation.

Comparison of the kinetic and inhibitor data of the purified dehydrogenase with those of rotenone-insensitive NADH oxidation in inside-out SMP from various plant tissues reveals several similarities. These include: a high $K_m$ for NADH (of the order of 100 $\mu$M; Refs. 2 and 3); a pH optimum of approximately 6.5 (3); inhibition by dicumarol and mersalyl (3, 26). A notable difference between the purified enzyme and that in SMP is the ability of the former to facilitate rapid rates of NADPH oxidation; this is most likely due to the removal of the enzyme from its hydrophobic environment (26).

The 43-kDa NAD(P)H dehydrogenase isolated here is probably the same as the 42-kDa enzyme previously isolated by Luethy et al. (14). Both activities have similar molecular masses and activities with different substrates, activators, and inhibitors. The most obvious difference between the two preparations is that the protein of Luethy et al. (14) appeared more sensitive to calcium and ADP. These differences could easily be explained by different ionic conditions in the assay. The identity of the 26-kDa protein isolated by Rasmussen et al. (16) remains unclear. However, it is interesting to note that the latter authors found a native molecular mass for their enzyme similar to that reported here, and their enzyme also had the ability to oxidize NADPH and reduce FeCN. It is possible, therefore, that the enzyme isolated by Rasmussen et al. (16) was identical to that reported here, but the protein was degraded to a lower apparent molecular mass during isolation or storage.

The enzyme purified here differs in molecular mass from the rotenone-insensitive internal NADH dehydrogenase of S. cerevisiae (7). However, similarities may become apparent when sequence data are available for the 43-kDa enzyme of plants.

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