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THE MECHANISM OF OXIDATION OF REDUCED NICOTINAMIDE DINUCLEOTIDE PHOSPHATE BY SUBMITOCHONDRIAL PARTICLES FROM BEEF HEART

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Summary

1. Oxidation of NADPH by various acceptors catalyzed by submitochondrial particles and a partially purified NADH dehydrogenase from beef heart was investigated. Submitochondrial particles devoid of nicotinamide nucleotide transhydrogenase activity catalyze an oxidation of NADPH by oxygen. The partially purified NADH dehydrogenase prepared from these particles catalyzes an oxidation of NADPH by acetylpyridine-NAD^+. In both cases the rates of oxidation are about two orders of magnitude lower than those obtained with NADH as electron donor.

2. The kinetic characteristics of the NADPH oxidase reaction and reduction of acetylpyridine-NAD^+ by NADPH are similar with regard to pH dependences and affinities for NADPH, indicating that both reactions involve the same binding site for NADPH. The binding of NADPH to this site appears to be rate limiting for the overall reactions.

3. At redox equilibrium NADPH and NADH reduce FMN and iron-sulphur center 1 of NADH dehydrogenase to the same extents. The rate of reduction of FMN by NADPH is at least two orders of magnitude lower than with NADH.

4. It is concluded that NADPH is a substrate of NADH dehydrogenase and that the nicotinamide nucleotide is oxidized by submitochondrial particles via the NADH—binding site of the enzyme.

Introduction

Oxidation of NADPH catalyzed by submitochondrial particles from beef heart is known to occur in the presence of various electron or hydrogen accep-

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tors other than oxygen. Danielsson and Ernster [1] demonstrated that the most active pathway in these particles is the nicotinamide nucleotide transhydrogenase reaction with NAD^+ as acceptor; NAD^+ can be replaced by analogues of this nucleotide [2]. Strong indications for a separate pathway was provided at an early stage by Rossi et al. [3] who showed that NADPH is a substrate of isolated NADH dehydrogenase although the rate of its oxidation in the presence of various acceptors is three to four orders of magnitude lower than that with NADH. However, similar results could not be obtained with NADH dehydrogenase in the intact membrane presumably due to the relatively low activity of the enzyme in situ as well as to leakage of electrons through the respiratory chain.

Subsequently, Ernster and coworkers [4] found that in the presence of KCN submitochondrial particles catalyze a slow and rotenone-insensitive oxidation of NADPH by either dichlorophenolindophenol or cytochrome *c*. At this stage no measurable activity could be demonstrated with either oxygen or ferricyanide [4]. Due to several kinetic and other similarities with the transhydrogenase reaction this NADPH dehydrogenase activity was proposed [4] to constitute a partial reaction of transhydrogenase. This conclusion was later supported by the finding [5] that NADPH dehydrogenase is sensitive to palmityl-CoA, an inhibitor of transhydrogenase [6].

Recently, Hatefi and coworkers [7—14] demonstrated that in the presence of KCN NADPH reduces most of the components of the respiratory chain in the absence of externally added acceptors. The redox pattern resembles that obtained with NADH except that FMN and iron-sulphur center 1 of NADH dehydrogenase are not reduced and that the rate of reduction of other components is markedly slower. This is consistent with the observed slow oxidation of NADPH by oxygen which is qualitatively similar to that of NADH with respect to sensitivity to rotenone and other respiratory chain inhibitors. Hatefi [7] and Hatefi and Hanstein [8] proposed that reducing equivalents from NADPH enter the respiratory chain at iron-sulphur center 2 thus bypassing iron-sulphur center 1, and that iron-sulphur center 2 may be involved in the transhydrogenase reaction. These conclusions were questioned by Rydström et al. [5,15] who provided evidence that in the absence of added NAD^+ the bulk of the NADPH oxidase activity is insensitive to palmityl-CoA and trypsin and therefore is unrelated to transhydrogenase. It was argued that all of the observed differences in redox patterns seen with NADPH and NADH can be accounted for by a slow oxidation of NADPH through the NADH-binding site of NADH dehydrogenase, in accordance with the results of Rossi et al. [3].

In more recent studies Ragan [16] and Hatefi and coworkers [17,18] concluded that, indeed, the differences in reduction of the components of NADH dehydrogenase (and Complex I) with NADH and NADPH are essentially quantitative and depend on the relative rates of entry and removal of electrons from these components. However, the evidence was indirect and the problem of the site of interaction between NADPH and NADH dehydrogenase, as well as the role of contaminating transhydrogenase, remained unsolved.

The present data show that soluble NADH dehydrogenase devoid of contaminating nicotinamide nucleotide transhydrogenase still catalyzes transfer of hydrogen from NADPH to acetylpyridine-NAD^+. The kinetic characteristics

of this reaction closely resemble those of the oxidation of NADPH by oxygen catalyzed by submitochondrial particles. Also, the results show that NADPH indeed is capable of reducing FMN and iron-sulphur center 1 of NADH dehydrogenase completely. In agreement with previous suggestions [5] it is thus concluded that in beef heart submitochondrial particles oxidation of NADPH and NADH by oxygen occurs through NADH dehydrogenase via the same binding site. A preliminary account of these results has been published elsewhere [19].

Materials and Methods

EDTA submitochondrial particles were prepared as described by Lee and Ernster [20]. The particles were washed twice with 0.25 M sucrose, suspended in the same medium and stored at −20°C at a protein concentration of 20 mg/ml or higher. Particle protein was determined by the biuret method. Partially purified NADH dehydrogenase devoid of transhydrogenase activity was prepared essentially as described by Ringler et al. [21] except that trypsin-treated submitochondrial particles were used. The NADH dehydrogenase recovered in the supernatant after phospholipase A treatment was devoid of cytochromes and was used as the source of enzyme. The properties of the trypsin-treated preparation, e.g. the NADH-ferricyanide reductase activity, were not significantly different from those [21] of the preparation obtained with control submitochondrial particles. Inactivation of nicotinamide nucleotide transhydrogenase was carried out as described by Juntti et al. [22] except that the concentration of trypsin was 100 μg/mg particle protein. Under these conditions the remaining transhydrogenase activity was less than 1% or 1 nmol/min per mg after 3 min of incubation; this low residual activity remained constant for prolonged times of incubation and was not inhibited by 10 μM palmityl-CoA, an inhibitor of transhydrogenase [6]. Also, palmityl-CoA (or lactate dehydrogenase plus pyruvate)-insensitive NADPH oxidase (cf. Results) was not inhibited by this trypsin treatment.

Reduction of NAD^+ and acetylpyridine-NAD^+ by NADPH was assayed spectrophotometrically with an Aminco-Chance DW-2 instrument essentially as described previously [23]. The same procedure was employed in the assay of reduction of acetylpyridine-NAD^+ by NADH except that the concentrations of NADH and acetylpyridine-NAD^+ were 200 and 200 μM, respectively. NADH oxidase and NADPH oxidase activities were estimated polarographically with an oxygen electrode. All enzyme activities were measured at 30°C.

Reduction of flavin (and iron-sulphur protein) was measured at 475—510 nm [7].

Reduction of iron-sulphur center 1 of NADH dehydrogenase in trypsin-treated submitochondrial particles was monitored at liquid nitrogen temperature with a Varian V-4502 ESR spectrometer equipped with 100 kHz field modulation. A modified microwave bridge enabled work at low microwave power (3 μW) to minimize microwave saturation. The nitrogen-saturated standard medium contained 0.25 M sucrose and 0.1 M Tris/acetate (pH 7.5). Reaction mixtures containing 50 mg of trypsin-treated submitochondrial particle protein per ml and 10 μg rotenone in a final volume of 0.2 ml were preincu-

bated for 1 min at 30°C. The mixture was preincubated for another minute in the presence of 5 mM succinate to achieve complete anaerobiosis. Subsequently, the reaction was initiated by the addition of either 1 mM NADH or 1 mM NADPH; in the latter case 2 mM pyruvate and 5 μg lactate dehydrogenase were added simultaneously in order to keep possible contaminations of NADH oxidized. After 2 min the mixtures were cooled in ice, transferred to the microcuvettes and kept in liquid nitrogen.

Lactate dehydrogenase (from rabbit muscle) and NADPH were obtained from Boehringer (Mannheim, Germany). All other biochemicals were purchased from Sigma (St. Louis, Miss., U.S.A.).

Results

As shown in Fig. 1A, beef heart submitochondrial particles catalyze an NADPH-dependent oxygen consumption the rate of which is increased substantially by the addition of alcohol dehydrogenase plus ethanol. The latter indicates that NADPH contains contaminating NAD^+ which is reduced by alcohol dehydrogenase and oxidized through the respiratory chain via NADH dehydrogenase. This effect of alcohol dehydrogenase was seen to various extents with all NADPH preparations tested that are commercially available; alcohol dehydrogenase plus ethanol alone did not cause any oxygen consumption, indicating that the submitochondrial particles per se contain negligible amounts of NAD(H) accessible to the surrounding medium (cf. ref. 24). It should be pointed out that the contaminating NAD(H) is not detectable with thin-layer chromatography (not shown). Obviously, the generation of NADH can also be accomplished by reduction of NAD^+ by NADPH catalyzed by nicotinamide nucleotide transhydrogenase present in these particles. Any contribution of the latter enzyme to the overall rate of oxygen consumption should be inhibited by either an additional system that keeps NAD^+ oxidized or agents that inhibit transhydrogenase. Lactate dehydrogenase plus pyruvate indeed diminishes the

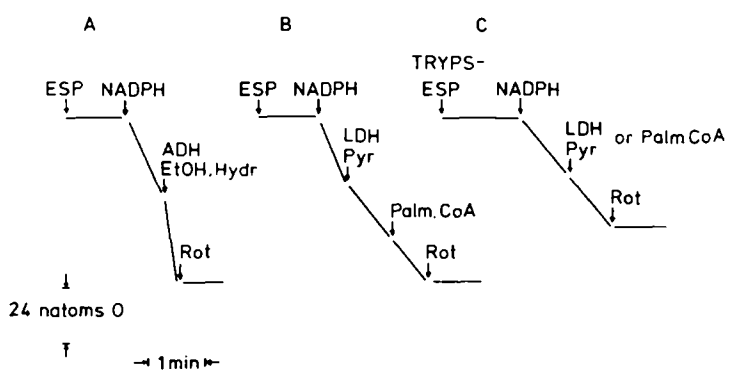

Fig. 1. NADPH oxidase activity of submitochondrial particles. Medium contained 0.25 M sucrose and 0.1 M Tris/acetate (pH 7.5). The amount of control (ESP) and trypsin-treated submitochondrial particles (TRYPS ESP) was 3 mg. The reaction initiated by 2 mM NADPH and terminated by 2 μg rotenone (Rot). Other additions were: A, 0.1 mg alcohol dehydrogenase (ADH), 0.1 M ethanol (EtOH) and 80 mM hydrazine (Hydr.) (neutralized with NaOH); B, 2 μg lactate dehydrogenase (LDH), 2 mM pyruvate (Pyr) and 10 μM palmityl-CoA (Palm CoA); C, same as in B. Final volume was 1 ml.

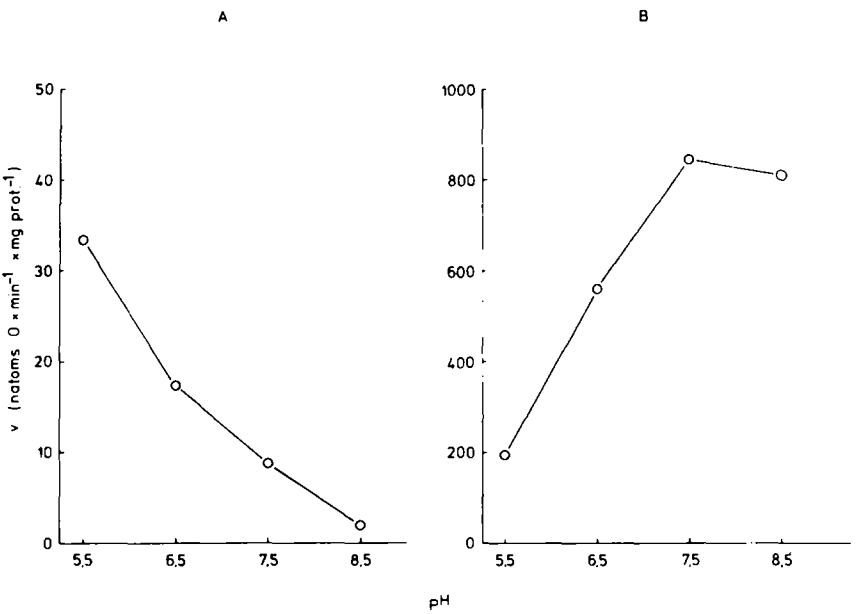

Fig. 2. NADPH oxidase (A) and NADH oxidase (B) activities of trypsin-treated submitochondrial particles as a function of pH. Medium contained 0.25 M sucrose, 0.1 M Tris/malate (pH 5.5—8.5). With NADPH as substrate the amount of protein was 2 mg and with NADH as substrate 50 μg. Reactions were initiated by the addition of 2 mM NADPH and NADH, respectively. Final volume was 1 ml.

rate of oxygen consumption (Fig. 1B); the remaining activity is not sensitive to palmityl-CoA. However, when the order of the two additions is reversed, palmityl-CoA diminishes the oxygen consumption whereas lactate dehydrogenase plus pyruvate has no effect (not shown). Inhibition of transhydrogenase by trypsin treatment (Fig. 1C) also diminishes the respiration to a rate which is close to that found in the presence of lactate dehydrogenase or palmityl-CoA; in this case lactate dehydrogenase or palmityl-CoA is without effect (cf. ref. 11). These findings show that transhydrogenase contributes substantially to the rate of respiration obtained with NADPH contaminated with only trace amounts of NAD(H). It may be calculated that a contamination of 0.05% NAD(H) approximately doubles the apparent NADPH oxidase activity at non-saturating concentrations of NADPH. In order to avoid the interfering NADH formation, in the studies of the mechanism of NADPH oxidase in submitochondrial particles, transhydrogenase was routinely inhibited by trypsin.

Fig. 2 shows the pH dependence of NADPH oxidase (Fig. 2A) and NADH oxidase (Fig. 2B), respectively, in trypsin-treated submitochondrial particles. Oxidation of NADH by oxygen is increasing with pH with a maximum at and above pH 7.5. In contrast, oxidation of NADPH by oxygen is decreasing with pH with no apparent pH optimum in the pH range investigated. Michaelis constants for the nicotinamide nucleotides and V values for NADH oxidase and NADPH oxidase at pH 7.5 were derived from the double reciprocal plots in Fig. 3. V for NADPH oxidase is 6.3 nmol/min per mg (Fig. 3A), whereas that for NADH oxidase is 1.3 μmol/min per mg (Fig. 3B). The corresponding K_m values are 570 and 8.3 μM, respectively.

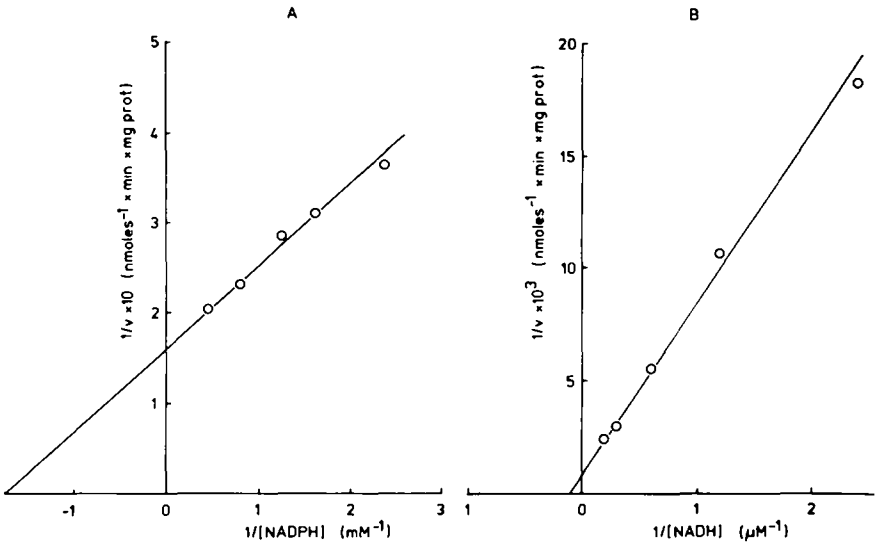

Fig. 3. Double reciprocal plots of NADPH oxidase (A) and NADH oxidase (B) activities of trypsin-treated submitochondrial particles at varying concentrations of nicotinamide nucleotides. The reactions were carried out at pH 7.5. Other conditions were as described in Fig. 2.

Partially purified NADH dehydrogenase catalyzes reduction of acetylpyridine-NAD$^+$ by both NADPH (Fig. 4A) and NADH (Fig. 4B), in agreement with the results of Rossi et al. [3] and Hatefi and Galante [18]. The soluble NADH dehydrogenase preparation used here was obtained from trypsin-treated sub-

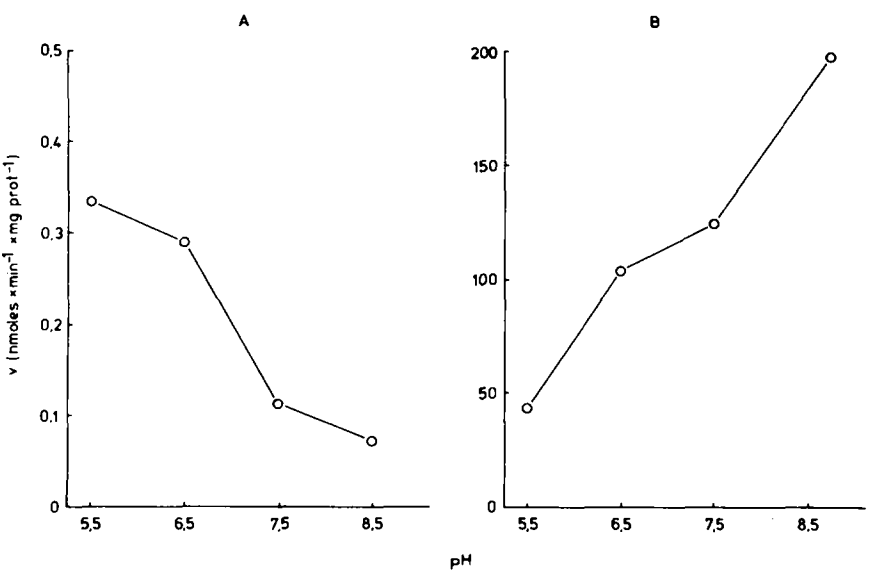

Fig. 4. Reduction of acetylpyridine-NAD$^+$ by NADPH (A) and NADH (B) as a function of pH, catalyzed by partially purified NADH dehydrogenase. Conditions were as described in Fig. 2 except that the amount of protein was 0.05 mg (A) and 0.5 mg (B), respectively. The concentration of acetylpyridine-NAD$^+$ was 200 μM.

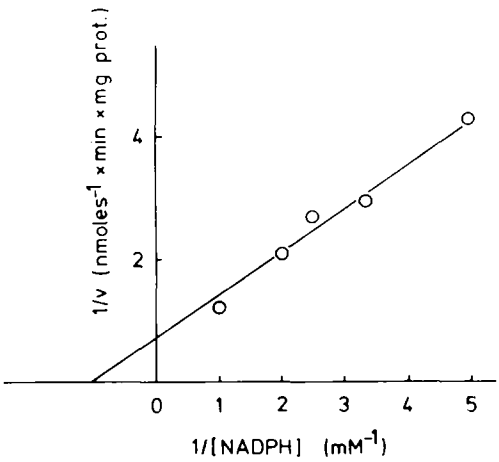

Fig. 5. Double reciprocal plots of reduction of acetylpyridine-NAD$^+$ by NADPH at varying concentrations of NADPH, catalyzed by partially purified NADH dehydrogenase. The reaction was carried out at pH 7.5. Other conditions were as described in Fig. 4.

mitochondrial particles devoid of nicotinamide nucleotide transhydrogenase. With NADPH as hydrogen donor (Fig. 4A) the activity is increasing with decreasing pH whereas with NADH as hydrogen donor (Fig. 4B) the activity is increasing with increasing pH. At pH 7.5, V for the former reaction was estimated to 1.3 nmol/min per mg protein (Fig. 5) and K_m for NADPH was calculated to 950 μM. The corresponding values for reduction of acetylpyridine-NAD$^+$ by NADH were not estimated (cf. ref. 3). In the experiments of Fig. 4 the concentration of acetylpyridine-NAD$^+$ was saturating.

A comparison of the data in Figs. 2—5 reveals that in those reactions where NADPH serves as hydrogen donor the pH dependences and Michaelis constants for NADPH are similar. The K_m values for NADPH are about 30—50 times higher than that derived earlier for nicotinamide nucleotide transhydrogenase [25]. Furthermore, the observed reduction of acetylpyridine-NAD$^+$ by either NADPH or NADH is insensitive to 10 μM palmityl-CoA, an inhibitor of nicotinamide nucleotide transhydrogenase (not shown). Thus, it appears highly unlikely that the observed NADPH-linked activities are related to nicotinamide nucleotide transhydrogenase. The alternative explanation that the NADPH-linked activities are catalyzed by NADH dehydrogenase would involve the assumption that NADH dehydrogenase accepts NADPH as a substrate. In the experiment of Fig. 6 reduction of the partially purified NADH dehydrogenase was measured spectrophotometrically at pH 7.5 employing either NADH or NADPH as reducing agent. NADH at saturating concentrations rapidly reduces the dehydrogenase (Fig. 6A). Reduction is also achieved with NADPH and the subsequent addition of NADH has virtually no effect (Fig. 6B). The rate of reduction with NADH is more than two orders of magnitude higher than that with NADPH. A low rate of reduction similar to that found with NADPH was achieved with very low concentrations of NADH (<0.1 μM) and an additional NADH-regenerating system (Fig. 6C).

Reduction of NADH dehydrogenase was further investigated by EPR spec-

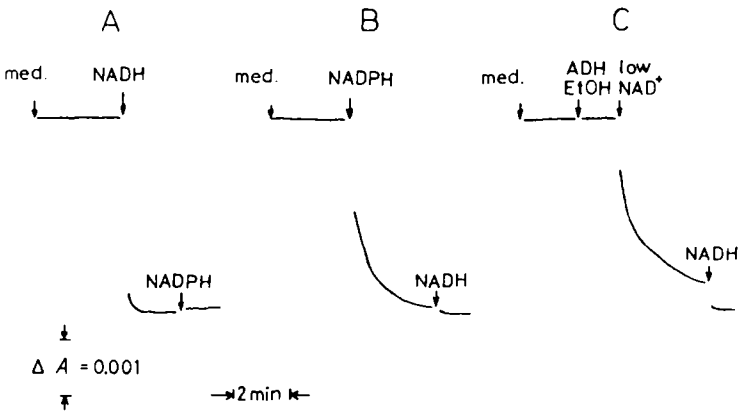

Fig. 6. Reduction of partially purified NADH dehydrogenase by NADH (A), NADPH (B) and a non-saturating concentration of NADH (C). Conditions were as described in Fig. 2 except that pH was 7.5, and that the medium contained 0.47 mg protein and 1 μg rotenone. In C the additions were 0.1 mg alcohol dehydrogenase, 0.1 M ethanol and 0.1 μM NAD^+.

troscopy using trypsin-treated submitochondrial particles as the source of enzyme. At pH 7.5 anaerobic, NADPH-treated submitochondrial particles show EPR signals at the *g* values 2.02, 1.94 and 1.91 (Fig. 7C). Essentially the

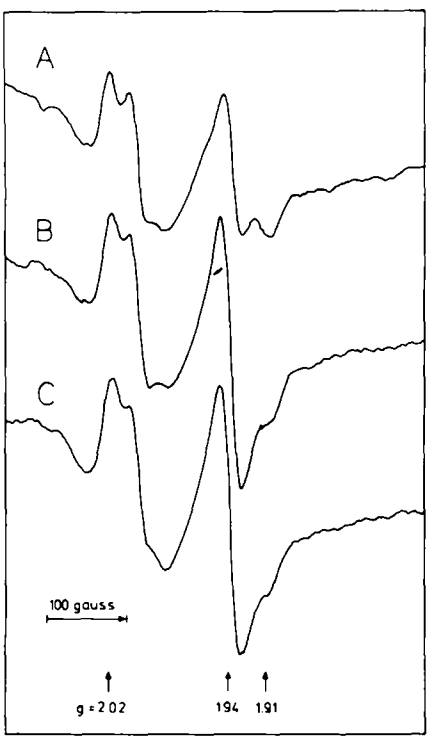

Fig. 7. EPR first derivative spectra of trypsin-treated submitochondrial particles in the absence of reducing agents (A), and in the presence of NADH (B) or NADPH (C). Conditions were as described in Materials and Methods.

same pattern was obtained with NADH (Fig. 7B) indicating that both nicotinamide nucleotides caused a reduction of iron-sulphur center 1 of the dehydrogenase. Iron-sulphur centers 2 and 3 plus 4 are not resolved at liquid nitrogen temperature [26] but has previously been shown to be reduced by NADPH [8]. In addition to reducing the components of NADH-ubiquinone reductase, NADPH has been shown to reduce cytochromes *b*, c_1, *c*, *a*, and a_3 in KCN-treated submitochondrial particles although at a lower rate [7]. These observations indicate that at or near redox equilibrium NADPH and NADH are equally capable of reducing all redox components of the respiratory chain.

Discussion

As demonstrated in the present paper a partially purified NADH dehydrogenase devoid of nicotinamide nucleotide transhydrogenase activity catalyzes the oxidation of both NADH and NADPH by acetylpyridine-NAD^+. The kinetic characteristics of these reactions, i.e. pH dependences, Michaelis constants and relative rates are similar to those of the NADH oxidase and NADPH oxidase reactions catalyzed by submitochondrial particles devoid of nicotinamide nucleotide transhydrogenase activity. The activities of the NADH-linked reactions increase with increasing pH whereas the activities of the NADPH-linked reactions increase with decreasing pH. Several other NAD-linked enzymes, e.g. lactate dehydrogenase [27] show a similar pH dependence for NADPH oxidation. Apparently, the negative charge of the 2'-phosphate group of NADPH is inhibitory for binding of the nicotinamide nucleotide to the enzyme. With NADH dehydrogenase the Michaelis constants for NADPH vary between approx. 600 and 1000 μM depending on the reaction catalyzed and are about two orders of magnitude higher than those for NADH. Approximately, the same relationship holds for the maximal velocities of these reactions, i.e. *V* for reduction of acetylpyridine-NAD^+ by NADPH at pH 7.5 is 1.25 nmol/min per mg protein whereas *V* for the corresponding NADH-linked reaction is higher than 120 nmol/min per mg protein [3]. Taken together these similarities suggest that NADPH is oxidized at a low rate directly by NADH dehydrogenase presumably via the NADH-binding site of the enzyme as advocated previously by Rydström et al. [5,15]. An implication of this suggestion is that both FMN and iron-sulphur centers 1, 2 and 3 plus 4 should be reduced by NADPH provided that electron transfer to oxygen is inhibited. Indeed, NADPH was found to reduce FMN completely in the partially purified preparation of NADH dehydrogenase. As expected, the rate of reduction of the flavine by NADPH is at least two orders of magnitude lower than by NADH, in agreement with the relative rates obtained for reduction of oxygen and acetylpyridine-NAD^+ by the two nicotinamide nucleotides. Reduction of iron-sulphur center 1 by NADPH was demonstrated with EPR spectroscopy using submitochondrial particles devoid of nicotinamide nucleotide transhydrogenase activity. Anaerobiosis was achieved by the presence of succinate. Under these conditions NADPH and NADH give identical levels of reduction of iron-sulphur center 1; the high potential centers 2 and 3 plus 4 were previously demonstrated to be reduced by NADPH [8]. Unsuccessful attempts by Hatefi and coworkers [7,8] to demon-

strate reduction of FMN and iron-sulphur center 1 in Complex I and submitochondrial particles appear to be due to leakage of electrons through the rotenone inhibition site. Such a minor leakage would not be detected with NADH as substrate where the rate of electron flux through the complex is at least two orders of magnitude higher as compared to the situation where NADPH serves as substrate. In a recent reinvestigation of this problem Hatefi and Bearden [17] demonstrated that indeed NADPH is capable of reducing iron-sulphur center 1 of Complex 1 partially, even when the bulk of the nicotinamide nucleotide transhydrogenase activity is inhibited by trypsin. The present data suggest that this incomplete reduction by NADPH was due to a slow oxidation of the iron-sulphur centers (mainly center 1) by contaminating cytochromes and oxygen. Other reports (refs. 16 and 18, cf. Introduction) claiming an almost complete reduction of FMN of Complex I by NADPH did not consider the possibility of NADH generation from contaminating NAD^+ plus nicotinamide nucleotide transhydrogenase.

The assumption that NADPH is accepted as a substrate by the NADH-binding site of NADH dehydrogenase implies, furthermore, that oxidation of NADPH is inhibited by NAD^+ or, conversely, that oxidation of NADH is inhibited by $NADP^+$. However, a 30-fold excess of NAD^+ brought about a slight stimulation rather than an inhibition of oxidation of NADPH by oxygen. At the present stage this effect cannot be explained satisfactorily but may indicate some type of nicotinamide nucleotide-dependent alteration of the enzyme (cf. ref. 3). It should be pointed out, however, that Rossi et al. [3] found a competitive relationship between NADPH and NAD^+ in the interaction of these nicotinamide nucleotides with highly purified NADH dehydrogenase. Inhibition of NADH oxidation by $NADP^+$ could not be estimated due to the presence of interfering NAD^+. in $NADP^+$.

In conclusion, the present investigation shows that in the absence of interfering NADH-producing nicotinamide nucleotide transhydrogenase activity, reduction of the components of the respiratory chain with NADH and NADPH does not differ in a qualitative sense. The rate of reduction of these components with NADPH is, however, considerably lower than with NADH. The simplest explanation for these findings is that NADPH is a substrate of NADH dehydrogenase and is oxidized by the NADH-binding site of the enzyme in accordance with the conclusion of Rossi et al. [3]. In the overall oxidation of NADPH by either acceptor, catalyzed by either submitochondrial particles or purified preparations of NADH dehydrogenase, the rate-limiting step appears to be the binding of NADPH to the enzyme. The available information does not support a possible structural and/or functional relationship between mitochondrial NADH dehydrogenase and transhydrogenase, or the existence of a specific NADP(H) binding site in NADH dehydrogenase, as proposed by Hatefi and coworkers [7—14].

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