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On the oxidation of reduced nicotinamide dinucleotide phosphate by submitochondrial particles from beef heart

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SUMMARY

The oxidation of NADPH catalyzed by submitochondrial particles from beef heart in the absence and presence of NAD^+ has been investigated. The data confirm earlier findings in this laboratory concerning the occurrence of an NADPH dehydrogenase with 2,6-dichlorophenolindophenol as the electron acceptor. This reaction is highly sensitive to palmityl-CoA, a feature further substantiating its possible relationship to nicotinamide nucleotide transhydrogenase. The particles also catalyzed a very low NADPH oxidase activity which probably proceeds *via* NADH dehydrogenase and is unrelated to transhydrogenase.

Submitochondrial particles obtained by sonication of heavy beef heart mitochondria[1] are known to catalyze a specific oxidation of NADPH in the presence of various acceptors[2]. As originally proposed by Kaplan and co-workers[3,4] and later demonstrated by Danielson and Ernster[5], the predominant pathway for this oxidation involves the nicotinamide nucleotide transhydrogenase reaction with NAD^+ as hydrogen acceptor. The kinetics of the transhydrogenase reaction and its functional relationship to the energy transfer system of the respiratory chain has been investigated extensively during the past years by Ernster and co-workers[6–8] and Rydström[9]. A second pathway involves the NADPH dehydrogenase described by Ernster *et al.*[2]. In this case the acceptor is either 2,6-dichlorophenolindophenol (DCIP) or externally added cytochrome *c*. On the basis of several common features of the NADPH dehydrogenase and transhydrogenase reactions, the authors[2] proposed that the former reaction involves a partial reaction of transhydrogenase.

Abbreviation: DCIP, 2,6-dichlorophenolindophenol.

Recently, Hatefi[10] showed that submitochondrial particles from beef heart catalyze a relatively rapid oxidation of NADPH in the absence of externally added acceptors. This oxidation resembled that of NADH by oxygen with respect to its sensitivity towards rotenone and other respiratory-chain inhibitors. From EPR measurements Hatefi[10] concluded that reducing equivalents from NADPH were fed into the respiratory chain at iron–sulphur center 2 of NADH dehydrogenase. In addition, it was proposed that this site might be involved in the transhydrogenase reaction. The present communication describes an attempt to distinguish between the above activities by using specific inhibitors.

Fig. 1A shows the oxidation of NADPH catalyzed by beef-heart submitochondrial particles with oxygen as acceptor. The addition of palmityl-CoA, which is a potent inhibitor of transhydrogenase[9], did not inhibit the reaction whereas rotenone brought about complete inhibition. In this experiment the concentration of NADPH was 0.17 mM, which is about three time the K_m as reported by Hatefi[10]. The V for the reaction was found to vary between 5–10 nmoles/min per mg protein, depending on the particle preparation used. When the experiment in Fig. 1A was carried out in the presence of 5 μM NAD^+ the rate of oxidation of NADPH was greatly accelerated (Fig. 1B) due to the contribution of the transhydrogenase reaction; the NADH formed was reoxidized by the respiratory chain. In this case, as expected, palmityl-CoA abolished the activating effect of NAD^+ and the subsequent addition of rotenone inhibited the oxidation of NADPH completely. To make sure that the transhydrogenase reaction was inhibited under the conditions employed in Fig. 1B, this experiment was repeated in the presence of rotenone and lactate dehydro-

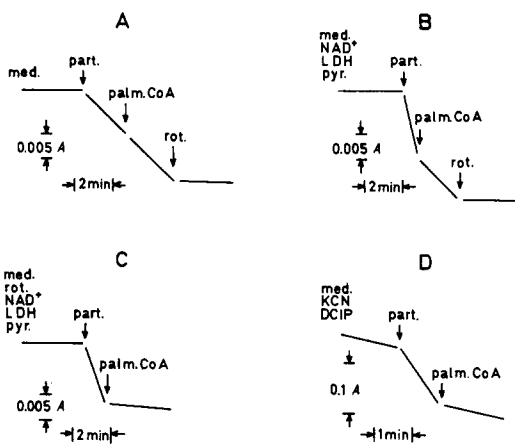

Fig. 1. Effect of palmityl-CoA on the oxidation of NADPH by beef-heart submitochondrial particles. The medium (med.) contained 0.25 M sucrose, 50 mM Tris–acetate, pH 7.5, and 0.17 mM NADPH. The additions were: (A–D), 0.4 mg of submitochondrial particles (part.) from beef heart mitochondria, obtained by sonication in the presence of either EDTA or Mg^{2+} *plus* ATP as described by Lee and Ernster[1]. 10 μM palmityl-CoA, 1 μg rotenone (rot.), 0.5 μg lactate dehydrogenase (LDH), 3.3 mM pyruvate (pyr.), 5 μM NAD^+, 50 μM 2,6-dichlorophenolindophenol (DCIP) and 1 mM KCN. The oxidation of NADPH with oxygen or NAD^+ as acceptor was measured at 366 nm using 3.1 mM^{-1} cm^{-1} as the extinction coefficient, and oxidation of NADPH with DCIP as acceptor was measured at 600 nm according to Ernster *et al.*[2]. All biochemicals used in this communication were of analytical grade and obtained from Sigma Chem. Co., U.S.A.

genase *plus* pyruvate as the NAD^+-regenerating system. As may be seen in Fig. 1C, palmityl-CoA caused a practically complete inhibition of the transhydrogenase reaction. In Fig. 1D the oxidation of NADPH by DCIP is demonstrated. Clearly, this oxidation was inhibited by palmityl-CoA as strongly as the transhydrogenase reaction in Fig. 1B.

Transhydrogenase has previously been shown[12] to be highly sensitive to trypsin, in contrast to NADH oxidase and NADPH dehydrogenase. Like the latter activities, the oxidation of NADPH with oxygen as acceptor was found to be essentially unaffected by a treatment with trypsin (*cf.* ref. 12 for assay) which inhibited transhydrogenase completely.

The presence of catalytic amounts of NAD(H) in commercial samples of NADPH or in submitochondrial particles leading to a transhydrogenation between NADPH and NAD^+, has previously been considered as a conceivable explanation for the observed oxidation of NADPH[2]. However, electrophoretic analysis indicated that the commercially available NADPH (and $NADP^+$ as well) was essentially free of NAD(H). This problem has now been reinvestigated making use of the high affinity of the energy-linked transhydrogenase for NAD^+ (ref. 8). A mixture containing submitochondrial particles and 2 mM NADPH was incubated together with enzyme systems that oxidized NADPH (and any NAD(H) present). After precipitation with acid and centrifugation, the remaining clear supernatant was used as medium for the energy-linked transhydrogenase reaction, assayed with the alcohol dehydrogenase system (*cf.* ref. 8). As may be seen in Fig. 2A, no transhydrogenation took place before NAD^+ was added to the system, the lower limit of detection in this case being about 0.015 μM. Since the analysis is a cyclic process, prolonged times of incubation can lower this value considerably. The present result indicates that the possible contamination of NADPH by NAD(H) was < 0.005 %. A different approach was recently used by Hatefi[10] who tried to demonstrate an increased oxygen uptake by the addition of β-hydroxybutyrate to a mixture containing particles, *via* the particle-bound NAD-specific β-hydroxybutyrate dehydrogenase. This system does not appear to be sufficiently active to reduce very low levels

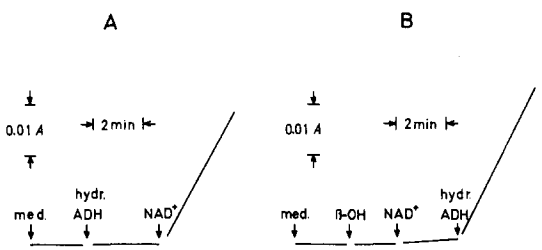

Fig. 2. Estimation of low amounts of NAD^+ by using the energy-linked transhydrogenase reaction. A mixture containing 6 mg of submitochondrial particles, 2 mM NADPH, 1 mM oxidized glutathione, 40 μg glutathione reductase, 0.5 μg lactate dehydrogenase and 3.3 mM pyruvate was incubated for 20 min at 30 °C in a final volume of 9 ml. Protein was precipitated by the addition of perchloric acid as described by Rydström *et al.*[6]. The resulting clear supernatant, adjusted to pH 7.5, was used as medium to which were added the components of the succinate-driven energy-linked transhydrogenase reaction[8] (except $NADP^+$ and NAD); the particles that were used in the assay were preincubated with succinate according to Nordenbrand and Ernster[11]. The additions were: (A and B), 6 mM hydrazine (hydr.), 60 μg alcohol dehydrogenase (ADH), 1 μM NAD^+ and 20 mM β-hydroxybutyrate (β-OH).

of NAD^+, as shown by the experiment of Fig. 2B. The rate of transhydrogenation following the addition of β-hydroxybutyrate was only about 5 % of that obtained with alcohol dehydrogenase under the same conditions.

The present data do not support the presence of a specific NADPH-oxidizing system in submitochondrial particles as proposed by Hatefi[10] which is different from the transhydrogenase reaction described by Kaplan and co-workers[3,4] or the NADPH dehydrogenase reaction described by Ernster *et al.*[2]. The low rate of rotenone-sensitive oxidation of NADPH by oxygen, which has frequently been observed in our laboratory, amounts to less than 1 % of that of NADH oxidase and is less than 20 % of the rate reported by Hatefi[10]. Since the NADH dehydrogenase activity is in large excess of the NADH oxidase[13], the rate of NADPH oxidation observed in our preparations may well be due to NADH dehydrogenase, which is known to catalyze a slow oxidation of NADPH[14] and which is not sensitive to palmityl-CoA[9] or trypsin[12]. Judging from the properties of the particle preparations made in our laboratory, any additional activity of rotenone-sensitive oxidation of NADPH may be explained by the presence of catalytic amounts of NAD^+. On the basis of the known Michaelis constants and maximal velocity for the transhydrogenase reaction from NADPH to NAD^+, it may be calculated that at saturating concentrations of NADPH, a ratio of NADPH to NAD^+ of 10^3 is sufficient to give a rate of 50 nmoles/min per mg protein, *i.e.* the rate of oxidation of NADPH reported by Hatefi[10]. The redox changes of the components of the respiratory chain which were observed by Hatefi[10] upon oxidation of NADPH in the presence of various respiratory inhibitors, are not inconsistent with a slow flux of reducing equivalents through the respiratory chain; exchange of NADPH for limiting amounts of NADH would most probably give a similar redox pattern.

The effect of palmityl-CoA on the oxidation of NADPH with DCIP as acceptor supports the proposal of Ernster *et al.*[2] that the bulk of the NADPH dehydrogenase reaction may be catalyzed by transhydrogenase. This proposal was based on several common features, *e.g.* both reactions involve the 4B-hydrogen of NADPH[2] and both are inhibited by agents such as Mg^{2+}, detergents and adenine nucleotides but not by rotenone or antimycin. The differential effects of trypsin on the two reactions could be explained by assuming that trypsin alters the NAD(H)-binding site of the transhydrogenase. This would not prevent an artificial acceptor like DCIP from interacting with the reduced enzyme or enzyme–substrate complex.

In conclusion the present data confirm and extend previous reports[2] from this laboratory concerning the occurrence of an NADPH dehydrogenase in submitochondrial particles from beef heart which may be related to nicotinamide nucleotide transhydrogenase. Our data do not indicate a direct connection between this enzyme and the respiratory chain, resulting in a rotenone-sensitive NADPH oxidase independent of the presence of NAD^+. Such a reaction has recently been postulated by Hatefi[10] on the basis of experiments in which, however, the presence of catalytic amounts of NAD^+ has not been convincingly eliminated.

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