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Evidence for two independent pathways of electron transfer in mitochondrial NADH: Q oxidoreductase. I. Pre-steady-state kinetics with NADPH

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The reduction of NADH: Q oxidoreductase by NADPH occurring in submitochondrial particles has been studied with the freeze-quench technique. It was found that 50% of the Fe-S clusters 2, 3 and 4 could be reduced by NADPH within 30 ms at pH 6.5. The remainder of the clusters, including cluster 1, were reduced slowly and incompletely; it was concluded that these clusters play no role in the NADPH oxidase activity. Nearly the same results were obtained at pH 8 under anaerobic conditions, demonstrating that the rate of reaction of NADPH with the enzyme was essentially the same at both pH values. The rate and extent of reduction of half of the clusters 2 by NADPH at pH 8 were not affected by the presence of O_2 of rotenone. This implies a pH-dependent oxidation of the enzyme as the cause for the absence of the NADPH oxidase activity at this pH. A dimeric model of the enzyme is proposed in which one protomer, containing FMN and the Fe-S clusters 1–4 in stoichiometric amounts, is responsible for NADH oxidation at pH 8. This protomer cannot react with NADPH. The other protomer, containing only FMN and the clusters 2, 3 and 4, is supposed to catalyse the oxidation of NADPH. The oxidation of this protomer by ubiquinone is expected to be strongly dependent on pH. This protomer might also catalyse NADH oxidation at pH 6–6.5.

Introduction

The pathway of electrons through NADH: Q oxidoreductase (EC 1.6.99.3, frequently called Complex I) is far from well known, in spite of extensive research on the electron-transferring groups, the Fe-S clusters and FMN [1], and on the role and location of the large number of subunits present in the purified enzyme [2–5]. Also efforts to resolve the topographical distribution of the clusters [6] did not help to understand the working mechanism of the enzyme. The molecular weight of purified Complex I is about 700 kDa per molecule of FMN [7]; the hydrodynamic properties [8] point to a dimeric structure.

In addition to the flavin, at least four different Fe-S clusters with characteristic EPR spectra are supposed to function in the enzyme [1]: cluster 1 ($g_{x,y,z} = 1.92, 1.94, 2.02$), cluster 2 ($g_{x,y,z} = 1.92, 1.92, 2.05$), cluster 3 ($g_{x,y,z} = 1.88, 1.94, 2.10$) and cluster 4 ($g_{x,y,z} = 1.86, 1.93, 2.04$). An EPR signal of yet another cluster ($g_{x,y,z} = 1.91, 1.95, 2.03$) has only been observed by Ohnishi and co-workers and was called N-1a [9] to distinguish it from the cluster 1 mentioned above, which was called N-1b. In contrast to the other clusters, which are fully reduced within 5 ms after mixing the enzyme with NADH [10,11], cluster N-1a cannot be reduced by NADH. It was only observed in redox titrations at potentials lower than −380 mV [9]. A signal of a possible sixth cluster ($g_{x,y,z} = 1.89, 1.92, 2.06$)

Abbreviations: AcPyADH, reduced 3-acetylpyridine adenine dinucleotide; Mes, 4-morpholineethanesulphonic acid.

could be evoked by NADH and is only observed at 4.2 K and high microwave power. The concentration of this sixth paramagnet in preparations of Complex I is less than 25% of that of the flavin. Clusters 2 and 4 are present in stoichiometric amounts to the flavin, whereas there is uncertainty about the concentration of cluster 3 which was found to be present in amounts of 0.5–1 times that of the flavin. Opinions disagree about the stoichiometry of cluster 1. According to initial reports of Orme-Johnson et al. [10,12] and Ohnishi et al. [13], its concentration in Complex I is (at least) 0.8 per FMN. In contrast, Albracht and co-workers determined this concentration as 0.4 per FMN and 0.5 per cluster 2 in Complex I [14] and as 0.5 ± 0.05 per cluster 2 in submitochondrial particles [15]. The latter data are in line with the idea that the enzyme might be a dimer [8].

Redox potentiometry [16] in the presence of electron-mediating dyes revealed a (pH-independent) midpoint potential of about -250 mV for the clusters 1, 3 and 4, whereas cluster 2 showed a midpoint potential of about -20 mV which was pH-dependent. During titration, all clusters behaved as $n = 1$ components.When titrated with the redox couple $NAD^+/NADH$ in the presence of the inhibitor rotenone, a different result was obtained. Although the apparent E'_m values for the clusters 1, 3 and 4 were only slightly lower (around -290 mV), during titration all clusters behaved as $n = 2$ components. This was assigned to the fact that NADH delivers two reducing equivalents at a time. From freeze-quench experiments of Complex I with AcPyADH it had earlier been concluded [10] that the sequence of redox potentials of the clusters might be: cluster 2 > clusters 3 and 4 > cluster 1.

Submitochondrial particles show a rotenone-sensitive NADPH oxidase activity with a rather sharp pH optimum around 6.0, which differs from the broad optimum of the NADH oxidase activity around pH 7 [17]. The K_m for NADPH in this reaction has been determined to be 550 μM, two orders of magnitude higher than the one for NADH, whereas the V_m of the NADPH oxidase measured at its optimal pH is about 60–70% of the V_m of the NADH oxidase at the same pH [17]. In 1965, Beinert et al. [18] showed that NADPH is able to completely reduce cluster 1 in soluble, high-molecular-weight (Type I) NADH dehydrogenase under anaerobic conditions, with a half-time of about 20 s. 10 years later Hatefi and co-workers [17,19] showed that aerobic reduction of Complex I with NADPH in the presence of rotenone resulted in incomplete reduction of clusters 1 and 3. The g_z peak of cluster 3 was shifted upfield compared to its position after reduction by NADH. Also the reduction of the flavin by NADPH, measured as the bleaching at the wavelength pair 475 minus 510 nm, was incomplete in Complex I as well as in rotenone-blocked, trypsin-treated submitochondrial particles [17,20]. Trypsin treatment at 0°C inactivates the energy-linked $NADH : NADP^+$ transhydrogenase activity; the NADPH-oxidation activity is hardly affected by such treatment [20,21]. So, on the score of incomplete reduction by NADPH of the flavin and Fe-S clusters 1 and 3 in Complex I, Hatefi et al. [17,21] suggested an alternative NADPH dehydrogenase pathway delivering reducing equivalents to the respiratory chain at the level of clusters 4 and 2, and by-passing the flavin and clusters 1 and 3. This suggestion was in agreement with their finding [20] that treatment of submitochondrial particles with trypsin at 30°C specifically inactivated the NADPH oxidation activity.

In 1976, Hatefi and Bearden [19] examined the differences in the reduction of Complex I by NADH, AcPyADH, NADPH and NADPH plus NAD^+ in more detail and concluded that these differences were essentially quantitative in nature, mainly reflecting the balance between the dehydrogenation rates of these nucleotides by Complex I and electron leakage from the complex by autooxidation. Simultaneously, Ragan [22,23] drew the same conclusion from the observation of the almost complete reduction of the chromophores bleaching at 450 nm, upon reduction with NADPH under closely anaerobic conditions. Thus, the conclusion was drawn that NADH and NADPH were oxidized at the same site, but with different and pH-dependent rates. The differences in pH optima for the oxidation of the two substrates was explained by Galante and Hatefi [24] as being caused by the required protonation of the 2′-phosphate group of NADPH with its pK_a of 6.1, which at low pH makes NADPH a closer electronic

analogue of NADH. One of us has questioned these conclusions some time ago [25], using the argument that the rate of oxygen uptake by submitochondrial particles in the presence of NADPH at pH 6.0 is at least 98% sensitive to rotenone. Thus the rate of auto-oxidation of Complex I is at most 2% of the dehydrogenation rate of NADPH and a higher level of reduction of cluster 1 (and of cluster 3) by NADPH might therefore be expected. It was suggested (as Hatefi and Hanstein had also initially concluded [17]) that part of these clusters might not be involved in NADPH oxidation. This was strengthened by preliminary results from freeze-quench experiments [25]. In the present report the reaction of submitochondrial particles with NADPH has been investigated in more detail. The results confirm the earlier proposal [25] of two independent pathways of electron transfer through NADH : Q oxidoreductase. A preliminary account of this work was presented at a recent congress [26].

Materials and Methods

NADPH, NADH and NAD^+ were purchased in the purest form available from Boehringer, Mannheim (F.R.G.). All other chemicals used were of analytical grade.

Bovine heart submitochondrial particles were prepared essentially as described in Ref. 27. Unless otherwise stated, the particles were treated with 0.1 mg of trypsin per mg of protein for 10 min at room temperature. Subsequently, the particles were centrifuged and washed twice with 0.25 M sucrose. Before and after the trypsin treatment the NAD(P)H oxidase activity and the NADPH-NAD^+ transhydrogenase activity (i.e., the stimulation of the NADPH oxidase activity at pH 6.2–6.5 upon addition of 1 mM NAD^+) were measured. The NADH and NADPH oxidase activities, measured in 0.25 M sucrose/50 mM potassium phosphate buffer (pH 8 and 6.5, respectively) and 2 mM substrate at 25°C, decreased by at most 5% and 9%, respectively, in accordance with the results obtained in Ref. 20. The transhydrogenase activity was destroyed for at least 99%.

Freeze-quench experiments were performed at room temperature (22°C), as described in Ref. 28. Three types of experiment were carried out: (i) reduction of untreated, aerobic submitochondrial particles with NADPH at pH 6.2; (ii) reduction of aerobic, trypsin-treated particles with NADPH at pH 6.5 or pH 8.0; (iii) reduction of anaerobic, trypsin-treated particles with NADPH at pH 8. The contents of the syringes, which were of equal size, was as follows.

(i) Syringe A contained submitochondrial particles (about 60 mg/ml) in 0.25 M sucrose/100 mM potassium phosphate buffer (pH 6.2), 30 μM rotenone and 1% (v/v) ethanol. Syringe B contained 10 mM NADPH or NADH in the absence or presence of 4 mM NAD^+ or $NADP^+$, all dissolved in the same medium as used in syringe A.

(ii) Syringe A contained trypsin-treated submitochondrial particles (45–60 mg/ml) in 0.25 M sucrose with 50 mM Tris-Mes buffer (pH 8.0) or 50 mM Mes-Tris buffer (pH 6.5) in the absence or presence of 50 μM rotenone and 2% (v/v) ethanol. Syringe B contained 10 mM NADH or NADPH in the absence or presence of 0.4 mM NAD^+, dissolved in a medium as in syringe A.

(iii) Trypsin-treated submitochondrial particles (60 mg/ml) suspended in 0.25 M sucrose, 50 mM Tris-Mes buffer (pH 8.0), 50 μM rotenone and 1% (v/v) DMSO were 10-times evacuated and gassed with H_2, in a glass vessel sealed with a rubber septum. The H_2 gas was freed from traces of O_2 by passage through a column with a Pd catalyst (Type E-236P, Degussa, Frankfurt, F.R.G.). Subsequently, the glass vessel was transferred via an air lock to a plexiglass glove box which was kept anaerobic by a steady flow of N_2 gas, inducing a slight overpressure inside the box. The syringes, filled with H_2 after evacuation and gassing five times, were likewise transferred to the glove box in which the mixing chamber, reaction hoses and spray nozzles were already present. The latter items were flushed with N_2 gas inside the glove box before use. A second rubber-sealed glass vessel, containing 10 mM NADPH or NADH dissolved in 0.25 M sucrose/50 mM Tris-Mes buffer (pH 8.0), was prepared in the same way. The H_2 gas was driven out of the syringes and the reactants were then transferred from the rubber-sealed glass vessels into the syringes with the aid of an hypodermic needle. When the syringes, the mixing chamber, the reaction hose and the spray

nozzle were assembled, the spray nozzle was placed in an opening in the bottom of the glove box, but was still separated from the air by a gas-tight stopper on the outside of the hole. The stopper was then removed and immediately replaced by a tight-fitting funnel containing cold isopentane (133 K) saturated with N_2 gas. A reaction was then initiated within 1 min. The distance between the spray nozzle and the isopentane was about 1 cm. A reference sample representing complete reduction was obtained by reaction of particles with 10 mM NADH for 30 ms, whereas the oxidised control sample was prepared by mixing with buffer. The quenching time (approx. 5 ms) has been included in the reaction times indicated in the figures. Each experiment, starting with two full syringes, was completed within 1 h and yielded 6–8 EPR samples.

EPR spectroscopy was performed as described earlier [29]. The relative amplitudes of the various Fe-S signals were obtained as follows: for cluster 1 from the trough of the $g = 1.94$ line measured at 45 K and 2 mW microwave power; for cluster 2 from the trough of the $g = 1.92$ line obtained at 17 K and 0.2 mW; for cluster 3 from the $g = 1.88$ line at 9 K and 20 mW; for cluster 4 from the trough of the $g = 1.86$ line at 9 K and 20 mW. The spectra of the clusters 3 and 4 had to be recorded under slightly saturating conditions in order to obtain an acceptable signal-to-noise ratio. No changes of the line shapes of the various clusters could be detected after incubation of the particles with trypsin for 30 min at room temperature.

Results

The partial reduction by NADPH of cluster 1 in Complex I previously observed [17,19] could also be reproduced with submitochondrial particles (Fig. 1). It can be seen from the $g_{x,y}$ lines of cluster 1 around $g = 1.94$ that NADPH reduced only 40% of this cluster. The broad trough around $g = 2$ is due to Cu_A of cytochrome *c* oxidase, which is not reduced. The degree of reduction of clusters 2, 3 and 4 by NADPH and NADH was the same (not shown). The same results were obtained at pH 6.2. Pretreatment of the particles with trypsin (0.1 mg/mg of particles) for 30 min at 0°C, whereby all transhydrogenase activity was already destroyed after 5 min, did not affect this reduction pattern, except for that of cluster 3. Reduction of this cluster diminished with NADPH as the reductant. Similar results were obtained at pH 7.3 and 6.2 (not shown). At the millisecond time scale this incomplete reduction could be followed in more detail. The reduction of cluster 1 (Fig. 2A) was, as expected, incomplete at $t = 100$ ms. The reduction of cluster 2 was biphasic. About half of this signal appeared within 10 ms, whereas the remaining half only fully developed after 100 ms. The reduction kinetics of cluster 4 could not be distinguished from first-order kinetics with a half-time of 10 ms (Fig. 2B), whereas the reduction kinetics of cluster 3 was similar to those of cluster 1. Fig. 3 shows EPR spectra of the g_x region of the clusters 3 and 4 during this reduction. Reduction of cluster 3, as seen from the trough at $g = 1.88$ in traces B and C, lagged behind that of cluster 4 with a trough at $g = 1.86$. Also visible in this figure is the overlap of signals of the clusters 2, 3 and 4. At the low-field side the trough of the $g = 1.88$ line of cluster 3 is superimposed on the $g_\perp$ of cluster 2, whereas at the high-field side overlap with the g_x of cluster 4 occurs. This made the determination of the signal

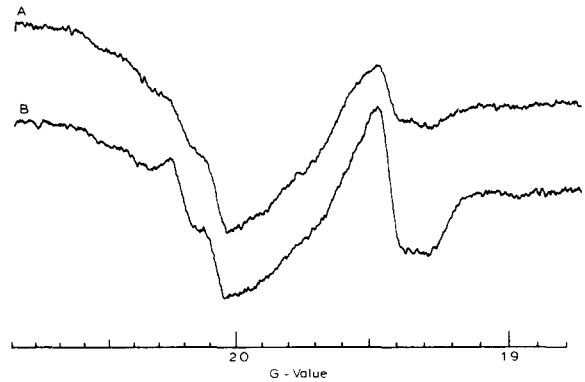

Fig. 1. EPR spectra of NADH:Q oxidoreductase present in submitochondrial particles reduced with NADPH or NADH. Submitochondrial particles (untreated) in 0.25 M sucrose/50 mM Tris-HCl buffer (pH 8) and 30 μM rotenone were mixed with 4 mM NADPH (A) or NADH (B). After 60 s at 0°C the samples were frozen in liquid nitrogen. EPR conditions: microwave frequency, 9239.7 MHz; temperature, 45 K; incident microwave power, 2 mW; modulation amplitude, 0.63 mT. The field-modulation frequency for this and other spectra in this paper was 100 kHz.

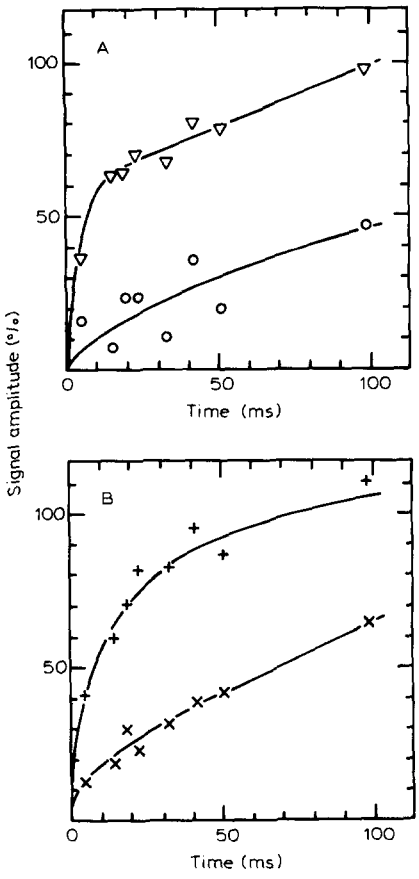

Fig. 2. Kinetics of reduction by NADPH of the 4 Fe-S clusters in NADH : Q oxidoreductase present in submitochondrial particles at pH 6.15 in the presence of rotenone. Submitochondrial particles (untreated) in 0.25 M sucrose/100 mM potassium phosphate buffer (pH 6.15) and 30 μM rotenone were mixed with 2.5 mM (final concentration) NADPH dissolved in the same medium. (A) O——O, cluster 1; ▽——▽, cluster 2. (B) ×——×, cluster 3; +——+, cluster 4.

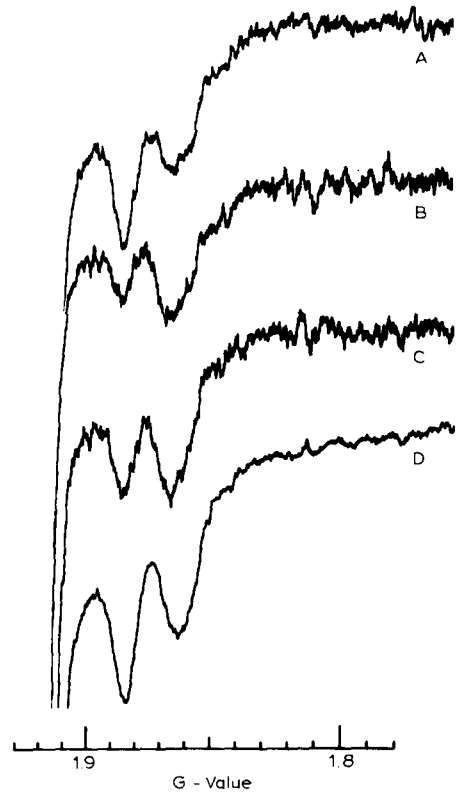

Fig. 3. EPR spectrum of the g_x region of clusters 3 and 4 after reduction with NAD(P)H. Reduction was carried out as in Fig. 2. (A) Reduction with NADH (2.5 mM) for 19 ms. (B), (C) and (D) Reduction with NADPH for 51 ms, 99 ms and 10 s, respectively. EPR conditions: microwave frequency, 9251 MHz; temperature, 11 K; microwave power, 20 mW; modulation amplitude, 1.25 mT.

amplitudes of cluster 3 somewhat ambiguous.

When NAD^+ was present in the reaction mixture, the reduction of cluster 2 (Fig. 4A) was complete within 10 ms. The initial rates of reduction of both cluster 1 and cluster 3 were accelerated and the final level of reduction was about 30–40%. The reduction of cluster 4 was biphasic, half of this signal appearing within 10 ms. The marked change in the reduction kinetics of the various clusters brought about by the presence of NAD^+ in this type of experiment, was presumably due to the production of NADH via the transhydrogenase activity of the particles and the subsequent redox potential set by the NAD^+/NADH couple. The reduction of all clusters by NADH was complete within the quenching time (5 ms) and was not affected by the presence of NADPH or $NADP^+$. Mixing with 1 mM (final concentration) NAD^+ gave the same results as mixing with buffer, demonstrating that the NAD^+ used was essentially free of NADH.

Also in the absence of added NAD^+ the transhydrogenase might interfere with the reduction kinetics of NADH : Q oxidoreductase present in the particles, since the absence of minute amounts of NAD^+ in the reaction mixture cannot be guaranteed. In order to exclude this possibility, the particles were treated with trypsin to destroy the transhydrogenase activity (see Materials and Methods section). Particles treated in this way

exhibited an NADPH oxidase activity of 330 nmol NADPH/min per mg of protein when measured under the same conditions as the freeze-quench experiments except for the addition of a catalytic amount of cytochrome *c* and the absence of the inhibitor rotenone. Since approx. 0.14 nmol of cluster 2 is present per mg protein [11,30], the inverse turnover number is about 25 ms. Thus, a cluster involved in NADPH oxidation is expected to become reduced within 25 ms.

The presteady-state kinetics of reduction of the clusters by NADPH in the presence of rotenone (and in the absence of added NAD^+) were only slightly changed after the trypsin treatment. The reduction of cluster 2 (Fig. 5A) still proceeded biphasically: 50% was reduced within 10 ms, while the remainder was reduced slowly. Cluster 1 was reduced incompletely even after 100 ms (Fig. 5A). The reduction of cluster 4 (Fig. 5B) was monophasic but incomplete (cf. Fig. 2B). Half of its

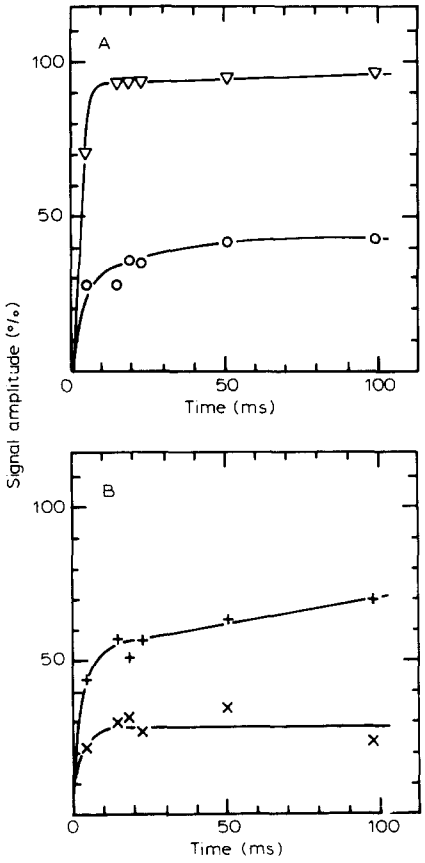

Fig. 4. Kinetics of reduction by NADPH of the Fe-S clusters in NADH:Q oxidoreductase in submitochondrial particles at pH 6.15 in the presence of rotenone and NAD^+. The experiment was carried out with the same batch of submitochondrial particles as in Fig. 2. Particles were mixed with 2.5 mM NADPH, 1 mM NAD^+ (final concentrations). The symbols used are the same as in Fig. 2.

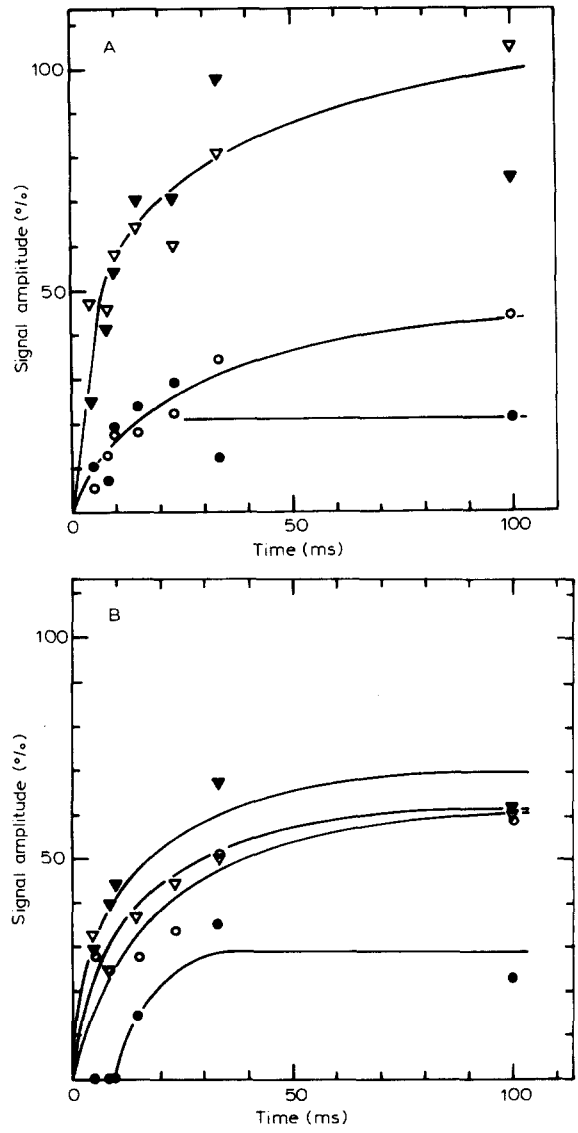

Fig. 5. Reaction of NADPH with trypsin-treated submitochondrial particles at pH 6.5 in the presence of rotenone. (A) O——O, cluster 1; ▽——▽, cluster 2. (B) O——O, cluster 3; ▽——▽, cluster 4. The closed symbols refer to the same experiment, but now in the presence of added NAD^+.

signal appeared within 10 ms, whereas the other half did not show up within 100 ms. Cluster 3 (Fig. 5B) showed similar reduction kinetics. In contrast to the findings with untreated particles (Figs. 2 and 4), addition of NAD^+ did not greatly affect the reduction kinetics of the clusters 2 and 4 in trypsin-treated particles (Fig. 5). This implies that the effects of NAD^+ in Fig. 4 were indeed due to rapid production of NADH catalysed by the transhydrogenase. The presence of NAD^+ had a slight, but pronounced effect on the reduction of clusters 1 and 3. Their level of reduction decreased and reduction of cluster 3 showed a lag phase (Fig. 5).

At pH 8.0, where hardly any NADPH oxidation occurs, the initial rates of reduction of the clusters by NADPH in the presence of rotenone were comparable with those obtained at low pH (Fig. 6). Both the rapid and the slow phases of the reduction of cluster 2 were slower (cf. Fig. 5A), whereas the rapid phase of the reduction of cluster 4 was accelerated (cf. Fig. 5B). Clusters 1 and 3 were only reduced by about 20–30%. In the absence of rotenone (Fig. 7). only about 50% of cluster 2 was rapidly reduced, whereas the second 50% remained oxidised, even after 5 s. Cluster 3

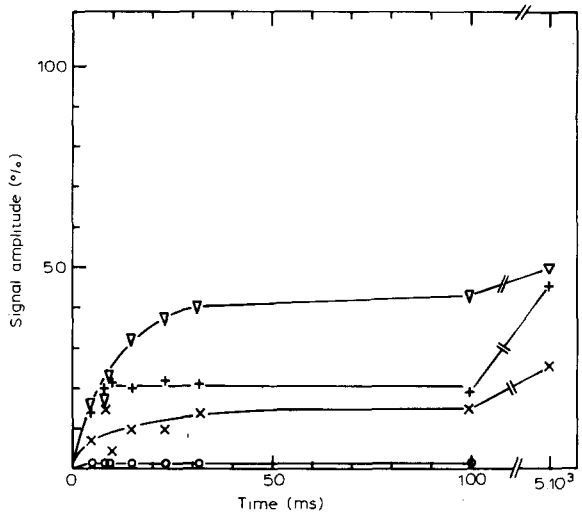

Fig. 7. Reduction by NADPH of trypsin-treated submitochondrial particles at pH 8.0 in the absence of an inhibitor. The symbols used are as in Fig. 6.

was reduced to a lower extent than in the presence of rotenone, whereas cluster 1 was not reduced at all. The reduction kinetics of cluster 4 were changed significantly when the inhibitor was omitted: within 10 ms a reduction level of 20%

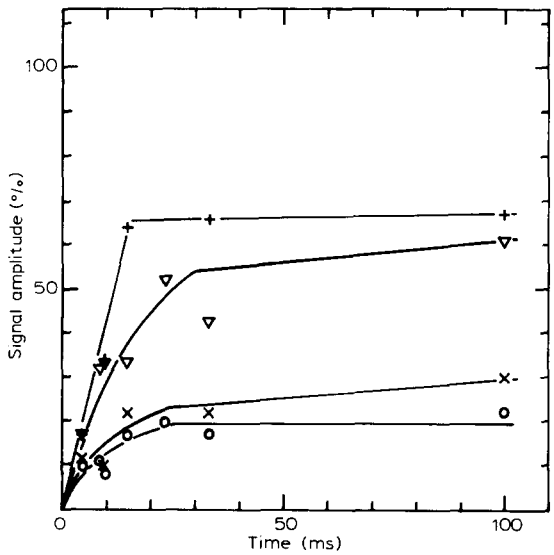

Fig. 6. Reduction by NADPH of trypsin-treated submitochondrial particles at pH 8.0 in the presence of rotenone. O——O, cluster 1; ▽——▽, cluster 2; × —— ×, cluster 3; + —— +, cluster 4.

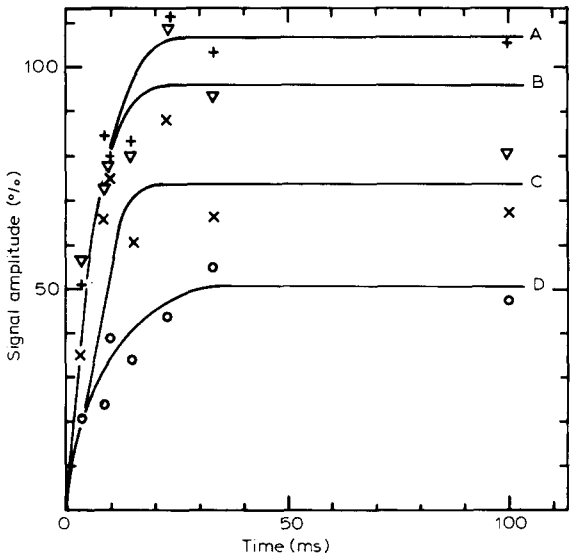

Fig. 8. Reaction of trypsin-treated submitochondrial particles with NADPH at pH 8.0 in the presence of both rotenone and NAD^+. The symbols used are as in Fig. 6.

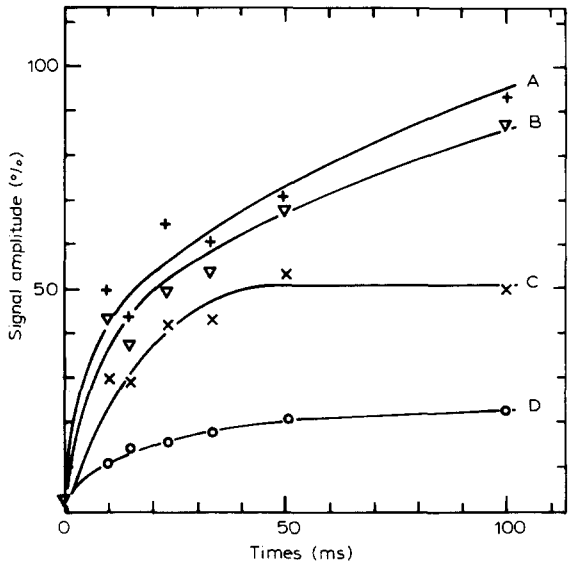

Fig. 9. Anaerobic reduction of trypsin-treated submitochondrial particles by NADPH at pH 8.0 in the presence of rotenone. The symbols used are those of Fig. 6.

was established which remained constant for at least 100 ms. After 5 s nearly 50% of this cluster was reduced. Ethanol (used as solvent for the inhibitor rotenone) had no effect on the reduction kinetics of any of the clusters.

The presence of NAD^+ caused a marked change in the kinetics of all clusters at pH 8 (Fig. 8). Clusters 2 and 4 were reduced completely within 20 ms (cf. Fig. 6). The levels of reduction of clusters 1 and 3 increased to 50% and 70%, respectively. When the experiment of Fig. 6 was carried out under anaerobic conditions, the pattern of reduction of clusters 2, 3 and 4 was also changed (Fig. 9). The rapid phases of reduction of clusters 2 and 4 were nearly unaffected, but the slow phases were accelerated. The extent of reduction of cluster 3 increased to 50%, whereas the rate of reduction did not change. Only the behaviour of cluster 1 was not affected by the absence of oxygen.

Discussion

The physiological significance of direct oxidation of NADPH, without the participation of the transhydrogenase, is not clear. The reaction has a high K_m for NADPH and its pH optimum is quite low. It has been proposed [19,22–24] that dehydrogenation of NADPH takes place at the site where also NADH is oxidised by the NADH : Q oxidoreductase. However, as yet no satisfactory explanation has been published for the incomplete reduction by NADPH of clusters 1 and 3. Moreover, since the pathway of electrons from NADH through the enzyme cannot be resolved by the freeze-quench technique, the pre-steady-state kinetics with NADPH might possibly provide more insight into the working mechanism of the enzyme.

The reduction of cluster 2 by NADH at pH 6.5 proceeded biphasically (Fig. 2A): 50% was reduced within 25 ms, which is the inverse turnover number of the enzyme under those conditions. The other 50%, however, was clearly reduced too slowly to be a component of the NADPH oxidase pathway. The latter criterion also holds for cluster 1, which after 100 ms is reduced by 50% only, and for 50% of clusters 3 and 4.

In the presence of both NADPH and NAD^+ (Fig. 4) clusters 1 and 3 and 50% of cluster 4 probably equilibrate rapidly with the NAD^+/NADH couple formed via the transhydrogenase activity of the untreated particles. The slow phase of the reduction of cluster 2 is accelerated due to formation of NADH. Cluster 2 is not in redox equilibrium with NAD^+/NADH couple [16]. The trypsin treatment removed this acceleration (Fig. 5) and the reduction kinetics of cluster 2 could no longer be distinguished from those in the absence of NAD^+, confirming that the transhydrogenase activity was destroyed effectively. Direct activity measurements indicated that the trypsin treatment removed the transhydrogenase activity by at least 99%. This means that the inverse turnover number per cluster 2, in the presence of NAD^+, is at least 2 s (and without added NAD^+ even longer). Thus the transhydrogenase could no longer interfere with our experiments on the millisecond time scale. The trypsin treatment only slightly affected the NAD(P)H oxidase activities, which is in agreement with results obtained by Hatefi et al. [20,21]. No alteration of the EPR line shapes could be observed, not even after treatment for 30 min at 22°C. Likewise, the treatment did not significantly change the reduction kinetics of clusters 1, 2 and 3 in the absence of added NAD^+. Only the

reduction kinetics of cluster 4 changed, becoming clearly biphasic.

The behaviour of cluster 3 was puzzling: 50% was reduced in the time required for a component involved in the oxidation of NADPH. The addition of NAD^+ lowered the level of reduction of this cluster and created a lag phase (Fig. 5). Although this might indicate a rapid equilibrium with NAD^+, this is not very likely. In that case an enhanced reduction of cluster 2 by the NADH formed (as in Fig. 4) might be expected, but this was not the case. It must be kept in mind that the actual reduction level of cluster 3 was sometimes rather difficult to determine due to overlap of signals (cf. Fig. 3). Given that the actual stoichiometry of cluster 3 is also still uncertain, its role in NADPH oxidation remains to be established.

The results reported in this and in a previous paper [25] have led to a tentative working hypothesis for NADH : Q oxidoreductase, presented in Fig. 10. The dimeric enzyme is thought to consist of two protomers, one with all known prosthetic groups in stoichiometric amounts (upper half of the model) and one which lacks cluster 1 and in which the presence of cluster 3 is as yet uncertain (lower part of the model). NAD(P)H donates its two reducing equivalents to the flavin, which in turn rapidly passes them on to two Fe-S clusters; no intense radical signals were ever observed in any of our experiments. A fifth cluster in the lower part as a counterpart of cluster 1 in the upper half of the model, cannot be excluded, although there is no evidence for such a component from the present data. NADH is capable of reducing all components within 5 ms, whereas only the lower part is rapidly reduced by NADPH (within 10 ms). The upper part is reduced too slowly by the latter substrate to be involved in NADPH oxidation. Reduction of this part is, however, more rapid than the estimated rate of NADH formation via the remainder of the transhydrogenase activity.

At pH 8.0 the reduction of part of the clusters by NADPH was almost as rapid as at pH 6.5 (Fig. 6). Even in the absence of rotenone, rapid reduction of nearly half of the clusters 2 and part of clusters 3 and 4 was observed at pH 8.0 (Fig. 7). These observations invalidate the original explanation for the pH dependence of the NADPH oxidase activity given by Galante and Hatefi [24], namely that the 2′-phosphate group of NADPH must be protonated before NADPH can be oxidised by the enzyme. Anaerobiosis accelerated the reduction of clusters 2 and 4 (Fig. 9) present in the upper part of the model. This is probably due to the absence of auto-oxidation. The reduction of cluster 1, however, was not affected. These results strongly suggest that the effect of pH on the NADPH oxidase activity is an effect on the oxidation of the enzyme (by ubiquinone), a topic further investigated in the accompanying paper [31]. It also implies that NADH oxidation at pH 8 proceeds exclusively via the upper protomer.

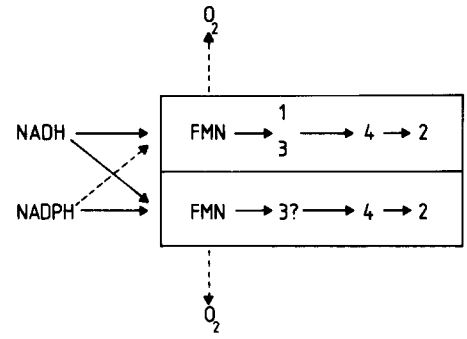

Fig. 10. Working hypothesis for the dehydrogenation of NADH and NADPH by NADH:Q oxidoreductase present in submitochondrial particles. The solid arrows indicate fast reactions, the dashed arrows slow reactions.

At present, the effect of added NAD^+ on the reduction kinetics of the clusters at pH 8.0 (Fig. 8) is not understood. Although an acceleration of the reduction of the cluster in the upper half of the model was observed, there was no effect of NAD^+ on the very small NADPH oxidation activity at that pH value.

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