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Biochimica et Biophysica Acta 1320 (1997) 256–264

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

A competitive inhibition of the mitochondrial NADH-ubiquinone oxidoreductase (Complex I) by ADP-ribose

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Received 3 January 1997; revised 24 February 1997; accepted 27 February 1997

Abstract

Considerable quantitative variations in the competitive inhibition of NADH oxidase activity of bovine heart submitochondrial particles (SMP) by different samples of NAD^+ were observed. ADP-ribose (ADPR) was identified as the inhibitory contaminating substance responsible for variations in the inhibition observed. ADPR competitively inhibits NADH oxidation with K_i values (25°C, pH 8.0) of 26 μM, 30 μM, and 180 μM for SMP, purified Complex I and three-subunit NADH dehydrogenase (FP), respectively. ADPR decreases NADH-induced flavin reduction and prolongs the cyclic bleaching of FP during aerobic oxidation of NADH. K_i for inhibition of the rotenone-sensitive NADH oxidase in SMP by ADPR does not depend on $\Delta\bar{\mu}_{H^+}$. The initial rate of the energy-dependent NAD^+ reduction by succinate is insensitive to ADPR. The inhibitor increases the steady-state level of NAD^+ reduction reached during aerobic succinate-supported reverse electron transfer catalyzed by tightly coupled SMP. The results obtained are consistent with the proposal on different nucleotide-binding sites operating in the direct and reverse reactions catalyzed by the mitochondrial NADH-ubiquinone reductase.

Keywords: NADH-ubiquinone reductase; Complex I; ADP-ribose; Competitive inhibition; Respiratory chain; (Bovine heart mitochondrion)

1. Introduction

Despite the significant progress in the structural studies on the proton-pumping NADH-quinone reductases (EC 1.6.99.3, Complex I) [1–3] the knowledge on the molecular mechanisms of the substrate dehydrogenation, intramolecular electron transfer and vectorial proton transfer is still at the primitive stage. The enzyme is composed of more than 40 distinct subunits, which are spatially arranged in such a way that the hydrophilic substrate (or product) interacts with the peripheral FMN-iron-sulfur containing three-subunit part (FP) [4–6]. The derived electrons reach intramembraneously located quinone reduction site(s) [7–10] travelling through a number of iron-sulfur clusters [11–13] presumably located in the connecting part (IP) [7,14].

Membrane-bound Complex I and simpler NADH dehydrogenase preparations (FP) derived from the original enzyme catalyze FMN-mediated stereospecific [4B-^{3}H] oxidation of NADH and rapid tritium-

Abbreviations: SMP, submitochondrial particles; FP, three subunit iron-sulfur-flavoprotein derived from Complex I; ADPR, ADP-ribose; FMN, flavin mononucleotide; rhein, 9,10-dihydro-4,5-dihydroxy-9,10-dioxo-2-anthracene carboxylic acid.

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PII S0005-2728(97)00029-7

H_2O exchange [15–17]. Significant variations in the apparent NADH affinity to the substrate binding site determined by the kinetic methods are scattered in the earlier literature [4,18–22]. The isolated respiratory chain-linked NADH dehydrogenase besides NADH oxidizes acetyl pyridine-NADH, pyridine aldehyde-NADH, thionicotinamide-NADH, deamino-NADH and NADPH [23]. Only deamino-NADH is oxidized at the rate comparable with that of NADH, and this was used to discriminate between energy-transforming and non-energy transforming bacterial NADH-quinone oxidoreductases [24,25]. Bovine heart submitochondrial particles (SMP) and the soluble partially purified NADH dehydrogenase are capable of NADPH oxidation with the pH-profile markedly different from that for NADH [26,27]. The catalytic sector of Complex I (FP) catalyzes NADPH $\rightarrow$ NAD^+ (acetyl-NAD^+) and NADH $\rightarrow$ acetyl-NAD^+ transhydrogenase reactions with the pH-profiles very similar to those for NADPH and NADH oxidation, respectively [28,29]. Two nucleotide-binding site model has been proposed to explain transhydrogenase activity of FP [29]. The observations on Fe-S clusters reduction by NADH or NADPH and studies on stoichiometry of the piericidin-induced inhibition of NADH and NADPH oxidation has led Albracht and associates to propose a heterodimeric structure for bovine heart Complex I which is composed of NADH-specific and NADPH-specific protomers [30–32]. The modified heterodimeric model with three separate pathways for oxidation of reduced pyridine nucleotides has been proposed to explain the inhibition pattern for thirteen different compounds mainly substituted carbocyanine dyes [33].

In addition to NADH oxidase activity tightly coupled bovine heart SMP catalyze the energy-dependent rotenone-sensitive stereospecific [4B-^{3}H] NAD^+ and $NADP^+$ reduction [16,26,34] which is believed to be a simple reversal of the proton-pumping NADH-ubiquinone reductase reaction.

The largest subunit of FP which contains FMN and N-3 iron-sulfur cluster has been identified as a NADH-binding subunit of mammalian and bacterial Complex I [35–38]. The conserved structural motifs of the primary structures of the bovine 51 kDa subunit [39], and its *(P.) denitrificans* [3] and *(N.) crassa* [40] counterparts has been recognized as the most likely candidate for the NADH-binding site. An additional segment of sequence that fits the nucleotide binding site has been identified in 39 kDa subunit of bovine Complex I [41] and in the homologous 40 kDa subunit of *(N.) crassa* complex [42].

Previous studies have revealed rhein as the only reversible inhibitor acting on the substrate side of the flavin [43,44]. Purely competitive inhibition with respect to substrate(s) has been documented for NADH-ferricyanide reductase and NADH $\rightarrow$ acetyl-NAD^+ transhydrogenase activities [44]. Rhein was shown not to affect NADH-acceptor reductase activity catalyzed by FP (low molecular mass NADH dehydrogenase); more complex inhibition than of purely competitive kinetics was found for NADH-ferricyanide reductase and ATP-driven reduction of NAD^+ by succinate which are catalyzed by the membrane bound Complex I [44]. It seems rather surprising that neither adenosine nor nicotinamide mononucleotide (oxidized or reduced) are the inhibitors of NADH oxidation while ATP, ADP, and AMP inhibit

Table 1
Kinetic parameters of ADPR-sensitive NADH oxidation by the respiratory chain-linked NADH dehydrogenase (25°C, pH 8.0)

Enzyme preparation	Electron acceptor	K_m^{NADH}(μM)	K_i^{NAD+}(mM)	K_i^{ADPR}(μM)
SMP (coupled) [a]	O_2 (NADH oxidase)	1.4	1.1	24
SMP (uncoupled) [b]	O_2 (NADH oxidase)	2.2	1.2	25
SMP	Ferricyanide (0.5 mM)	14	1.2	26
Complex I	Ferricyanide (0.5 mM)	31	1.2	30
FP	Ferricyanide (0.5 mM)	47	2.0	1.250
FP + 70 mM guanidine/Cl^-	Ferricyanide (0.5 mM)	28	–	180
FP	Hexammineruthenium III (0.5 mM)	180	–	800

The precision of the values given was about 10%.
[a] Particles were preincubated in the presence of oligomycin (0.6 nmol per mg protein) as described in Fig. 6.
[b] The same as before ([a]), gramicidin (0.1 μg/ml) was added to the assay mixture.

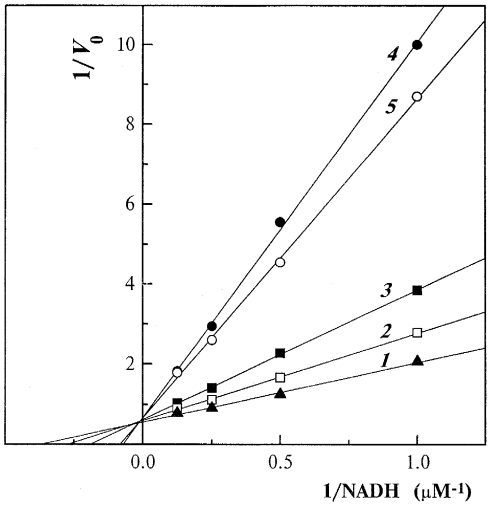

Fig. 1. Inhibition of the NADH oxidase by NAD^+. The initial rates (v_0, μmol per min per mg of protein) of NADH oxidation by SMP in the presence of gramicidin (uncoupled respiration) were measured after 'activation' of NADH-Q reductase by the addition and complete oxidation of 5 μM NADH. 1, no NAD^+ was added (control, 5 μM NAD^+ formed from NADH added for activating pulse was present). The additions of 1 mM NAD^+ were: 2, Sigma, grade V; 3, Sigma, grade III C; 4, Reanal; 5, as 2, the stock solution of 100 mM NAD^+ in water was kept for 48 h at room temperature.

at very high concentrations (about 10^{-2} M) [45]. Moreover, NAD^+, which appears to be the closest analogue of NADH, is a weak competitive inhibitor of NADH oxidation (K_i is about 1 mM) [4,22,46] whereas it is the substrate for the energy-dependent reverse reaction with the apparent affinity in micromolar range [46]. The uncompetitive inhibition of FP by low concentration of NAD^+ ($K_i = 25$ μM) has been reported, which was dependent on the nature of an artificial electron acceptor [47].

No clear-cut model for the arrangement of nucleotide-binding site(s) in Complex I can be constructed yet. The use of the competitive substrate-binding site inhibitors seems to be a powerful tool for further understanding of Complex I reaction mechanism. In this report we will characterize ADP-ribose (ADPR) as a competitive inhibitor of NADH oxidation. Inability of ADPR to inhibit the reverse reaction is considered as an indication for the presence of two different nucleotide-binding sites operating in $\Delta\bar{\mu}_{H^+}$-generating NADH oxidation and in $\Delta\bar{\mu}_{H^+}$-consuming NAD^+ reduction.

2. Materials and methods

Submitochondrial particles [48], Complex I [49] and soluble three subunits NADH dehydrogenase (FP) [5] were prepared according to the published procedures and stored in liquid nitrogen. Protein content was determined with the biuret reagent [50].

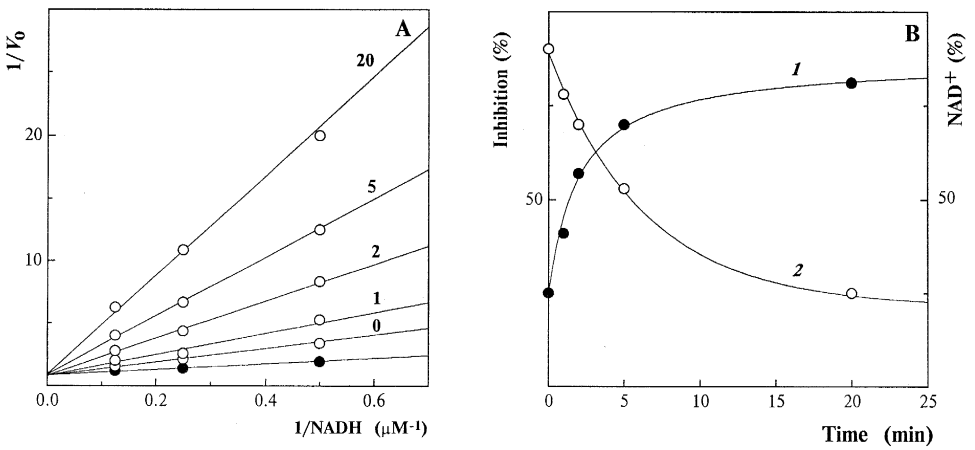

Fig. 2. Effect of NAD^+ hydrolytic decomposition on the efficiency of NADH oxidase inhibition. A: 100 mM freshly prepared aqueous solution of NAD^+ was kept at 100°C for the time (min) indicated by the figures on the lines and NADH oxidase was assayed as described in Fig. 1 in the presence of 1 mM NAD^+ (assumed concentration). (●) no NAD^+ was added. B: inhibitory effect of NAD^+ (curve 1, left ordinate) and actual NAD^+ content (curve 2, right ordinate) as a function of NAD^+ hydrolysis time-course. 8 μM NADH was used as the substrate.

Enzymatic activities were measured at 25°C in the standard reaction mixture containing: 0.25 M sucrose, 0.2 mM EDTA, and 20 mM Tris/Cl^- (SMP) or 20 mM potassium phosphate (Complex I and FP), pH 8.0. The rates of NADH oxidation or NAD^+ reduction were followed at 340 nm ($E_{340}^{mM} = 6.2$) or at 420 nm (ferricyanide reductase, $E_{420}^{mM} = 1.0$). NADH-ubiquinone reductase in SMP was pulsed aerobically with 5 μM NADH to activate the enzyme before the NADH oxidase and reverse electron transfer activities were measured [48]. All the activities are expressed as the initial rates of NADH oxidation or NAD^+ reduction (v_0, μmol per min per mg protein). Other details of the assays are indicated in the legends to figures and Table 1.

Chromatographic purification of NAD^+ hydrolysis products was performed on DEAE-cellulose column [51]. Salt-free NAD^+ hydrolysis products were collected using Sephadex G-10 column [52]. The homogeneous products of NAD^+ hydrolysis were identified by thin-layer chromatography on Silica gel plates with a butanol/acetic acid/H_2O mixture (5:2:3, v/v) as the solvent. The enzymatic hydrolysis of NAD^+ was carried out as follows: 1 unit of NAD^+ glycohydrolase (NADase EC 3.2.2.5, Sigma N-5263) was added to 1 ml of 0.1 M solution of NAD^+ (pH 7.5) and the mixture was incubated at 37°C for 1.5 h. The reaction was followed by measuring the cyanide-adduct formation at 327 nm ($E_{327}^{mM} = 5.9$) in the mixture containing 50 mM Tris/Cl^- and 1 M KCN, pH 9.0 [53]. The concentration of NAD^+ was measured enzymatically with yeast alcohol dehydrogenase (EC 1.1.1.1) [54].

The sources of the chemicals were as follows: NADH, NAD^+, rotenone, oligomycin, gramicidin, NADase, ADP-ribose were from Sigma (USA); EDTA was from Merck (Germany); DEAE-cellulose and Silica gel plates were from Whatman (USA); Sephadex G-10 was from Pharmacia (Sweden). Some samples of NAD^+ were from Reanal (Hungary). Other chemicals were of the highest quality commercially available.

3. Results

The original aim of these studies was to quantitate relative affinities of pyridine nucleotides to the substrate-binding site(s) of Complex I as revealed in different assay systems. We were particularly puzzled by the significant quantitative difference between the apparent K_i^{NAD+} for competitive inhibition of NADH oxidation (millimolar range) and the apparent K_m^{NAD+} for the reverse electron transfer (micromolar range) [46]. This prompted us to obtain closer insight into the kinetic pattern of the inhibition by NAD^+. Fig. 1 demonstrates that the inhibition of NADH oxidase is indeed purely competitive. However, we have noticed that the apparent K_i values were variable depending on the particular samples of NAD^+. Although possible effects of NAD^+ impurities on the kinetics of several NADH/NAD^+-dependent dehydrogenases have been emphasized and extensively discussed [55],

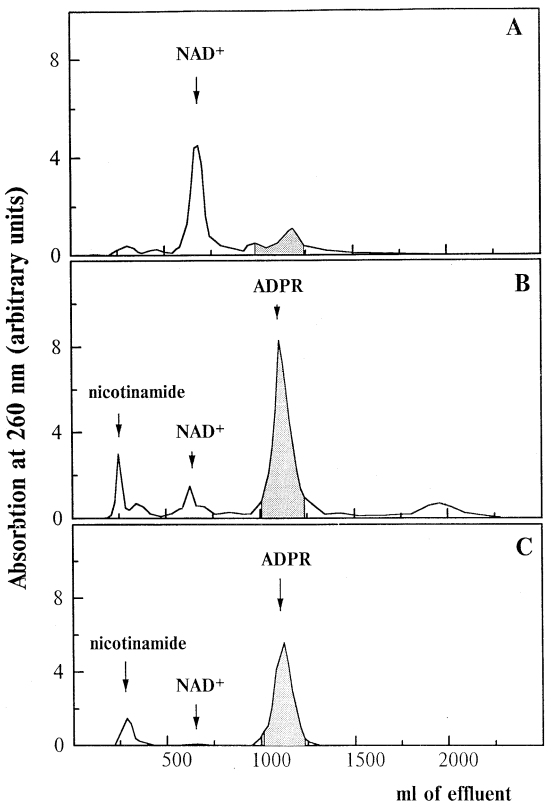

Fig. 3. Elution profile for the chromatography of NAD^+ samples on DEAE-cellulose. A: 1 ml freshly prepared 100 mM NAD^+ solution was applied on the column 30×3,5 cm and eluted with NaCl [51]. B: as (A) after 60 min hydrolysis at 100°C. C: as (A) after treatment with NAD^+-glycohydrolase for 90 min at 37°C. The compounds indicated were identified by thin-layer chromatography with the appropriate standards. Shadowed areas indicate the fractions which showed significant inhibitory effect on NADH oxidase of SMP.

to our knowledge, no special attention toward this problem had been paid to the respiratory chain-linked NADH dehydrogenases. When aqueous NAD^+ solution adjusted to pH 7.0 was stored for 2 days at 20°C, the apparent K_i for such samples gradually decreased. The inhibitory potency increased substantially in parallel with NAD^+ content decrease when the sample of NAD^+ was heated at 100°C for 1 h (Fig. 2). To identify the inhibitory substance(s), the heated samples of NAD^+ were subjected to ion-exchange chromatography as depicted in Fig. 3. Very similar chromatographic patterns were found for 'heated' NAD^+ and NAD^+ subjected to treatment with NAD^+ glycohydrolase (EC 3.2.2.5). The only fraction in hydrolyzed NAD^+ preparations which showed significant inhibitory effect was that corresponding to ADPR. Thus the inhibition pattern for ADPR was studied in greater detail. Fig. 4 shows that the inhibition of the NADH oxidase activity of SMP by ADPR is purely competitive with respect to substrate. The K_i value derived from Dixon plot was 26 μM.

It was of interest to examine whether simpler enzyme preparations oxidizing NADH are also inhibited by ADPR. Qualitatively the same kinetic patterns as shown in Fig. 4 (purely competitive inhibition) for SMP, purified Complex I, and FP (with hexammineruthenium III as electron acceptor) were obtained. A slight deviation from purely competitive inhibition by both NAD^+ and ADPR for FP in the presence of ferricyanide as an electron acceptor (mixes type inhibition) was noted. The quantitative kinetic parameters for NADH oxidation and inhibition by ADPR are summarized in Table 1. For comparison, the data on the competitive inhibition by chromatographically pure NAD^+ are also presented. It is worth noting that very close K_i values were found for SMP and Complex I whereas significantly higher K_m^{NADH} and K_i^{ADPR} were determined for FP. This is in agreement with the earlier findings on a drastic decrease of the apparent affinity of NADH to the substrate binding site after solubilization of FP [29,45,56]. In agreement with the results of Hatefi et al. [29,45], both K_m^{NADH} and K_i^{ADPR} decreased in the presence of guanidine.

The effect of ADPR on the NADPH oxidase activity of SMP at pH 6.5 was tested. A noncompetitive inhibition ($K_i^{ADPR} \approx 30$ μM) was observed. Further detailed studies are needed for the meaningful interpretation of this phenomenon because of markedly different pH-profiles and relative turnovers for NADH and NADPH oxidation and also because of possible involvement of transhydrogenase (EC 1.6.1.1) in the overall NADPH oxidation by SMP.

Further evidence for competition of NADH and ADPR for the substrate binding site which is equivalent to the inhibition of electron transfer between NADH and flavin was obtained by the direct mea-

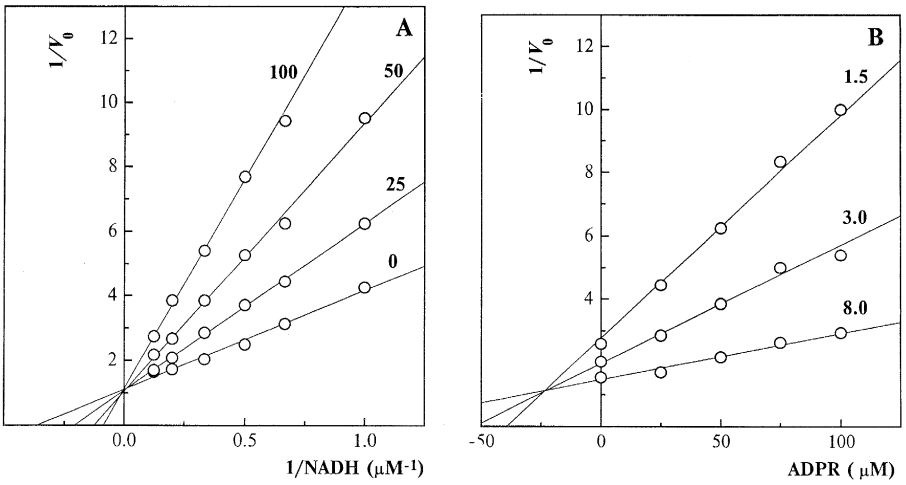

Fig. 4. Competitive inhibition of NADH oxidase activity of SMP by the chromatographycally pure ADPR. A: double-reciprocal plots for NADH oxidation in the presence of different ADPR concentration (μM, indicated by the figures on the lines). B: Dixon plot; concentrations of NADH (μM) are indicated by the figures on the lines.

surements of FMN redox cycle in FP after the addition of a limited amount of NADH. It has been documented that FP is capable of NADH oxidation coupled with oxygen → superoxide one-electron reduction at much lower rate than those observed with other artificial electron acceptors [47,57]. Thus the redox dependent cyclic bleaching of flavin can be followed at the relatively high protein concentration suitable for the direct spectrophotometric registration. When limited amount of NADH (20 μM) was added to the aerobic transparent solution of 0.56 μM FP (in terms of FMN content) an immediate decrease of absorption at 445 nm was observed followed by complete reoxidation after a certain time interval (Fig. 5). The cycle could be repeated by further addition of NADH (not shown). The presence of

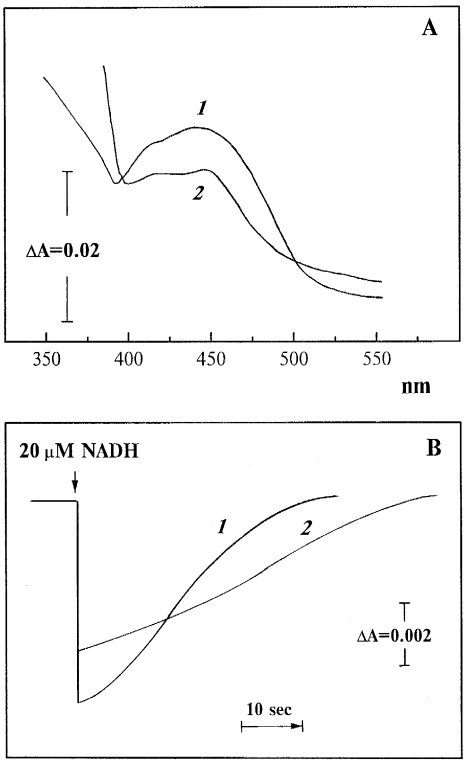

Fig. 5. Three-subunit FP chromophore bleaching by NADH. A: absorption spectra of the enzyme (0.064 mg/ml) as prepared (curve 1) and in the presence of 50 μM NADH (curve 2). B: time course of the NADH-induced bleaching under aerobic conditions. 20 μM NADH was added to 0.48 μM FP solution in 50 mM potassium phosphate (pH 8.0), 1 mM EDTA and 70 mM guanidine; the absorbance change at 445–500 nm was followed in the control sample (curve 1) and in the presence of 0.7 mM ADPR (curve 2). The addition of ADPR to the enzyme did not induce any spectral changes.

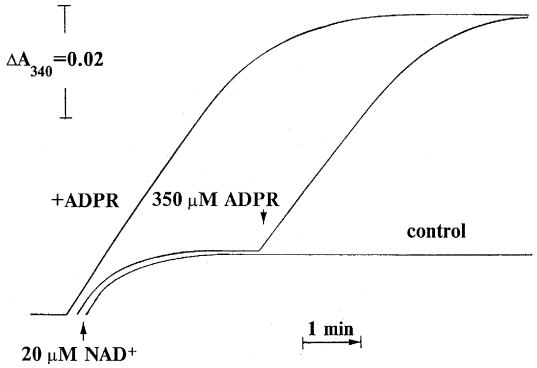

Fig. 6. The effect of ADPR on the time-course of the succinate-supported NAD^+ reduction catalyzed by tightly coupled SMP. SMP (10 mg/ml) were preincubated for 1.5 h at 25°C with oligomycin (0.6 nmol per mg of protein) in the mixture containing: 0.25 M sucrose, 20 mM Tris-Cl^-, 0.2 mM EDTA, 2 mM malonate (potassium salts), pH 8.0. 100 μg of SMP were added to 2 ml of standard mixture and Complex I was pulsed with 5 μM NADH before the initial rates were measured after simultaneous addition of NAD^+ and 10 mM succinate. All the activities were completely blocked by rotenone (2 μM) or gramicidin (0.1 μg/ml). The final NAD^+ concentration was 25 μM (5 μM left after pulse activation and 20 μM added simultaneously with 10 mM succinate where indicated). 350 μM ADPR was present in the assay mixture where indicated (left curve).

ADPR decreased the steady-state level of flavin reduction and increased the time needed to complete the redox cycle as expected for the inhibition between NADH and flavin.

We have also examined the effect of ADPR on $\Delta\bar{\mu}_{H^+}$-dependent reverse reaction catalyzed by the membrane-bound Complex I. The apparent K_m^{NAD+} for this reaction was 15 μM, the value which closely corresponds to those previously reported [46,58]. Unexpectedly, the initial rates of the succinate-supported NAD^+ reduction were negligably affected by ADPR at any NAD^+ concentration. In fact a slight increase of the initial rates (~20%) was observed in the presence of ADPR. In the separate experiments it was shown that ADPR (up to 1 mM concentration) did not affect succinate oxidation rate. An interesting 'activating' effect of ADPR was observed when the steady-state level of NAD^+ reduction was followed during the aerobic rotenone- and uncoupler-sensitive reverse electron transfer (Fig. 6). The presence of ADPR significantly increased the extent of NAD^+ reduction: the ratios $NADH/NAD^+$, reached during oxidation of succinate by tightly coupled SMP (respiratory control ratio of 5.0) in the presence of 25

μM NAD^+ added were 0.07 and 0.37 with and without ADPR respectively. The addition of ADPR after the steady-state level of NAD^+ reduction has been reached brought about further reduction up to the level seen in the presence of the inhibitor of NADH oxidation.

4. Discussion

The present experiments show that ADPR is a potentially useful compound for studies of the enzyme substrate-binding site(s). The structural analogy of ADPR with both NADH and NAD^+ is evident and the kinetic data strongly suggest that the inhibitor binds with the same catalytic site as does NADH. We have emphasized earlier [46] that being the substrate for the reverse electron transfer (K_m is about 20 μM) NAD^+ is known to be a very weak competitive inhibitor of NADH oxidation [4,22]. The low affinity of NAD^+ to the substrate binding site of NADH oxidase is somehow puzzling because NAD^+ seems to be the closest analogue of NADH. It may be speculated that the positive charge in oxidized nicotinamide moiety plays crucial role in the overall NADH oxidation catalysis providing the repulsive force for the product release from the enzyme active site thus contributing to the reaction energetic profile. Perhaps the most interesting finding reported here is that ADPR a competitive inhibitor of NADH oxidation does not inhibit and actually stimulates the $\Delta\bar{\mu}_{H^+}$-dependent reverse reaction (Fig. 6). Several possible mechanisms for such a kinetic behaviour merit brief discussion. It is expected that some properties of any enzyme functioning as an energy transducing device must be changed when $\Delta\bar{\mu}_{H^+}$-on the coupling membrane is built up. It is thus conceivable that the affinity for ADPR (and/or for NADH) is $\Delta\bar{\mu}_{H^+}$-dependent. Such a proposal, however, seems unlikely because neither apparent K_m^{NADH} nor K_i^{ADPR} are significantly different for 'coupled' and 'uncoupled' NADH oxidase (Table 1). Another possible explanation for the absence of ADPR inhibitory effect on the energy-dependent NAD^+ reduction is that the precise kinetic mechanisms of the direct and reverse reactions may not be identical [58]. If an ordered reaction mechanism is operating during NADH oxidation where the only substrate-free intermediate bears oxidized flavin NADH and ADPR would compete for the same binding site, providing that only the FMN_{ox}-form of the enzyme has high affinity for NADH or ADPR. The ordered mechanism for $\Delta\bar{\mu}_{H^+}$-dependent reverse reaction can be easily constructed where the only substrate-free intermediate is the FMN_{red}-form which has high affinity for NAD^+ and low or no affinity for ADPR. Should such an explanation be correct the thermodynamic consequence follows that the substrate (product or competitive inhibitor) binding per se should strongly influence the redox properties of flavin. Worth noting is that the dramatic difference in the redox behaviour of flavin in Complex I titrated either by artificial redox dyes or NAD^+/NADH couple has been documented [59]. It would be of interest to titrate the enzyme redox components in the presence of ADPR. Such an experimental approach may be helpful in studies of possible long distance conformational changes induced by the substrate binding. It should be noted that the difference in the precise kinetic mechanisms as discussed can hardly explain the stimulating effect of ADPR on the reverse electron transfer (Fig. 6).

The simplest explanation for unidirectional inhibitory effect of ADPR is that at least two nucleotide-binding sites (NADH- and NAD^+-specific) exist in the functionally operative membrane-bound Complex I, and only NADH-specific site is sensitive to ADPR. The heterodimeric structure of Complex I within the membrane postulated earlier [32] may serve as a structural basis for such a two-site model. It is also worth noting that two nucleotide-binding motifs, one in 51 kDa subunit [39] and another one in 39 kDa subunit [41], were found in the sequence studies on Complex I. The dramatic difference in the sensitivity of direct and reverse electron transfer to rotenone (the other highly specific inhibitor of Complex I) [60,61] has led us to propose a two-site model (for quinone and quinol respectively) on the oxygen side of Complex I. In light of the findings reported here the similar two binding site arrangement most likely operates on the substrate side of the enzyme. The final conclusion on the number and functional role of the pyridine nucleotide binding sites in Complex I should await further detailed studies of the effects of ADPR and other catalytically inert nucleotide analogues on all NAD^+/NADH and

NADH/NADPH-dependent reactions catalyzed by the intact and resolved enzyme preparations. These studies are currently underway in our laboratory. Taken together with the results presented in this report the data presently available [58,60,61] suggest that $\Delta\bar{\mu}_{H^+}$-dependent reverse electron transfer catalyzed by Complex I is not a simple reversal of $\Delta\bar{\mu}_{H^+}$-generating NADH oxidation.

5. Note added in proof

After the manuscript was accepted two papers appeared which are relevant to the present studies. Based on some controversies about the stoichiometry of the EPR-detectable iron-sulfur clusters in Complex I, a monomeric structure of the enzyme comprising 2 FMN and 8 different Fe-S clusters has been proposed (S.P.J. Albracht, A.M.P. de Jong, Biochim. Biophys. Acta 1318 (1997) 92–106). Studies on NADH- and NADPH-induced lipid peroxidation in submitochondrial particles and the effect of rhein on those reactions have led to the conclusion that Complex I may distinguish between electron input from NADH and NADPH by differences in the substrate binding site(s) (M.A. Glinn, C.P. Lee, L. Ernster, Biochim. Biophys. Acta 1318 (1997) 246–254).

Acknowledgements

This work was supported by National Institute of Health (USA) Fogarty International Research Collaborative Grant R03 TW00140-01A2 to Dr. T. Ohnishi (Department of Biochemistry and Biophysics, School of Medicine, University of Pennsylvania and A.D.V. The authors were partially supported by the Russian Foundation for Fundamental Research (grant 96-04-48185) to A.D.V. We are grateful to Dr. T. Ohnishi and Dr. V. Grivennikova for the constructive criticizm.

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