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Kinetics of the Transhydrogenase Reaction Catalyzed by Mitochondrial NADH:Ubiquinone Oxidoreductase (Complex I)

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Abstract—The kinetics of the NADH→3′-acetylpyridine adenine dinucleotide ($APAD^+$) transhydrogenase reaction (DD-reaction) catalyzed by different preparations of mitochondrial NADH-dehydrogenase (submitochondrial particles (SMP), purified Complex I, and three-subunit fragment of Complex I (FP)) have been studied. Complex I (in SMP or in purified preparation) catalyzes two NADH→$APAD^+$ reactions with different rates and nucleotide affinities. Reaction 1 has high affinity to $APAD^+$ (K_m = 7 μM, for SMP) and low rate (V_m = 0.2 μmol/min per mg protein, for SMP) and occurs with formation of a ternary complex. Reaction 2 has much higher rate and considerably lower affinity for oxidized nucleotide (V_m = 1.7 μmol/min per mg protein and K_m = 160 μM, for SMP). FP catalyzes only reaction 1. ADP-ribose inhibits reaction 1 with mixed type inhibition (competitive with non-competitive) with respect to NADH and $APAD^+$. Rhein competes with both substrates. The results suggest that at least two nucleotide-binding sites exist in Complex I.

Key words: NADH:ubiquinone oxidoreductase, transhydrogenase reaction, kinetics, Complex I, submitochondrial particles, three-subunit flavoprotein

NADH:ubiquinone oxidoreductase (Complex I, NADH-dehydrogenase, EC 1.6.5.3) is an exceedingly complex component of the mitochondrial respiratory chain. The enzyme catalyzes the oxidation of NADH by ubiquinone coupled with the vectorial transfer of four protons from mitochondrial matrix into intermembrane space [1]. The molecule of the enzyme consists of at least 42 subunits encoded by the mitochondrial and nuclear genomes (the overall molecular weight is about 1,000 kD) [2, 3], and contains 5-7 iron-sulfur centers [4, 5], tightly bound FMN [6], and at least two types of bound ubiquinone [7].

The structures of homologous procaryotic proton-translocating NADH:quinone oxidoreductases (NDH-1) are significantly simpler (the *nqo* genome operon of *Paracoccus denitrificans* consists of 14 genes encoding the polypeptides of the Complex and six open reading frames) [8]. There are 14 homological polypeptides among mitochondrial Complex I subunits. It is considered that functioning mechanisms of the Complexes in mitochondrial and procaryotic membranes are the same (or very similar). Thus, it can be supposed that 14 of 42 (or more) mitochondrial enzyme subunits form the minimal structure essential for catalysis; the function of the other 28 subunits is unknown.

The characteristics of Complex I nucleotide-binding active center(s) are poorly investigated. Until recently it was generally accepted that the enzyme has the single specific binding center for NADH and/or NAD^+, associated with it 51 kD flavin-containing subunit [9, 10]. The amino-acid sequences analysis of the enzyme subunits confirms the presence of typical nucleotide-binding motif in 51 kD subunit [2] and revealed the existence of another possible site of nucleotide binding in 39 kD subunit [2]. A number of results (briefly listed below) are not in accordance with existence of the single nucleotide-binding center in Complex I.

1. Differences in Fe-S clusters reduction, during the NADH and NADPH oxidation, led Albracht et al. to the hypothesis suggested that NADH:ubiquinone oxidoreductase was a heterodimer [11] or a monomer with eight Fe-S centers and two FMN [12].

2. The kinetics of the superoxide-radical generation by Complex I are characterized with two K_m, both for NADH and NADPH [13, 14].

3. Depending on concentration, NAD^+ affects as competitive or non-competitive inhibitor with respect to NADH [15, 16].

Abbreviations: SMP) submitochondrial particles; FP) flavoprotein, three-subunit fragment of Complex I; APAD(H)) 3′-acetylpyridine adenine dinucleotide, an NAD(H) analog; DD) hydride ion transfer between NADH (or its analogs) and NAD^+ (or its analogs), DPNH→DPN^+-exchange; DMSO) dimethyl sulfoxide.

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4. The substrate photo-affinity analogs label several subunits of the enzyme [17-19].

5. The enzyme affinities to NAD^+ and NADH, during NADH oxidation and $\Delta\bar{\mu}_{H^+}$-dependent NAD^+ reduction by ubiquinone, are essentially different [20].

6. ADP-ribose competes with NADH during oxidation, catalyzed by Complex I in SMP, but do not affect the reverse reaction [21]. The treatment of SMP by aryl-azido-β-alanine ADP-ribose results in 20-fold inhibition of the first activity, whereas the second one decreased just 2.5-fold [22].

All of the data mentioned indicate that the amount of nucleotide-binding centers still remains unknown. Nevertheless, the determination of the quantity of nucleotide-binding centers operating in NADH: ubiquinone oxidoreductase is necessary for understanding of the enzyme operation mechanism. The capability of Complex I to catalyze the DD-transhydrogenase reaction (the hydride ion reversible transfer from NADH to oxidized 3′-acetylpyridine adenine dinucleotide ($APAD^+$)) [23-25] represents one of the possibilities for the enzyme nucleotide-binding characteristics study. The kinetic analysis of this bi-substrate reaction can serve as indirect but quite reliable instrument for this purpose. In this connection, the investigation of the kinetic mechanism of the NADH→$APAD^+$ transhydrogenase reaction embodies the expediency, because it can lead to the determination of the enzyme substrate-binding centers quantity.

In the previous brief publication [26], we revealed that DD-transhydrogenase reaction, catalyzed by three different preparation of mitochondrial NADH-dehydrogenase, passed with a formation of the ternary complex. This suggests the participation of at least two nucleotide-binding centers in this reaction. The more detailed analysis of DD-transhydrogenase reaction catalyzed by SMP, isolated Complex I and FP is presented in this paper. The possible scheme of the transhydrogenase reaction catalysis by the enzyme is discussed.

MATERIALS AND METHODS

SMP [27], Complex I [15], and FP [28] were prepared according to the published methods. Protein content was determined with biuret reagent (SMP and Complex I) or by the Lowry procedure (FP).

The transhydrogenase activity was measured at 26°C. The reaction was followed as an increase in absorption at 375 nm ($E^{375}_{mM} = 5.1$), due to the simultaneous NADH oxidation with $APAD^+$ reduction [29]. The assay mixture for SMP contained 0.25 M sucrose, 20 mM Tris-HCl, 0.2 mM EDTA, pH 8.0, and 4 μM rotenone. The Complex I activity was measured in the reaction mixture containing 20 mM Tris-HCl, 0.2 mM EDTA, pH 8.0, 0.5 mg/ml of BSA, 0.15 mg/ml of azolectin (the soy bean phospholipid mixture), and 4 μM rotenone. Complex I (0.5 mg/ml) was pre-incubated in the assay mixture without rotenone and with 1 mg/ml of azolectin, before using (1 h, room temperature). The FP activity was measured in the reaction mixture containing 20 mM Hepes, 0.2 mM EDTA, pH 8.0. SMP (10-30 μg/ml), Complex I (1-3 μg/ml), or FP (0.5 μg/ml) were added to assays during the activity measurements. The Complex I and FP solutions were kept on ice during the time of experiments.

For mitochondrial nicotinamide nucleotide transhydrogenase (H^+-transhydrogenase, EC 1.6.1.1) inactivation, SMP were subjected to the weak trypsinolysis [30]. The suspension of SMP (40-50 mg/ml) was diluted to 20 mg/ml by the medium containing 0.25 M sucrose, 20 mM Tris-HCl, pH 8.0, 0.2 mM EDTA, and trypsin (0.1 mg per 1 mg of SMP). The suspension was incubated 30 min at 0°C and then diluted with the same medium, but without trypsin, for the stock solution of SMP preparation (1-3 mg/ml). This solution was kept on ice during the follow experiment.

The reduced 3′-acetylpyridine adenine dinucleotide (APADH) was obtained with alcohol dehydrogenase reaction [31, 32]. $APAD^+$ water solution (80-100 mM, 0.1 ml) was supplemented with 0.5 ml of 100 mM Tris (pH 9.4), containing 103 mM ethanol, 110 mM semicarbazide, and 10 mg/ml of alcohol dehydrogenase (25 U/mg). The mixture was incubated for 10-15 min at 4°C. The reduction of $APAD^+$ entailed with increase of absorbance at 363 nm (5-10 μl of the reaction mixture was put into 2-ml cuvette for absorbance measurements). On the reaction completion, the mixture was boiled for 2 min to denature the alcohol dehydrogenase, then quenched and centrifuged. The APADH concentration in the supernatant was determined using the coefficient $E^{363}_{mM} = 9.1$ [33].

The $APAD^+$ and NAD^+ concentrations in solutions used were determined with alcohol dehydrogenase [33]. The nucleotides (20-50 μM) were added to the reaction mixture containing 100 mM Tris (pH 8.7), 103 mM ethanol, 73 mM semicarbazide, and 5 or 50 U of alcohol dehydrogenase, in case of NAD^+ and $APAD^+$, correspondingly. $E^{340}_{mM} = 6.22$ and $E^{363}_{mM} = 9.1$ were used for calculations, correspondingly [33]. The NADH preparation used (Sigma, USA, No. 8129, prepared enzymatically) did not contain NAD^+ impurity.

ADP-ribose and rhein concentrations were determined using $E^{260}_{mM} = 15.4$ and $E^{435}_{mM} = 11.1$, correspondingly.

NADH, NAD^+, $APAD^+$, ADP-ribose, Hepes, EDTA, rotenone, and DMSO were from Sigma, alcohol dehydrogenase from Sigma and Reanal (Hungary). Tris (base) was from Merck (Germany), rhein was from Aldrich (USA).

The calculations and curve fittings were performed using Microcal Origin version 4.0, computer program.

RESULTS

The Kinetics of DD-Transhydrogenase Reaction Catalyzed by Complex I in SMP

The NADH→APAD$^+$ reaction. The dependences of initial rate on both substrates concentrations for the transhydrogenase reaction, catalyzed by Complex I in SMP, were studied for the kinetic mechanism establishment (Fig. 1). The direct plots dependences obtained looked like curves with plateaus (Fig. 1, a and b). Whereas the shape of double reciprocal plots curves suggested the complicated kinetics of the reaction (Fig. 1, c and d). The reaction rate dependences on NADH concentrations were linear at any APAD$^+$ concentration in double reciprocal plots. However, the lines obtained at low and high APAD$^+$ concentrations intercepted in different points (Fig. 1c). The reaction rate dependences on APAD$^+$ concentration were not linear in double reciprocal plots (Fig. 1d). It is obviously, that different interception points for curves on Fig. 1c are the consequences of the non-hyperbolic dependences of the reaction rate on APAD$^+$ concentration. The data presented suggest that there are two NADH→APAD$^+$ reactions occur, different in rates and affinities to APAD$^+$. One of them (further named the reaction 1) has high affinity to APAD$^+$ and comparatively low V_m; another one (further named the reaction 2), on the contrary, has low affinity to APAD$^+$ and high V_m.

The interception of lines (in all range of NADH concentrations and extrapolated for low APAD$^+$ concentrations) serves as diagnostic test for the ternary complex mechanism for reaction 1. In the case of binary complex mechanism ("ping-pong"), such dependences would look like the series of parallel lines. In turn, the formation of the ternary complex during the reaction 1 is an indication that at least two nucleotide-binding centers participate in it. The V_m^{app} double reciprocal dependences on fixed substrates concentrations (Fig. 1, e and f), obtained from Figs. 1c and 1d data, allow to determine the true K_m and V_m for reaction 1 (Table 1, in the case of reaction 2 the approximate values are given).

The other difficulty in kinetics of DD-transhydrogenase reaction, catalyzed by Complex I in SMP, is that there is double substrate inhibition in wide ranges of NADH and APAD$^+$ concentrations (Fig. 2). The inhibition by each nucleotide is better visible at low concentrations of another substrate. Both substrates inhibit the reaction in concentrations one order higher then corresponding K_m (Fig. 2, a and b). In further investigations we used both nucleotides in non-inhibiting concentrations.

The kind of the dependences obtained and quantity parameters of the reactions did not change in the presence of myxothiazol instead of rotenone, and did not depend on SMP coupling. The treatment of SMP with trypsin [30], specifically inactivating the mitochondrial proton-translocating nicotinamide nucleotide transhydrogenase (EC 1.6.1.1), also did not lead to the DD-reaction kinetics alterations.

The APADH→NAD$^+$ reaction. When APADH and NAD$^+$ were used as substrates, the rate dependences on the oxidized nucleotide concentration were also biphasic (data not shown). Two APADH→NAD$^+$ reactions are about 4 times slower and have significantly lower affinities to the reduced nucleotide in comparison with corresponding NADH→APAD$^+$ reactions (Table 1).

The Kinetics of DD-Transhydrogenase Reactions Catalyzed by Isolated Complex I and FP

Complex I. The kinetics of the reaction catalyzed by isolated Complex I is qualitatively similar to the SMP one (data not shown). K_m for nucleotides obtained were close to values calculated for SMP, whereas corresponding V_m were about 3-5 times higher in the case of isolated enzyme (as expected because of essential enzyme purification) (Table 2). The double substrate inhibition by high concentrations of nucleotides was also observed in reaction, catalyzed by isolated Complex I.

FP. As it was mentioned, FP (fragment of Complex I) consists of just three subunits and represents the minimum structure capable to catalyze both NADH oxidation and transhydrogenase reaction. The complicated kinetics of reaction, described in preceding section, suggests the presence of more than one nucleotide-binding center consisting of native enzyme. Some of these centers can be located in hydrophilic subunits of the enzyme. In this connection, the kinetic investigation of DD-reaction, catalyzed by FP, was of particular interest.

As shown on Fig. 3, NADH→APAD$^+$ reaction, catalyzed by three-subunit flavoprotein (FP), has simple hyperbolic rate dependences on both substrate concentrations, in the range of 40-200 and 3-100 μM, for NADH and APAD$^+$ correspondingly (Fig. 3, a and b). These dependences are linearized in double reciprocal plots (Fig. 3, c and d). The intercepting lines suggest the formation of the ternary complex during the reaction. As already mentioned, this requires at least two nucleotide-binding centers. It is obviously that three-subunit fragment of Complex I capable to catalyze the reaction 1: $K_m^{APAD^+}$ determined is close to values obtained for SMP and isolated Complex I (Table 2). The dependences for reaction catalyzed by FP in presence of guanidine (FP activator [28, 34, 35]) were analogous to those obtained in its absence (data not shown), but reaction in this case had differing affinities to substrates and 4 times higher velocity (Table 2).

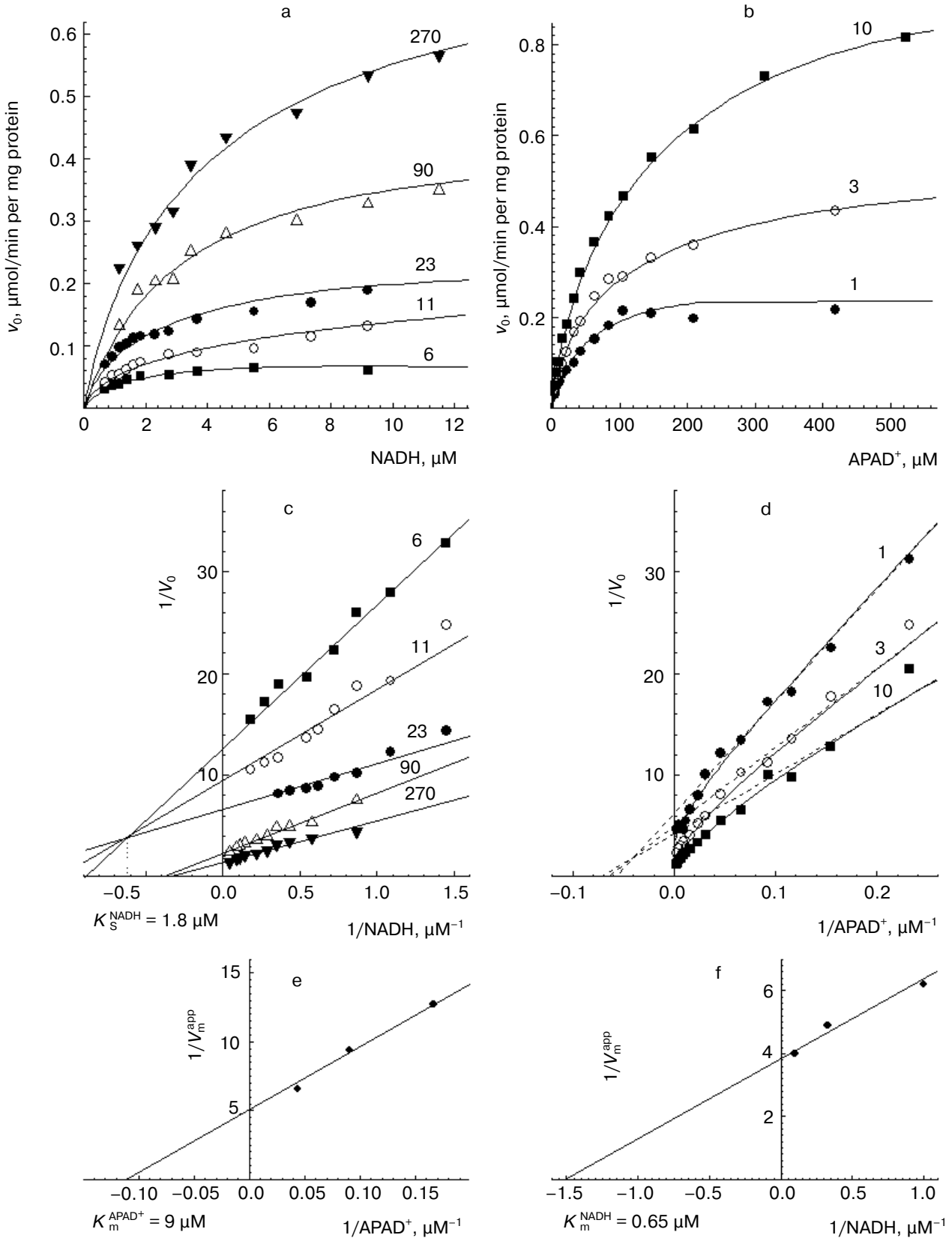


Fig. 1. The kinetics of NADH→APAD$^+$ transhydrogenase reaction catalyzed by SMP (26°C, pH 8.0): a, b) direct plots; c, d) double reciprocal plots. Figures on the lines are the concentrations of another substrate; e, f) V_m^{app} dependences on substrate concentrations (on (c) and (d) data).

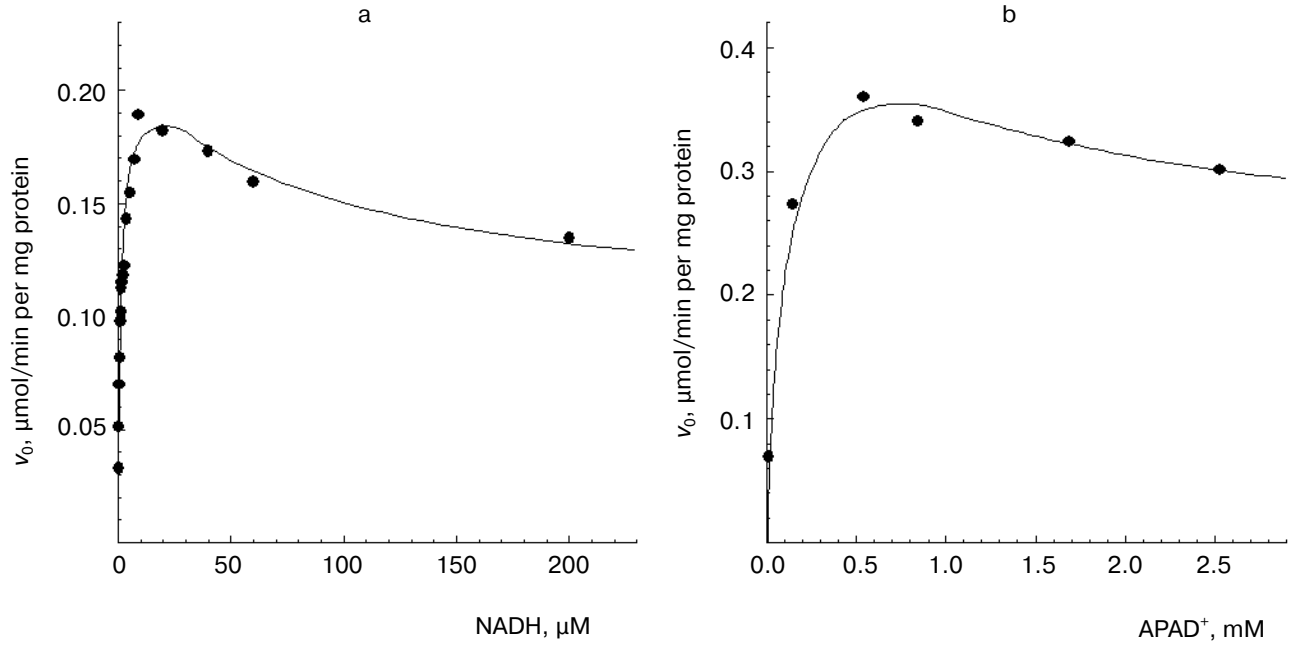


Fig. 2. The substrate inhibition of NADH→$APAD^+$ transhydrogenase reaction (SMP) by high substrates concentrations. The reaction rate dependences on nucleotide concentrations in wide range: a) on NADH concentration in the presence of 50 μM $APAD^+$; b) on $APAD^+$ concentration in the presence of 4 μM NADH.

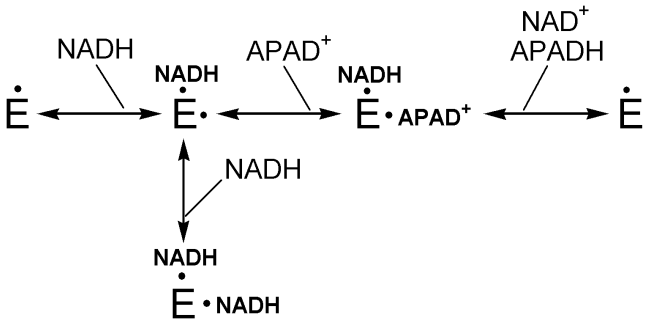
Table 1. The parameters of DD-reactions catalyzed by Complex I in SMP (pH 8.0, 26°C)

	Reaction 1 (ternary complex)	Reaction 2
NADH → $APAD^+$		
K_m^{NADH} (μM)	0.65	7
$K_m^{APAD^+}$ (μM)	9	500
V_m (μmol/min per mg protein)	0.22	2
APADH → NAD^+		
K_m^{APADH} (μM)	50	250
$K_m^{NAD^+}$ (μM)*	4	70
V_m (μmol/min per mg protein)	0.05	0.4

* The apparent K_m values found in the presence of 100 μM APADH are given. The discrepancy of presented values obtained in several experiments was at most 10%.

As for the native enzyme, the reaction catalyzed by FP is inhibited by high concentrations of NADH (Fig. 3a). This inhibition disappear in the presence of high $APAD^+$ concentration, this suppose the competition between nucleotides. Guanidine did not prevent the substrate inhibition. No inhibition by high concentrations of $APAD^+$ was observed for FP.

The simple kinetics of reaction catalyzed by three-subunit NADH-dehydrogenase, allowed to compare the theoretical dependences for chosen model steady-state kinetics (see below) with experimentally obtained results. The presented kinetic scheme of reaction was used as model:



The following expression describes the steady-state initial rate solution for this model:

$$v = \frac{V_m}{1 + \frac{K_m^A}{A} + \frac{K_m^B\,(1 + \frac{A}{K_i})}{B} + \frac{K_s \cdot K_m^B\,(1 + \frac{A}{K_i})}{A \cdot B}},$$

where K_m^A is Michaelis constant for NADH; K_m^B is Michaelis constant for APAD$^+$; K_s is substrate constant determined for NADH; K_i is substrate inhibitor constant for NADH; A is NADH concentration; B is APAD$^+$ concentration; V_m is maximal rate; v is initial rate of the reaction. The substrate inhibition by NADH, competitive with respect to APAD$^+$, is represented by multiplication of K_m^B to $(1 + A/K_i)$ in the expression mentioned.

Figure 4 (a and b) shows that at 3 μM APAD$^+$ theoretical curves (solid lines) are well coinciding with experimental data at K_m^{NADH}, K_s^{NADH}, $K_m^{APAD^+}$, and V_m determined (Table 2) and K_i for NADH adjusted (500 and 50 μM, in the absence or presence of 75 mM guanidine, correspondingly).

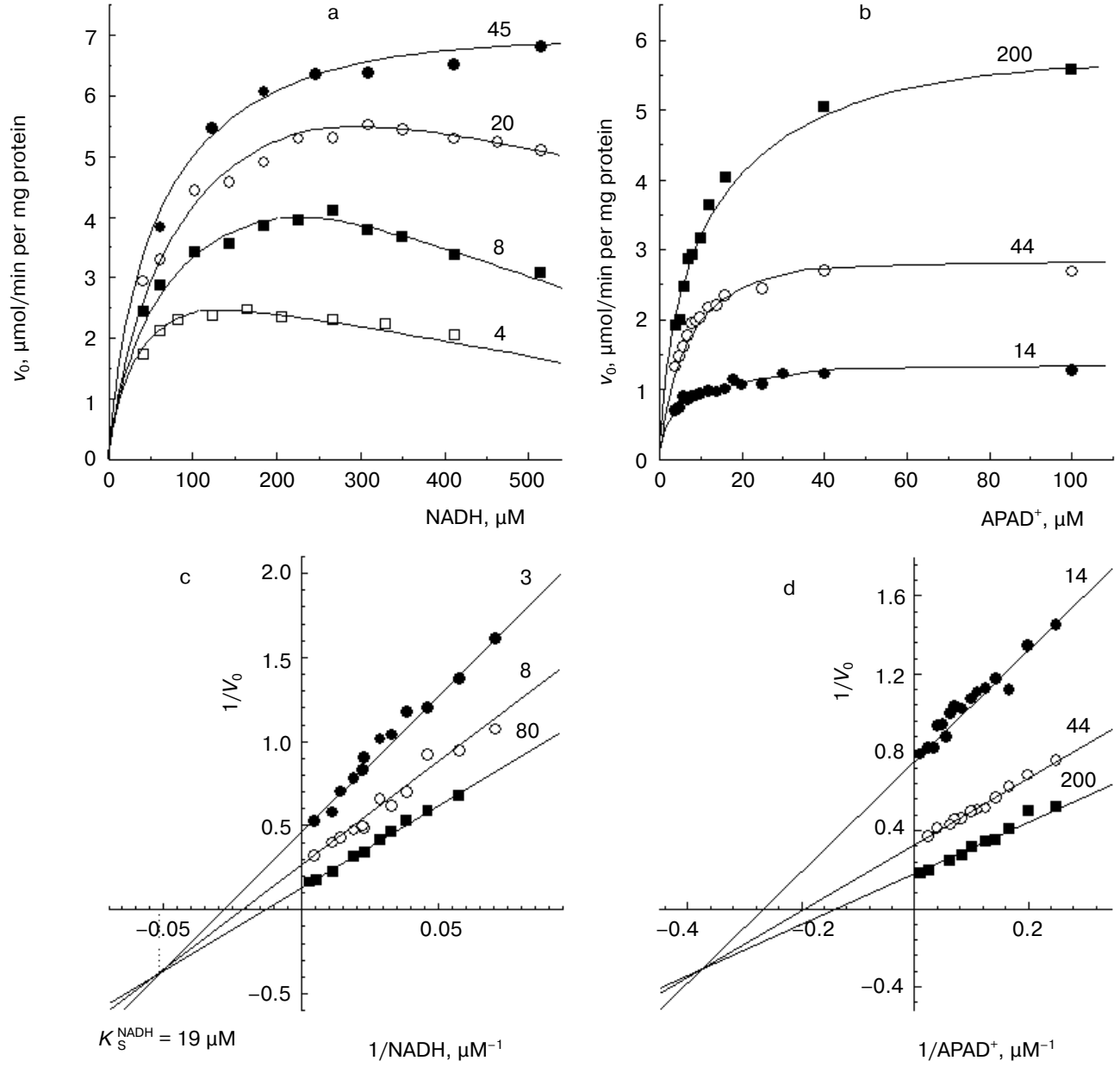


Fig. 3. The kinetics of NADH→APAD$^+$ transhydrogenase reaction catalyzed by FP (26°C, pH 8.0): a, b) direct plots; c, d) double reciprocal plots. Figures on the lines are the concentrations of another substrate.

Table 2. The parameters of NADH→APAD$^+$ transhydrogenase reaction 1 catalyzed by SMP, Complex I, and FP (pH 8.0, 26°C)

Parameter	Preparation			
	SMP	Complex I	FP	
			– guanidine	+ 75 mM guanidine
K_m^{NADH} (μM)	0.65	1.4	60	5
K_s^{NADH} (μM)	1.8	2.1	20	1.2
$K_m^{APAD^+}$ (μM)	9	14	9	18
V_m (μmol/min per mg protein)	0.22	0.56	8	35
K_i^{NADH} (μM)*			500	50
$K_i^{ADP\text{-}ribose}$ (μM)				
with respect to NADH	40 (C + NC)**	110 (C + NC)	1800 (C)	110 (C + NC)
with respect to APAD$^+$	150 (C + NC)	190 (C + NC)	4200 (NC)	110 (C + NC)
K_i^{rhein} (μM)				
with respect to NADH	10 (C)			
with respect to APAD$^+$	16 (C)			

* The constant for NADH substrate inhibition, theoretically determined values (expression on p. 656).

** Parenthetically pointed the type of inhibition: C, competitive; NC, non-competitive. The discrepancy of presented values obtained in several experiments was at most 10%.

The Effects of ADP-Ribose and Rhein on DD-Reaction 1

The essential difference in affinities to oxidized nucleotide of two NADH→APAD$^+$ reactions, catalyzed by Complex I, allows the study of inhibitory effects on reaction 1.

ADP-ribose. ADP-ribose is an effective inhibitor of reactions catalyzed by different preparations of the mito-

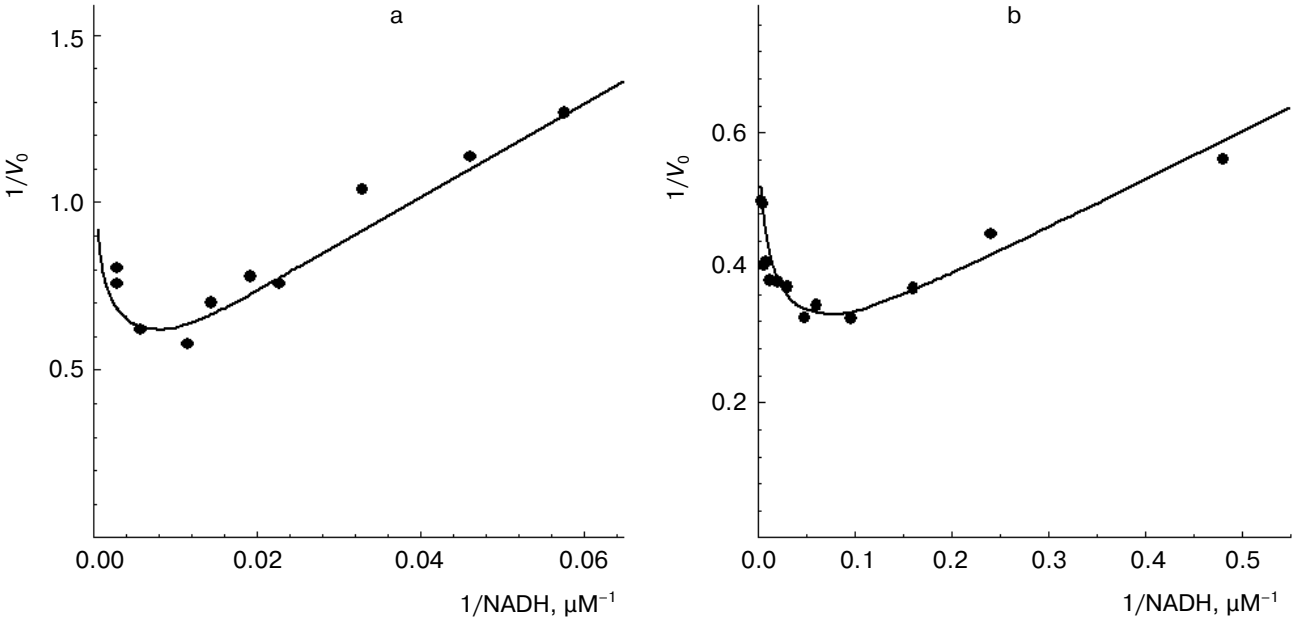


Fig. 4. The double reciprocal dependences of NADH→APAD$^+$ transhydrogenase reaction rate (FP) on NADH concentration in wide range (APAD$^+$, 3 μM): a) in the absence of guanidine; b) in the presence of 75 mM guanidine.

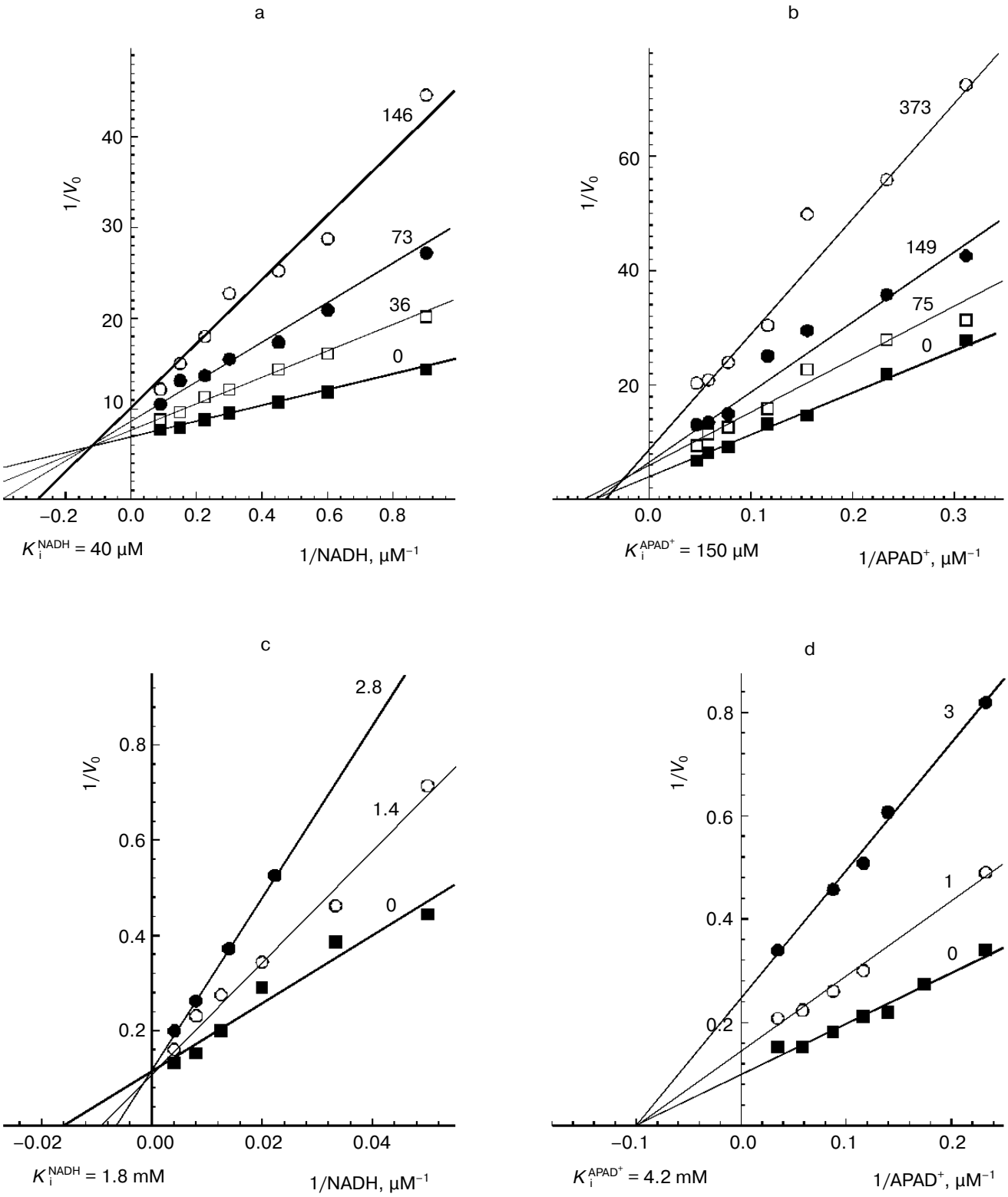


Fig. 5. The effect of ADP-ribose on NADH→APAD$^+$ transhydrogenase reaction 1 catalyzed by SMP (a, b) and FP in the absence of guanidine (c, d) (double reciprocal plots): a, c) with respect to NADH (20 and 80 μM of APAD$^+$, correspondingly); b, d) with respect to APAD$^+$ (8 and 200 μM NADH, correspondingly). Figures indicate the ADP-ribose concentrations. K_i was determined by Dixon method.

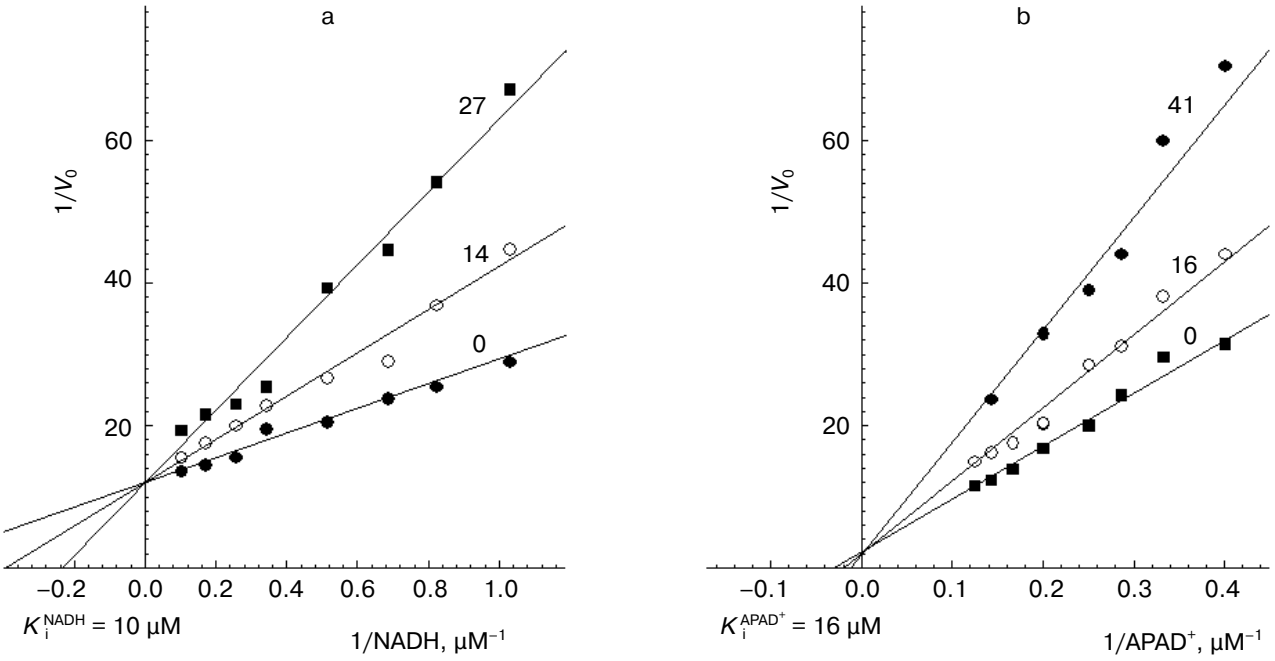


Fig. 6. The effect of rhein on NADH→APAD$^+$ transhydrogenase reaction 1 catalyzed by SMP (double reciprocal plots): a) with respect to NADH (APAD$^+$, 7 μM); b) with respect to APAD$^+$ (NADH, 8 μM). Figures indicate the rhein concentrations. K_i was determined by Dixon method.

chondrial NADH-dehydrogenase, competitive with respect to NADH [21]. It was shown in our laboratory that this inhibitor blocks the reactions of NADH oxidation, not affecting on the reverse electron transfer. It was of obvious interest to study the ADP-ribose effect on NADH→APAD$^+$ transhydrogenase reactions catalyzed by different enzyme preparations.

Figure 5 (a and b) demonstrates the results obtained for the ADP-ribose effect on reaction 1 catalyzed by Complex I in SMP. The straight lines intersection in double reciprocal plots (fourth quadrant) suggests the mixed type of inhibition with respect to both substrates of the reaction. The analogous results were obtained for isolated Complex I (data not shown). $K_i^{ADP\text{-}ribose}$ found with Dixon plots for SMP and Complex I are very similar (Table 2). They many-fold higher then values obtained for NADH-oxidase and NADH-ferricyanide reductase reactions (25 μM) catalyzed by SMP [21].

The ADP-ribose influence on the reaction, catalyzed by FP in the presence of 75 mM guanidine, was analogous to those observed for the native enzyme preparations (Table 2). In the absence of guanidine the types of inhibition convert into competitive with respect to NADH and non-competitive with respect to APAD$^+$ (Fig. 5, c and d), the affinity to the inhibitor significantly decreasing (Table 2).

Rhein. Rhein (1,8-dihydroxy-9,10-anthraquinone-3-carboxylic acid) is an effective inhibitor of NADH oxidation, catalyzed by Complex I, competitive with respect to NADH [36-38]. The intrinsic redox-activity of rhein (as an artificial electron acceptor) is an imperfection of this compound, which interferes the study of its effects on reaction catalyzed by isolated Complex I and FP. Therefore, we just investigated the effect of rhein on DD-reaction 1, catalyzed by Complex I in SMP, where the redox-activity of the compound is not essential. In accord with data presented on Fig. 6, rhein effectively inhibits reaction 1, competitively with respect to both substrates (Table 2).

DISCUSSION

We have shown that NADH→APAD$^+$ transhydrogenase reaction, catalyzed by SMP, has a complicated kinetics. Formally, the results obtained can be interpreted as the preparation simultaneously catalyzes two reactions with different kinetic parameters.

The inner mitochondrial membrane (and SMP, consequently) contains the proton-translocating nicotinamide nucleotide transhydrogenase (H^+-transhydrogenase, EC 1.6.1.1), catalyzing the reversible hydride-ion transfer between NADH and NADP$^+$. In our previous paper was shown that treatments with trypsin and palmitoyl-CoA, selectively inhibiting H^+-transhydrogenase, did not alter the kinetics of DD-transhydrogenase reaction catalyzed by SMP at pH 8.0 (in the absence of NADPH) [26]. Thus, no one of reactions supposed does

not concerned with nicotinamide nucleotide transhydrogenase activity.

There are some more NAD(P)H-dependent enzymes in mitochondria (in matrix and outer membrane), which potentially capable to catalyze the transhydrogenase reaction [39, 40]. Lipoyl dehydrogenase (the lipoyl dehydrogenase component of the α-keto-acids dehydrogenases) catalyzes the hydride-ion detachment in 4B position, as Complex I does, during reverse reaction, NADH oxidation and lipoic acid reduction [39]. However, even if these enzymes consist in SMP, they are inside of locked vesicles impenetrable for nucleotides. The possibility of their presence in isolated Complex I and FP is highly improbable. The absence of qualitative differences in kinetics of NADH→APAD$^+$ reactions, catalyzed by SMP and isolated Complex I, confirms that the measured activity concerned only with NADH: ubiquinone oxidoreductase operations.

According to the data presented, two DD-transhydrogenase reactions, catalyzed by mitochondrial NADH:ubiquinone oxidoreductase, differ in rates and affinities to the oxidized nucleotide. Reaction 1 has high affinity to the oxidized form of the nucleotide, comparably low V_m and occurs with a formation of the ternary complex, i.e., with participation of at least two nucleotide-binding centers. Only this reaction retains during the Complex I fractioning (FP purification). For all of the NADH-dehydrogenase preparations used, close $K_m^{APAD^+}$ values were obtained for reaction at low APAD$^+$ concentrations (Table 2), and V_m increased proportionally with FMN concentration. The substrate inhibition by high NADH concentrations, competitive with respect to APAD$^+$, was shown for reaction 1.

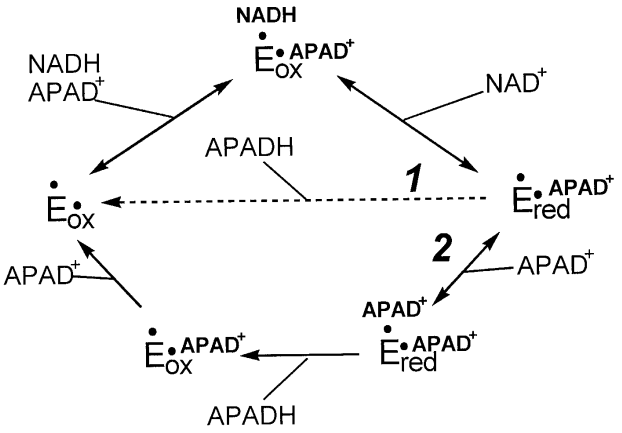


Fig. 7. Scheme illustrating the participation of two nucleotide-binding centers in NADH→APAD$^+$ transhydrogenase reactions 1 and 2, catalyzed by Complex I. Active centers of oxidized and reduced enzyme (E_{ox} and E_{red}, correspondingly) are indicated by the points. For further explanations see text.

Reaction 2 has worse affinity to the oxidized nucleotide and higher velocity (Table 1). The data obtained do not allow the establishment of this reaction mechanism, since it cannot be measured separately, without reaction 1. If reaction 2 would occur with the ternary complex formation, NADH:ubiquinone oxidoreductase should have at least three nucleotide-binding centers then. However, in the case this reaction occurs with "ping-pong" mechanism, either individual center(s) or one of the reaction 1 centers can participate in it. The center binding APAD$^+$ in reaction 1, obviously, do not participate in reaction 2: the affinities to the oxidized nucleotide are considerably different in those reactions. Consequently, among centers involved in reaction 1, only one, binding NADH, could take part in the second reaction. In such a case the summarized reaction of DD-transhydrogenation could be described by the scheme presented on Fig. 7. In this scheme the path *1* corresponds to reaction 1, occurring with the ternary complex formation and possessing high affinity to the oxidized nucleotide and low rate. According to this scheme, reaction 2 is possible in the case of APAD$^+$ reduction via NADH binding center (path *2*). In that way, both reactions catalyzed by Complex I can theoretically occur with involvement of just two nucleotide-binding centers. However FP, containing both centers participating in reaction 1, does not catalyze reaction 2, even in the presence of guanidine, which makes FP behavior similar to the native enzyme. This can suggest the presence of individual center(s) for reaction 2 in native Complex I. According to Chen and Guillory data [18], 98% of Complex I transhydrogenase activity disappear under parting of 42-, 39.5-, and 30.5-kD subunits. All of these three subunits are labeled by photo-affinity analogs of nucleotides [18, 19]. Our data is that, the rate of reaction 2, disappearing while fractioning, is one order higher then rate of remaining reaction 1. It is in good agreement with Chen and Guillory data. Thus, the reaction 2 can proceed with a center(s) locating on 42-, 39.5-, or 30.5-kD subunits.

In this paper, the ADP-ribose and rhein (the reversible inhibitors of Complex I active center) effects on NADH→APAD$^+$ reaction 1, occurring with the ternary complex formation, have been investigated. Using these inhibitors we planed to reveal whether substrates interact with enzyme during the reaction by the ordered mechanism or not. The employment of other approaches for this purpose (products inhibition, alternative substrates, direct and reverse reactions constants ratio) is difficult in the case of DD-reaction: the substrates of such a reaction are its products.

The use of ADP-ribose (mixed competitive with non-competitive type of inhibition with respect to both substrates) did not permit to establish whether the reaction 1 is ordered. It should be noted that ADP-ribose merely competes with NADH in others reactions of Complex I. Moreover, it does not affect on the reverse electron transfer reaction [21].

The competition of rhein with NADH and $APAD^+$ in reaction 1 could be the consequence of its binding in two centers operating in this reaction. On the other hand, if rhein binds with enzyme only in one of the nucleotide-binding centers, its competition with both substrates would suggest the ordered mechanism of NADH→$APAD^+$ reaction 1. Rhein competes with NADH in other reaction of Complex I as well [38]. In case the NADH binding center is the only place of rhein interaction with enzyme, it would imply that DD-reaction 1 has ordered mechanism, and NADH is the first substrate. This assumption is confirmed by the close values of K_i^{rhein} obtained with respect to NADH and $APAD^+$ (Table 2). Though, the results of this investigation cannot allow the univocal conclusion of whether DD-reaction 1 is ordered, without reliable data concerning amount of rhein binding places in Complex I [38].

Nevertheless, the investigation of the transhydrogenase reaction 1 ordering, as well as the establishment of mechanism of the second NADH→$APAD^+$ reaction, catalyzed by mitochondrial NADH:ubiquinone oxidoreductase, seems to be essential for determination of this enzyme nucleotide-binding centers functions.

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