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Kinetic Mechanism of Mitochondrial NADH:Ubiquinone Oxidoreductase Interaction with Nucleotide Substrates of the Transhydrogenase Reaction

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Abstract—The effects of Tinopals (cationic benzoxazoles) AMS-GX and 5BM-GX on NADH-oxidase, NADH:ferricyanide reductase, and NADH → $APAD^+$ transhydrogenase reactions and energy-linked NAD^+ reduction by succinate, catalyzed by NADH:ubiquinone oxidoreductase (Complex I) in submitochondrial particles (SMP), were investigated. AMS-GX competes with NADH in NADH-oxidase and NADH:ferricyanide reductase reactions (K_i = 1 μM). 5BM-GX inhibits those reactions with mixed type with respect to NADH (K_i = 5 μM) mechanism. Neither compound affects reverse electron transfer from succinate to NAD^+. The type of the Tinopals' effect on the NADH → $APAD^+$ transhydrogenase reaction, occurring with formation of a ternary complex, suggests the ordered binding of nucleotides by the enzyme during the reaction: AMS-GX and 5BM-GX inhibit this reaction uncompetitively just with respect to one of the substrates ($APAD^+$ and NADH, correspondingly). The competition between 5BM-GX and $APAD^+$ confirms that NADH is the first substrate bound by the enzyme. Direct and reverse electron transfer reactions demonstrate different specificity for NADH and NAD^+ analogs: the nicotinamide part of the molecule is significant for reduced nucleotide binding. The data confirm the model suggesting that during NADH → $APAD^+$ reaction, occurring with ternary complex formation, reduced nucleotide interacts with the center participating in NADH oxidation, whereas oxidized nucleotide reacts with the center binding NAD^+ in the reverse electron transfer reaction.

Key words: NADH:ubiquinone oxidoreductase, Complex I, nucleotide-binding centers

NADH:ubiquinone oxidoreductase of mitochondria (Complex I, NADH-dehydrogenase, EC 1.6.5.3) has an exceedingly complicated structure. The enzyme of about one million daltons molecular weight consists of at least 42 different subunits and contains bound FMN, iron-sulfur clusters, and ubiquinone [1-6]. The key function of the complex is oxidation of NADH (electrons flow into the respiratory chain) and $\Delta\bar{\mu}_{H^+}$ generation in the first electrochemical coupling site. Complex I and other preparations of NADH-dehydrogenase can catalyze NADH oxidation by different electron acceptors: oxygen (superoxide-radical generation) [7-10], ferricyanide, hexammineruthenium (III), ubiquinone analogs [11-17], and oxidized nucleotides (transhydrogenase reaction) [18-20]. Besides, the enzyme in tightly coupled membranes (of mitochondria or submitochondrial particles) catalyzes the reverse electron transfer or energy-dependent NAD^+ reduction by ubiquinol [21-24].

Until recently it was considered that the only nucleotide-binding center of Complex I was located on a 51-kD subunit belonging to the smallest catalytically active fragment of the enzyme—a three-subunit flavoprotein (FP) [2, 25]. However, the embedding of photoaffinity substrate analogs in several subunits of NADH:ubiquinone oxidoreductase [26, 27], different effects of ADP-ribose on reactions of direct and reverse electron transfer [28, 29], and other data [9, 30, 31] imply two or more nucleotide-binding centers in Complex I.

Our studies of the kinetic mechanism of the NADH → $APAD^+$ transhydrogenase reaction, catalyzed by Complex I, resulted in the suggestion of the functioning of at least two nucleotide-binding centers even in FP [32]. It is obvious that one of the centers is located on the 51-kD subunit [2]. The second one can be formed by amino acid residues of the other two or all three FP subunits.

Abbreviations: APAD(H)) 3′-acetyl pyridine adenine dinucleotide, an NAD(H) analog; deamino NAD(H)) nicotinamide hypoxanthine dinucleotide, an NAD(H) analog; DMSO) dimethyl sulfoxide; FP) flavoprotein, three-subunit fragment of Complex I; SMP) submitochondrial particles.

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Investigation of the interaction of Complex I with nucleotide substrates is a necessary step in understanding how it operates. In particular, it is of interest to establish substrate binding order during the transhydrogenase reaction, the only one of the reactions catalyzed by the enzyme in which two nucleotides participate.

Our previous work showed that NADH:ubiquinone oxidoreductase catalyzes two NADH → $APAD^+$ transhydrogenase reactions [33]. One (reaction 1) has high affinity to nucleotides. This fact allows study of reaction 1 features separately from the second reaction catalyzed by the enzyme, which has more than one order of magnitude lower affinities to the substrates. There are two substrate-binding centers participating in reaction 1 (it occurs with ternary complex formation). To investigate the order of the NADH → $APAD^+$ transhydrogenase reaction, it is convenient to use reversible inhibitors that are not substrates or products of the reaction. Different inhibition types with respect to reduced and oxidized substrates would suggest the ordered mechanism of transhydrogenation. The Tinopals AMS-GX and 5BM-GX (cationic benzoxazoles, fluorescent whitening agents, components of some photographic emulsions and laundry detergents), inhibitors of different NAD^+-dependent dehydrogenases [34-36], were used for this purpose in this investigation. The reasons why Tinopals were chosen as the study instruments were as follows. First, as it will be shown in this paper, they serve as effective inhibitors of initial steps of NADH oxidation by Complex I. Second, the use of traditional Complex I inhibitors of the initial steps of NADH oxidation (ADP-ribose and rhein) did not reveal whether NADH → $APAD^+$ reaction 1 is ordered or not [33].

The data presented in this paper show nucleotide binding order during transhydrogenase reaction 1: 1) the enzyme binds nucleotides with an ordered mechanism; 2) NADH is the first substrate interacting with the enzyme. The results suggest functional difference of the two nucleotide-binding centers of the enzyme: one of them is specific for reduced forms of pyridine nucleotides, and another one for oxidized forms. These data coincide with the hypothesis suggesting different mechanisms of direct and reverse reactions catalyzed by Complex I [1, 28, 31].

MATERIALS AND METHODS

SMP [24], Complex I [37], and FP [25] were prepared by the published procedures.

The transhydrogenase reaction was followed as a change in optical absorption at 375 nm ($E^{375}_{mM} = 5.1$) due to the simultaneous NADH oxidation with $APAD^+$ reduction [38], under the same conditions that were used for the reaction kinetics study [33].

NADH, deamino NADH, and APADH oxidase activities of SMP were followed as decreases in optical absorption at 340 nm (NADH and deamino NADH oxidation, $E^{340}_{mM} = 6.22$) and at 363 nm (APADH oxidation, $E^{363}_{mM} = 9.1$) [39]. SMP were added to the concentration of 20-50 μg/ml into the cuvette with medium (26°C) containing 0.25 M sucrose, 20 mM Tris-HCl, pH 8.0, 0.2 mM EDTA, and BSA (1 mg/ml, BSA was excluded from essay medium for the study of the Tinopals' effects). Complex I was activated by the oxidation of 5 μM NADH (deamino NADH or APADH). The corresponding reduced nucleotides with known concentrations were added after that, and the initial reaction rates were then registered.

The reverse electron transfer was followed as an increase in optical absorption at 340 nm (NAD^+ and deamino NAD^+ reduction, $E^{340}_{mM} = 6.22$) and at 363 nm ($APAD^+$ reduction, $E^{363}_{mM} = 9.1$) [24, 39] in the same medium (26°C). SMP (2 mg/ml) were preincubated in assay medium for 1 h at 26°C with oligomycin (0.6 nmol per mg protein) and malonate (2 mM). Then 10-20 μg/ml were supplemented with the assay medium in cuvettes, and Complex I was activated by oxidation of 5 μM NADH (deamino NADH or APADH). The reactions were started with addition of corresponding oxidized nucleotides and 10 mM succinate.

For observation of SMP NADH:ferricyanide reductase activity, 0.5 mM K^+-ferricyanide and 4-40 μM NADH were added in the medium. The reaction was started by SMP (2-5 μg/ml) addition and was followed as decrease in optical absorption at 420 nm (due to one-electron reduction of ferricyanide, $E^{420}_{mM} = 1$) [15].

NAD^+, deamino NADH, and APADH concentrations were determined by published methods [33, 39].

NADH, NAD^+, $APAD^+$, deamino NADH, deamino NAD^+, Hepes, EDTA, BSA, succinate, rotenone, oligomycin, and DMSO were from Sigma (USA); alcohol dehydrogenase was from Sigma and Reanal (Hungary), and Tris (base) was from Merck (Germany). Tinopals AMS-GX and 5BM-GX (Ciba-Geigy Corporation, USA) were received from Dr. W. M. Anderson as a gift (Indiana University School of Medicine, USA). The other reagents were produced in Russia (or USSR).

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

RESULTS

Effects of Tinopals (AMS-GX and 5BM-GX) on reactions catalyzed by NADH:ubiquinone oxidoreductase. *NADH oxidation and reverse electron transfer.* Tinopals (the cationic benzoxazoles shown in Fig. 1) have a number of features complicating the use of these compounds as specific inhibitors of reactions with the participation

of NADH and APADH. First, the Tinopals absorption spectra are similar to those of pyridine nucleotides (absorption maximum at 348 nm, E^{340}_{mM} is about 50 and 33, for AMS-GX and 5BM-GX, respectively [35, 36]). Second, Tinopals decrease NADH and APADH absorption, evidently due to the formation of weak complexes of these compounds with the nucleotides (data not shown). This feature of Tinopals when used as inhibitors could affect the value of K_i determined, since its concentrations (and uncombined nucleotides concentrations) would be understated. However, AMS-GX and 5BM-GX at 20 μM (or lower) concentrations have practically no affect on the spectra of the nucleotides: the maximal NADH and APADH absorptions are 10-15% diminished, whereas the absorptions at 375 nm (observation of NADH → $APAD^+$ reaction) are at most 5-7% decreased (data not shown). Therefore, Tinopals AMS-GX and 5BM-GX, in concentrations less than 20 μM, can be used as inhibitors of reactions with involving NADH and APADH.

Figure 2 shows double reciprocal rate dependences on NADH concentrations obtained for NADH-oxidase and NADH:ferricyanide reductase reactions in SMP in the presence or absence of AMS-GX and 5BM-GX. The intercepts of the curves on the ordinate axis show competition of AMS-GX with NADH (Fig. 2, a and c). The value of K_i determined with the Dixon method was 0.9-1.4 μM. 5BM-GX inhibited both reactions with mixed, competitive with non-competitive, type (Fig. 2, b and d), with K_i of 4.4-5.2 μM.

AMS-GX and 5BM-GX have practically no effect on the electron transfer from succinate to NAD^+ (Fig. 3, a and b) and on succinate oxidation in coupled and uncoupled SMP (data not shown). AMS-GX at 20 μM concentration decreases the rate of the succinate-oxidase reaction at most by 15%, which clearly accounts for insignificant slowdown of reverse electron transfer in the presence of this compound (Fig. 3a). Thus Tinopals, as well as ADP-ribose [28], are unidirectional inhibitors for Complex I: they significantly decrease the rate of the direct reaction and do not affect the reverse reaction. The mechanism of substrate interaction with Complex I can be revealed from studies of the effects of AMS-GX and 5BM-GX on transhydrogenase reaction 1, which occurs with formation of a ternary complex and, hence, with the participation of two enzyme active centers.

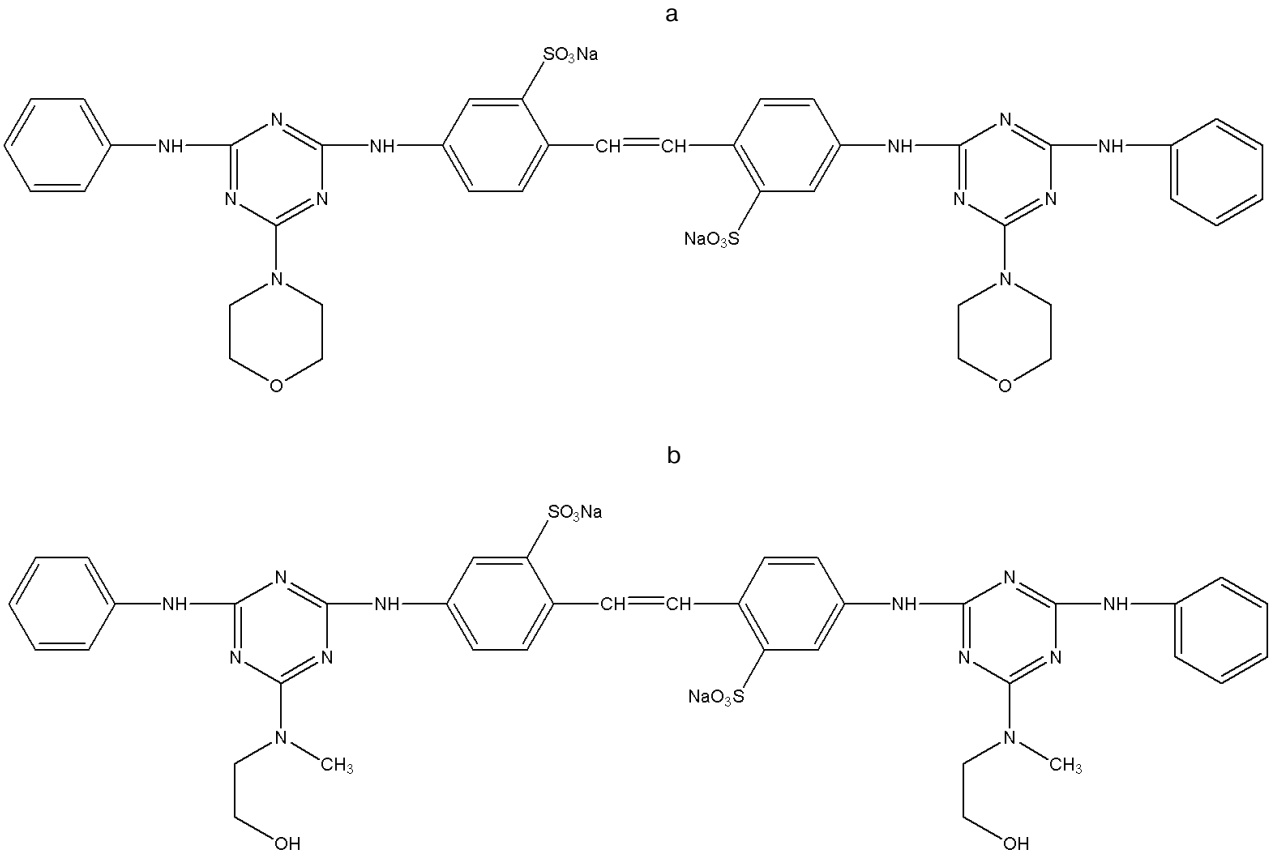

Fig. 1. Structures of cationic benzoxazoles (Tinopals) AMS-GX (a) and 5BM-GX (b).

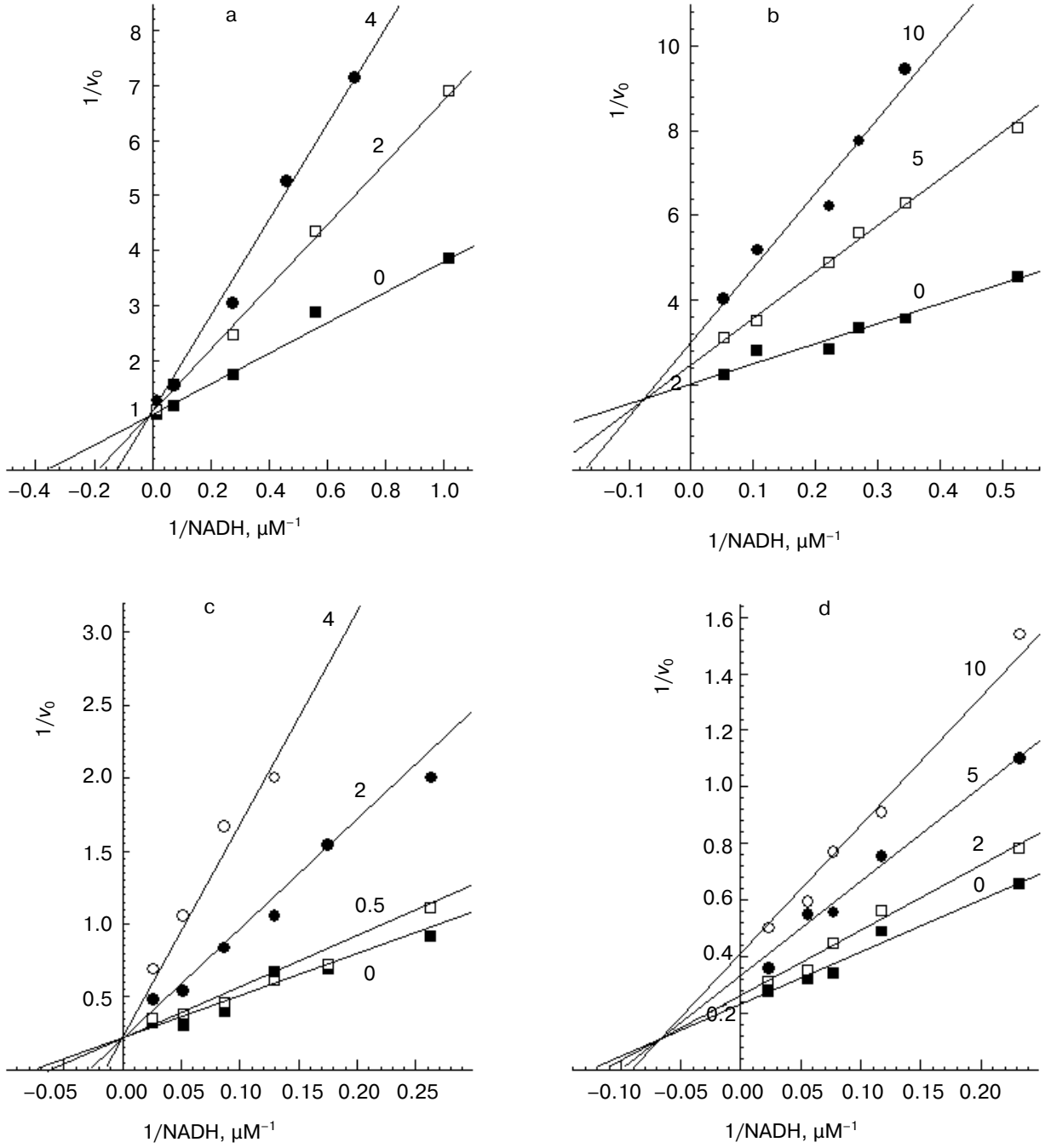

Fig. 2. Effects of Tinopals on NADH-oxidase (a, b) and NADH:ferricyanide reductase (c, d) reactions in SMP (double reciprocal plots, v_0 on ordinate axis is expressed in μmol/min per mg protein): a, c) effect of AMS-GX; b, d) effect of 5BM-GX. The inhibitor concentrations (μM) are indicated by the figures on the lines. K_i was determined by the Dixon method.

NADH → APAD$^+$ reaction 1. As mentioned above, Complex I catalyses two transhydrogenase reactions denoted as reaction 1 and reaction 2 [33]. There are two nucleotide-binding centers participating in the first reaction. It can be analyzed separately from the second reaction because of significantly higher affinities to the nucleotide substrates [32, 33]. The double reciprocal plots shown in Fig. 4 demonstrate the effects of Tinopals on NADH → APAD$^+$ reaction 1 catalyzed by Complex I in SMP. The parallel lines obtained (Fig. 4b) imply uncompetitive effect of AMS-GX with respect to APAD$^+$. Also, it is evident that inhibition type changes when the oxidized nucleotide concentration increases: the effect of AMS-GX on reaction 2 is different from its effect on

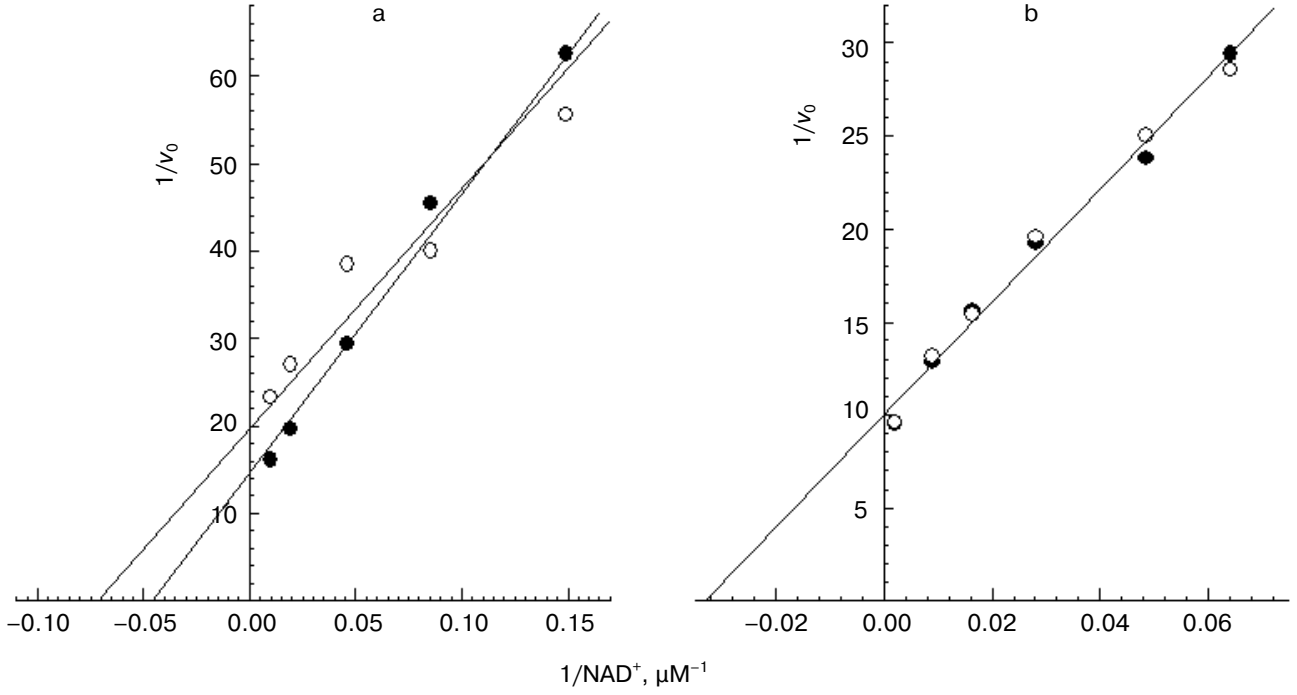

Fig. 3. Effects of Tinopals on energy-dependent NAD^+ reduction by succinate in SMP (double reciprocal plots, v_0 on ordinate axis is expressed in μmol/min per mg protein): solid circles are the reaction in the absence of inhibitors, open circles are the reaction in the presence of 10 μM AMS-GX (a) and 10 μM 5BM-GX (b).

reaction 1. The type of inhibition with respect to NADH in reaction 1 is non-competitive (Fig. 4a). The $K_i^{AMS\text{-}GX}$ values were 5.8 and 5 μM, with respect to NADH and $APAD^+$, respectively.

5BM-GX affects the reaction uncompetitively with respect to NADH (Fig. 4c), whereas a simple competition with respect to $APAD^+$ was obtained (Fig. 4d). The values of $K_i^{5BM\text{-}GX}$ were 13 and 16 μM, respectively.

The results suggest that NADH → $APAD^+$ reaction 1, catalyzed by NADH:ubiquinone oxidoreductase, occurs with ordered binding of substrates: NADH is the first substrate interacting with the enzyme during the reaction.

Oxidation and reduction of nucleotides in direct and reverse electron transfer reactions catalyzed by Complex I. One of two possible nucleotide-binding centers participating in NADH → $APAD^+$ reaction 1, occurring with formation of the ternary complex, is obviously located on the 51 kD subunit [2]. NADH probably interacts with just this center. We suppose that $APAD^+$, in the transhydrogenase reaction, and NAD^+, in reverse electron transfer, interact with the enzyme at the same center. The suggestion comes from the similar $K_m^{APAD^+}$ and $K_m^{NAD^+}$ values found for these reactions (9 and 7.2 μM, respectively) and competition of NADH with oxidized nucleotides in both cases with similar K_i (50 and 40 μM, respectively) [31, 33]. In the case when the oxidized nucleotides interact with the enzyme at the same place in these reactions (NADH → $APAD^+$-transhydrogenase and reverse transfer), the kinetic parameters for $APAD^+$ and NAD^+ reduction in reverse electron transfer must coincide. K_m and V_m values obtained for reverse electron transfer reactions with NAD^+ and its analogs are given in table. The $K_m^{APAD^+}$ value is close to that obtained for NAD^+, and V_m values, obtained with the two nucleotides as substrates, are congruous. $K_m^{APAD^+}$ in reverse electron transfer reaction coincides with $K_m^{APAD^+}$ in transhydrogenase reaction 1 (11 μM (table) and 9 μM [33], respectively).

A similar experiment was performed with the reduced nucleotide analogs in direct reaction. K_m^{APADH} values for oxidase and transhydrogenase (1) reactions were found to be similar (125 μM (table) and 50 μM [33], respectively) and were more than one order of magnitude higher than K_m^{NADH} values in both reactions (2.7 μM (table) and 0.7 μM [33], respectively).

Effect of NAD^+ on NADH → $APAD^+$ transhydrogenase reaction 1. NAD^+ in millimolar concentrations competes with NADH in all of the reactions catalyzed by Complex I [40]. Previously we found that $APAD^+$ in millimolar concentrations competed with NADH in transhydrogenase reaction 1 as well [32, 33]. At the same time, being a substrate of the transhydrogenase reaction, $APAD^+$ had one order of magnitudes higher affinity than as inhibitor of NADH oxidation [33]. Since the results given above suggest the participation of a common center in reverse electron transfer and transhydrogenase

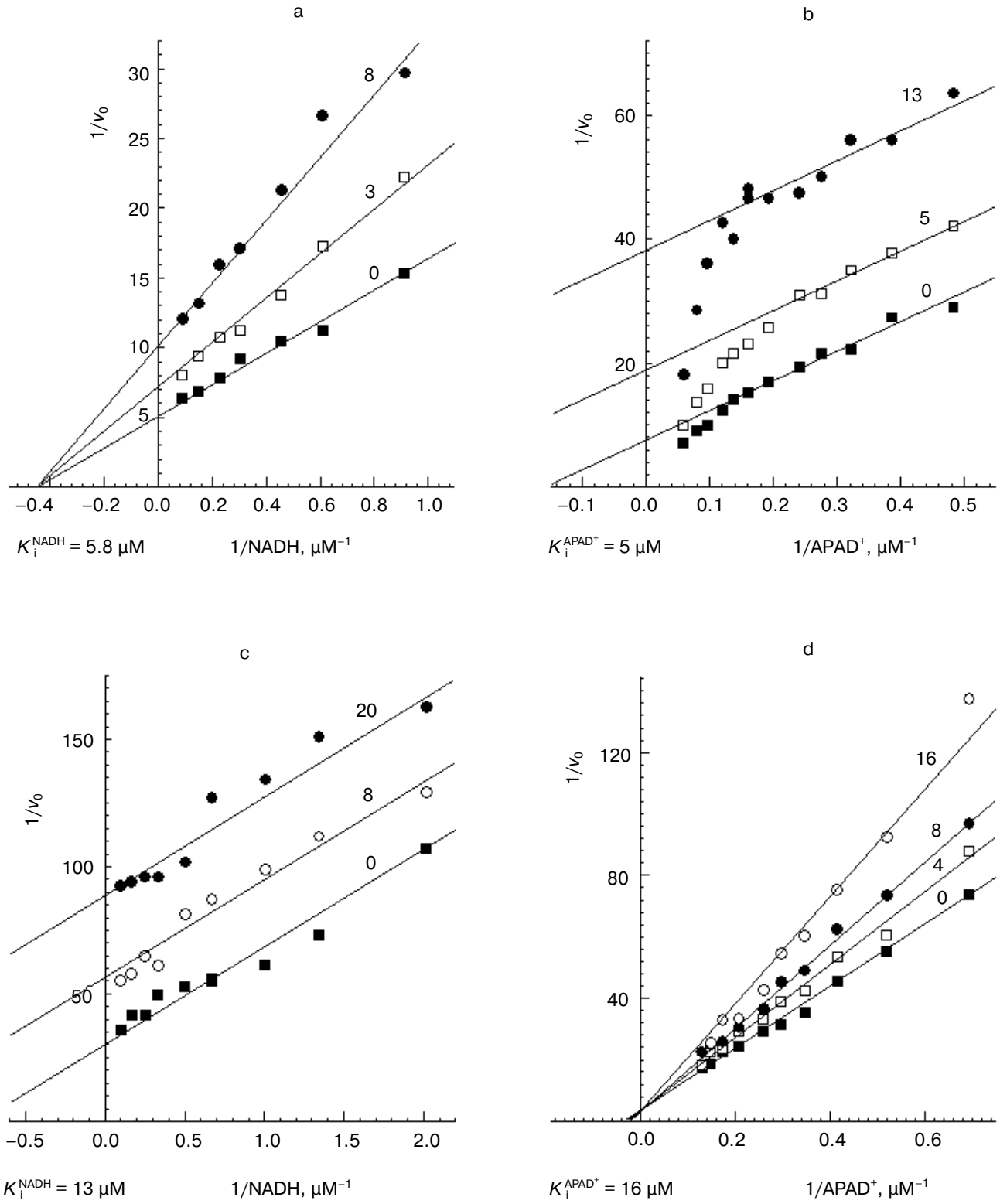

Fig. 4. Effects of Tinopals AMS-GX (a, b) and 5BM-GX (c, d) on NADH → $APAD^+$ transhydrogenase reaction 1 in SMP (double reciprocal plots, v_0 on ordinate axis is expressed in μmol/min per mg protein): a, c) with respect to NADH ($APAD^+$, 18 and 7 μM, respectively); b, d) with respect to $APAD^+$ (NADH, 8 μM). The inhibitors concentrations (μM) are indicated by the figures. K_i values were determined by the Dixon method (for (a) and (d)) or calculated with the equation for uncompetitive inhibition (for (b) and (c)).

Parameters of nucleotide oxidation and reduction in direct and reverse electron transfer reactions catalyzed by Complex I in SMP (pH 8.0, 26°C)

Substrate-nucleotide	Reaction			
	direct electron transfer*		reverse electron transfer**	
	K_m, μM	V_m, μmol/min per mg protein	K_m, μM	V_m, μmol/min per mg protein
NAD(H)	2.7	1.15	7	0.14
APAD(H)	125	0.55	11	0.14
Deamino-NAD(H)	0.4	0.80	33	0.14

* Oxidase reaction catalyzed by uncoupled SMP.
** Energy-dependent reduction of NAD^+ by succinate. The range of presented values obtained in several experiments was at most 10%.

reaction 1, which binds NAD^+ and $APAD^+$ with similar affinities (table), it can be supposed that NAD^+ must compete with $APAD^+$ in NADH → $APAD^+$ reaction 1 with K_i magnitude about 10 μM (commensurable with $K_m^{APAD^+}$ in NADH → $APAD^+$ reaction 1). NAD^+ in such concentrations could not affect the apparent affinity for NADH.

The data represented on Fig. 5b indicate that NAD^+ competes with $APAD^+$ in NADH → $APAD^+$ reaction 1 catalyzed by Complex I with K_i value about 18 μM. The inhibition with respect to NADH (Fig. 5a) proved to be mixed, close to non-competitive type (K_i 40 μM). Similar NAD^+ effects were observed for transhydrogenase reactions catalyzed by isolated Complex I and FP (Fig. 5, c and d). $K_i^{NAD^+}$ values for the three preparations, determined by the Dixon method, were very similar (40-70 and 8-23 μM with respect to NADH and $APAD^+$, respectively).

DISCUSSION

On studying effects of the cationic benzoxazoles Tinopals AMS-GX and 5BM-GX we revealed the ordered interaction of the nucleotide substrates with mitochondrial NADH:ubiquinone oxidoreductase in NADH → $APAD^+$ transhydrogenase reaction 1. Both Tinopals used, AMS-GX and 5BM-GX, were uncompetitive inhibitors of this reaction with respect to one of the substrates (to $APAD^+$ and NADH, respectively, Fig. 4), which is possible only for the ordered interaction of enzyme with substrates. The kind of inhibition of NADH → $APAD^+$ transhydrogenase reaction 1 by these two compounds suggests that AMS-GX interacts only with the enzyme/$APAD^+$ complex, whereas 5BM-GX interacts only with the enzyme/NADH complex. The effects of the two Tinopals on NADH → $APAD^+$ reaction 1 can be described by the scheme represented in Fig. 6, which is consistent with the experimental data. According to the scheme, 5BM-GX interacts with the complex of the enzyme with NADH, in the center for $APAD^+$ binding, and thus competes with the second substrate. It follows that NADH is the first substrate of the reaction (otherwise 5BM-GX would not compete with $APAD^+$). AMS-GX obviously interacts with the ternary enzyme/substrates complex (not affecting NADH binding). Hence, the ternary complex forming during NADH → $APAD^+$ reaction 1 is sufficiently steady and capable of interacting with AMS-GX. The ordered mechanism of NADH → $APAD^+$ reaction 1 suggests that the oxidized nucleotide binding center becomes accessible only after the enzyme binds NADH. Thus the oxidized nucleotide-binding center can be formed as a result of conformational changes in Complex I under NADH binding [41].

It is of interest to clarify the mechanism of FMN participation in NADH → $APAD^+$ reaction 1. It is not clear from the data obtained when its reduction take place during the reaction. Besides, the reaction without FMN participation cannot be eliminated completely. Additional investigations are necessary for elucidation of these details.

The mentioned results suggest that two centers participating in transhydrogenase reaction 1 correspond to different centers taking part in direct and reverse reaction catalyzed by NADH:ubiquinone oxidoreductase. These centers have different specificity to nucleotides. The nicotinamide part of the reduced nucleotide molecule is obviously essential for binding in the center participating in the oxidase reaction: the enzyme binds NADH and deamino-NADH with high affinities, whereas it has two orders of magnitude lower affinity to APADH. However,

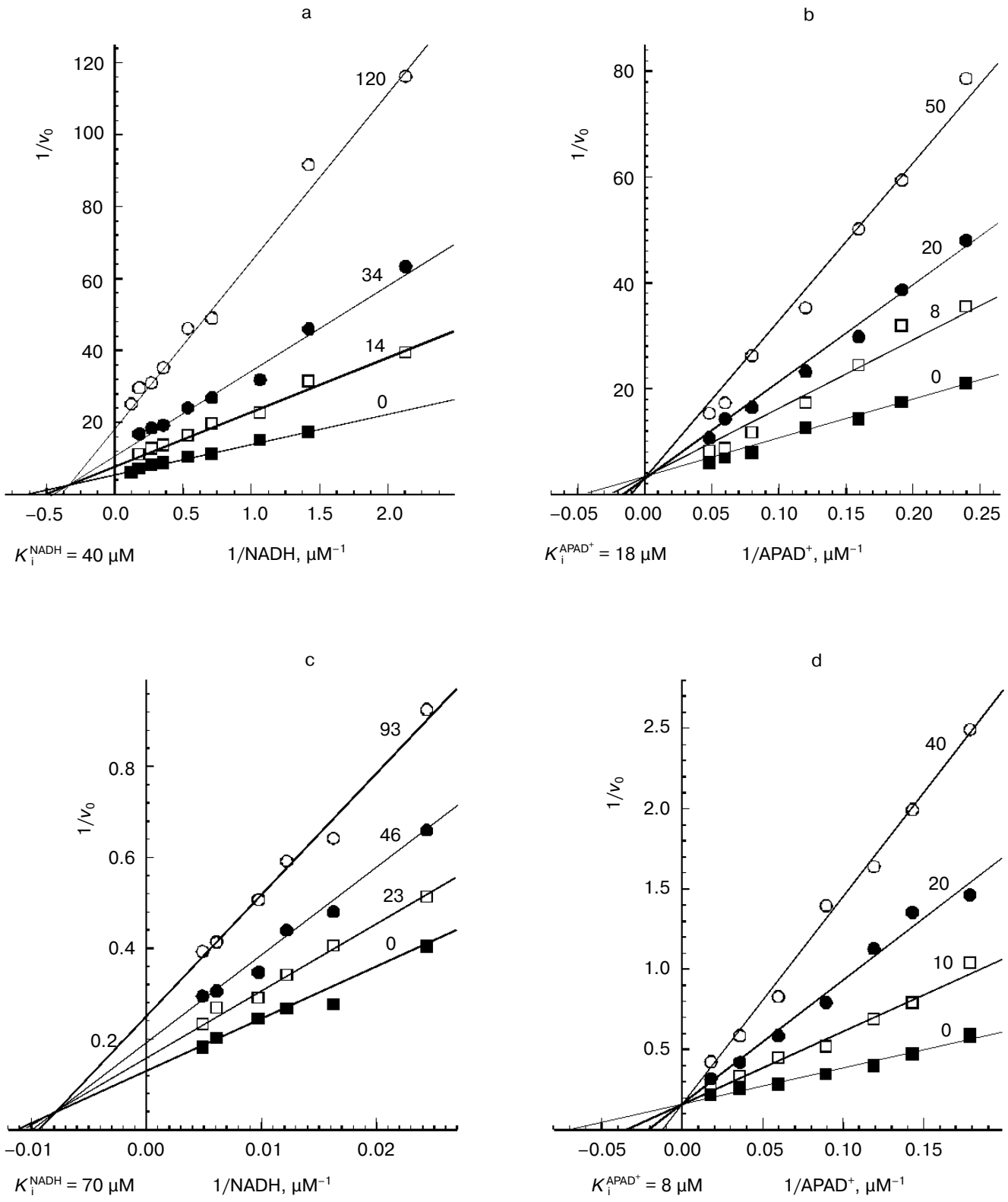

Fig. 5. Effect of NAD^+ on NADH → $APAD^+$ transhydrogenase reaction 1 catalyzed by SMP (a, b) and FP (c, d) (double reciprocal plots, v_0 on ordinate axis is expressed in μmol/min per mg protein): a, c) with respect to NADH ($APAD^+$, 20 and 80 μM, respectively); b, d) with respect to $APAD^+$ (NADH, 10 and 200 μM, respectively). NAD^+ concentrations (μM) are indicated by the figures. K_i values were determined by the Dixon method.

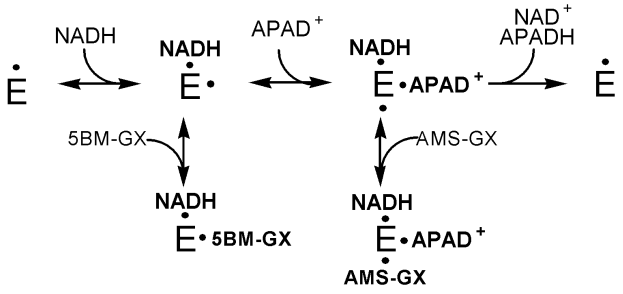

Fig. 6. Schematic illustration of nucleotide and Tinopal binding by Complex I. The active centers of the enzyme (E) are indicated with the circles. The explanations are given in the text.

the center participating in reverse electron transfer reaction is less specific to substrates and has similar affinities to NAD^+, $APAD^+$, and deamino-NAD^+. Both centers are evidently involved in transhydrogenase reaction 1 and located in FP. The data presented confirm the hypothesis about different pathways of electron transfer during NADH oxidation and NAD^+ reduction catalyzed by the enzyme [1, 28, 31, 42].

Besides the similar K_m values for oxidized nucleotides, transhydrogenase (1) and reverse electron transfer reactions (table) [33] have practically coincident rates (about 0.2 μmol/min per mg protein for Complex I in SMP). This fact can be considered to suggest resemblance of electron transfer paths in the two cases.

The competition of NAD^+ with $APAD^+$ in transhydrogenase reaction 1 is a consequence of a common nucleotide-binding center participating in this reaction and in the reverse electron transfer. NAD^+, being a member of the transhydrogenase reaction, could not be used as an inhibitory instrument for investigation of the reaction order. In the NADH → $APAD^+$ reaction it can be regarded as a product and/or alternative substrate. It is obvious that NAD^+, in millimolar concentrations, would compete with NADH (substrate inhibition by $APAD^+$ at higher than 1 mM concentrations was shown for this reaction [32, 33]). The results are in good agreement with Avraam and Kotlyar's data, who had shown that low concentrations of NAD^+ inhibit the oxidation of NADH by ferricyanide, 2,6-dichlorophenol-indophenol, and oxygen, catalyzed by FP, and NADH-oxidase reaction, catalyzed by isolated Complex I [30]. They suggest that such an effect of NAD^+ can be seen in reactions which occur with rates close to that of the transhydrogenase reaction 1 or slower.

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