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Kinetics of the mitochondrial three-subunit NADH dehydrogenase interaction with hexammineruthenium(III)

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Abstract

The steady-state kinetics of the NADH dehydrogenase activity of the three-subunit flavo-iron-sulfur protein (FP, Type II NADH dehydrogenase) in the presence of the one-electron acceptor hexammineruthenium(III) (HAR) were studied. The maximal catalytic activities of FP with HAR as electron acceptor calculated on the basis of FMN content were found to be approximately the same for the submitochondrial particles, Complex I and purified FP. This result shows that the protein structure responsible for the primary NADH oxidation by FP is not altered during the isolation procedure and the lower (compared with Complex I) catalytic capacity of the enzyme previously reported was due to the use of inefficient electron acceptors. Simple assay procedures for NADH dehydrogenase activity with HAR as the electron acceptor are described. The maximal activity at saturating concentrations of HAR was insensitive to added guanidine, whereas at fixed concentration of the electron acceptor, guanidine stimulated oxidation of low concentrations of NADH and inhibited the reaction at saturating NADH. The inhibitory effect of guanidine was competitive with HAR. The double-reciprocal plots $1/v$ vs. $1/$[NADH] at various HAR concentrations gave a series of straight lines intercepting on the ordinate. The plots $1/v$ vs. $1/$[HAR] at various NADH concentrations gave a series of straight lines intercepting in the fourth quadrant. The kinetics support the mechanism of the overall reaction where NADH is oxidized by the protein-$Ru(NH_3)_6^{3+}$ complex in which positively charged electron acceptor is bound at the specific site close to FMN, thus stabilizing the flavosemiquinone intermediate.

Keywords: NADH dehydrogenase; Assay condition; Flavoprotein; Electron transfer; Hexammineruthenium(III); Steady-state kinetics; (Bovine heart mitochondria)

1. Introduction

NADH-ubiquinone oxidoreductase (Complex I, EC 1.6.99.3) of bovine heart mitochondria is the most complex complex of the respiratory chain and is composed of 42 distinct polypeptides [1,2]. In intact mitochondria or submitochondrial particles, the enzyme catalyzes the rotenone-sensitive reduction of bulk ubiquinone by NADH, coupled with the vectorial translocation of 2–5 H^+ per 2 e^- transferred through coupling site I [2,3]. The only purified preparation retaining most of the catalytic properties of the intact membrane-bound enzyme is Complex I [4]. It catalyzes rotenone-sensitive NADH-Q reductase and a number of rotenone-insensitive NADH-artificial acceptor reductase activities (for selected aspects, such as preparation procedure [4], structure [5], iron-sulfur clusters [6,7], and molecular biology of the enzyme [8,9] see references indicated). Although several mechanistic proposals on the intramolecular electron transfer coupled with H^+-translocation have been published [10–13], the energy coupling mechanism at Site I remains a 'black box'.

Numerous attempts to resolve the structure–function relationship within the mammalian coupling Site I have

Abbreviations: FP, the soluble 3-subunit iron-sulfur flavoprotein fraction derived from Complex I; Menadione, 2-methyl-1,4-naphthoquinone; DCIP, 2,6-dichlorophenolindophenol; WB, Wurster's Blue (semiquindiimine radical of *N,N,N',N'*-tetramethyl-*p*-phenylenediamine); Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HAR, hexammineruthenium(III).

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resulted in the isolation of several type of soluble NADH dehydrogenases originating from Complex I [14,15]. Among these, the soluble three-subunit iron-sulfur flavoprotein (FP fraction) [16] appears to be the simplest catalytically competent fragment of Complex I. FP catalyzes the rotenone-insensitive oxidation of NADH by ferricyanide, menadione [16–18], DCIP, cytochrome *c* [18], WB [19,20], and oxygen [21,22]. It is also capable of FMN-mediated [4B-^{3}H]NADH–H_2O exchange [23] and NADH-acetylpyridine adenine dinucleotide transhydrogenation [17]. The enzyme bears the NADH binding site on the 51 kDa subunit [24] and two iron-sulfur clusters (one binuclear, $E_m = -465$ mV, and one tetranuclear, $E_m = -410$ mV) [25]. FMN is most probably bound to the 51 kDa subunit where iron-sulfur center 3 (Ohnishi's nomenclature [6]) is also apparently located [26,27]. Another binuclear iron-sulfur cluster is formed by the conserved cysteine residues in the 24 kDa subunit [28,29]. EPR spectral lineshape and midpoint redox potentials of the iron-sulfur clusters in FP significantly differ from the counterparts in intact Complex I, apparently due to modification caused by the chaotropic agents used for the enzyme solubilization [25].

The catalytic activity of FP determined on the basis of FMN content using ferricyanide or menadione as electron acceptors was found to be decreased after purification, suggesting some modification of protein and/or prosthetic group(s) which participate in the initial steps of NADH oxidation [17,18]. Moreover, the enzyme turnover number can be specifically 'improved' (V_{max} increase and K_m^{NADH} decrease) by guanidinium salts [17,30]. No clear explanation for this phenomenon exists, although an interesting mechanistic hypothesis on the stimulatory effect of guanidine has been offered [17].

Taken together, the data on the catalytic and structural properties of FP suggest that this relatively simple subfraction of Complex I may serve as a useful preparation for studies on the initial steps of NADH oxidation, particularly for elucidation of the role of FMN in the enzyme mechanism. The catalytic properties of any oxidoreductase are strongly dependent on the nature of the artificial electron acceptor used for the assay of diaphorase activity. Recently, interaction of one-electron acceptor hexammineruthenium(III) (HAR) with the mitochondrial NADH-ubiquinone reductase (submitochondrial particles and Complex I) have been studied, and the apparent affinities of NADH and NAD^+ to the enzyme active site have been determined by the steady-state kinetics approaches [31]. It seemed to be of interest to apply this acceptor for studies on simpler system, such as FP. In this paper we will show that HAR is the most efficient electron acceptor which reveals almost the same turnover numbers for particulate and resolved preparations of NADH dehydrogenase. A simple model for FP/HAR/NADH interaction, explaining the stimulatory effect of guanidine on the diaphorase activities of the enzyme, will be discussed in relation to the properties of FMN as the enzyme-bound primary electron acceptor.

2. Materials and Methods

FP [32] and Complex I [4] were prepared according to the published procedures and stored in liquid nitrogen. Protein content was determined by the Lowry et al. procedure [33]. Submitochondrial particles were prepared as described previously [34]. All enzymatic assays were carried out at 26° C in the standard mixture containing: 20 mM Hepes (pH 8.0) and 0.2 mM EDTA using a Hitachi 200 spectrophotometer. The reaction was followed by a decrease of absorption at 340 nm ($\varepsilon_{mM,340}^{NADH} = 6.22$). When high concentrations of NADH were used (> 0.2 mM) the reaction was followed at 380 nm ($\varepsilon_{mM,380}^{NADH} = 1.23$). When ferricyanide was used as electron acceptor the reaction was followed at 420 nm ($\varepsilon_{mM,420}^{ferri.} = 1.0$). The details of the assays are described in the legends to the figures and tables. The sources of the chemicals were as follows: NADH, EDTA, NAD^+, Hepes, were from Sigma (USA); menadione was from Calbiochem (USA); $Ru(NH_3)_6Cl_3$ was from Strem Chemicals (USA). Other chemicals were of the highest quality commercially available.

3. Results

3.1. General characteristics of NADH-HAR reductase activity of FP and the assay procedures

Fig. 1 shows the time course of NADH oxidation catalyzed by FP in the presence of 100 μM HAR. At high

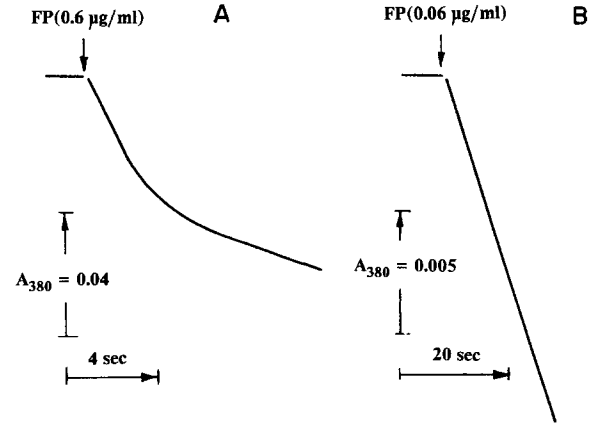


Fig. 1. Time-course of NADH oxidation in the presence of hexammineruthenium (HAR) and different amounts of the enzyme. The standard reaction mixture contained 1 mM NADH and 100 μM $Ru(NH_3)_6Cl_3$. See text for further explanations.

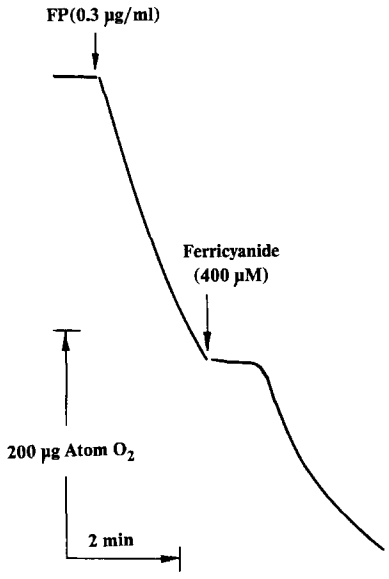


Fig. 2. NADH oxidase reaction catalyzed by FP in the presence of HAR. The standard reaction mixture contained 1 mM NADH and 100 μM HAR.

enzyme concentration and relatively low sensitivity of the instrument (curve A) the reaction was biphasic: the initial rapid phase was followed by the slower zero-order NADH oxidation. At low enzyme concentration and high sensitivity of the instrument the reaction proceeded at a constant rate for several minutes (curve B). Only the first rapid phase of curve A showed the same specific activity as was estimated from curve B. Simple calculations revealed that the inflection point in curve A corresponds to the reduction of approximately 100 μM HAR and the further slower rate was apparently due to autoxidation of the reduced HAR. This was directly shown by following the NADH oxidase activity of FP in the presence of HAR as demonstrated in Fig. 2. Addition of the enzyme caused almost immediate oxygen consumption (note the difference in time scale in Figs. 1 and 2) which proceeded with the slowly decreasing rate (decrease of oxygen concentration). The addition of ferricyanide instantly stopped oxygen consumption, presumably due to the efficient competition with oxygen for the reduced HAR and/or with oxidized HAR for the reduced enzyme. Indeed, the inhibition was spontaneously relieved after about 1 min, when added ferricyanide was reduced and the system returned to the initial conditions. Although FP was shown to react directly with oxygen [21,22], no measurable NADH oxidase was observed in the absence of HAR under the experimental conditions employed in Figs. 1 and 2. It was shown in the separate spectrophotometric assay that the specific NADH oxidase activity of FP in the absence of HAR was less then 1% of that observed in the presence of 1 mM electron acceptor. The results described in Figs. 1 and 2 showed that HAR is an efficient electron acceptor which can be used for both spectrophotometric and polarographic (with an oxygen electrode) assays of NADH dehydrogenase activity. In the latter case some precautions should be taken because the autoxidation rate for the reduced HAR is not very high and may become rate-limiting.

Table 1
Effect of guanidine on the catalytic activities of NADH dehydrogenase (26° C, Hepes buffer, pH 8.0)

Electron acceptor (100 μM)	Activity [a] (μmol NADH oxidized/min per mg protein)	
	− guanidine	+ guanidine
Ferricyanide	46	117
Menadione	230	284
Hexammineruthenium	1300	736

[a] Initial rates of 1 mM NADH oxidation at 380 nm (menadione and hexammineruthenium as electron acceptors) or ferricyanide reduction at 420 nm were measured. 50 mM guanidine chloride was added to the standard assay mixture where indicated.

The comparative reactivities of some artificial electron acceptors in the NADH dehydrogenase reaction catalyzed by FP and the effect of guanidine on the diaphorase activities are depicted in Table 1. At equal concentrations of the electron acceptors (100 μM), the relative rates of NADH oxidation in the absence of guanidine were: 1.0; 0.15 and 0.03 for HAR, menadione and ferricyanide, respectively. It worth noting that the relative activities given in Table 1 do not represent the maximal catalytic capacity of FP revealed with different electron acceptors, because the apparent K_m values for the latter are quite different.

Table 2
Effect of guanidine on the NADH-ferricyanide reductase activity of FP at different levels of the substrate and electron acceptor (26° C, Hepes buffer, pH 8.0)

NADH (μM)	Ferricyanide (mM)	Activity [a] (μmol NADH oxidized/min per mg protein)		Stimulation by guanidine: (B)/(A)
		− guanidine (A)	+ guanidine (B)	
50	0.1	9.5	130	14
50	1.0	34	250	7
190	0.1	30	175	6
190	1.0	82	370	4

[a] Initial rates of NADH oxidation were measured. 50 mM guanidine chloride was added to the assay mixture where indicated.

Remarkably, guanidine, which is known to activate all previously studied diaphorase activities of FP [17] significantly inhibited the NADH-HAR reductase in contrast to a more than 2.5-times increase of NADH-ferricyanide and slight activation of NADH-menadione reductases. A much less pronounced activating effect of guanidine on menadione reductase as compared to that on ferricyanide reductase is in accord with the observation of Hatefi and Galante [17]. They have reported that with ferricyanide as electron acceptor guanidine significantly decreased K_m^{NADH} and increased V_{max} whereas with menadione as acceptor the decrease in K_m^{NADH} was much more pronounced than the increase in V_{max}. The high, almost saturating amount of NADH (1 mM) was present in the assay system described in Table 1. With various concentrations of NADH and ferricyanide the stimulatory effect of guanidine was dependent on both the substrate and electron acceptor concentrations (Table 2). Further analysis of guanidine effect on NADH-HAR reductase activity will be presented below.

We were unable to reveal measurable activity of FP with HAR as the electron acceptor with NADPH or α-NADH at pH 8.0. The NADH-HAR reductase was only slightly pH-dependent within the interval of 6.5–9.0 and the activity was only 1.2-times higher at 37° C as compared with that routinely measured at 26° C. The NADPH-HAR reductase activity of FP in the presence of high concentrations of the nucleotide and electron acceptor (1 mM each) was substantially stimulated by lowering pH. At pH 6.3 it was about 1% of that observed with 1 mM NADH and 1 mM HAR. Guanidine had negligible effect on the NADPH-HAR reductase activity observed at pH 6.3. No further studies on the NADPH-HAR reductase activity of FP were undertaken. The enzyme showed perfect stability in NADH-HAR reductase assay when stored aerobically on ice for 3–5 h.

3.2. Catalytic turnover of FP within intact Complex I and in the isolated soluble form

It has been shown that compared to Complex I the soluble FP has considerably lower NADH dehydrogenase activity per mole of FMN and higher K_m for NADH [17]. Since HAR is able to accept electrons from particulate preparations of Complex I [31] as well as from soluble FP (this paper), the comparison of FMN turnover numbers in different types of preparation seemed worthwhile. Table 3 summarizes the catalytic properties of NADH dehydrogenase in the particulate and soluble forms as revealed by extrapolation to infinite HAR concentration. The calculated turnover numbers for submitochondrial particles, Complex I and FP are very close, if not identical, taking into consideration that the precise molar content of active Complex I in submitochondrial particles is hard to determine. It might be expected that a fraction of 'right-side-out' submitochondrial particles will contribute to the FMN content but not to the activity because the permeability barrier for the substrates. Indeed, two morphologically different membraneous populations have been separated after sucrose gradient centrifugation of EDTA-submitochondrial particles [37,38]. However, the absence of latent and/or atractylate-sensitive ATPase activity in both fractions [38] make the presence of significant fraction of 'right-side-out' sealed vesicles in the preparations of EDTA- or similar AS-submitochondrial particles quite unlikely.

3.3. The steady-state kinetics of NADH-HAR reductase

Fig. 3 shows the effect of varying concentrations of electron acceptor at different levels of NADH on the initial rates of NADH-HAR reductase. For a wide range of electron acceptor concentrations (20 μM–1 mM) the double-reciprocal plot deviated from linearity indicating either a mixed-type kinetic mechanism of the overall reaction or heterogeneity of the enzyme preparation (Fig. 3A). Since the nonlinearity was dependent on NADH concentration, the latter possibility seems unlikely. At relatively high concentrations of the electron acceptor the double-reciprocal plots were linear (Fig. 3B) at various NADH concentrations and intercepted at the fourth quadrant at -2 mM^{-1}.

Table 3
Catalytic activity of the soluble FP and particulate preparations of NADH dehydrogenase (26° C, pH 8.0)

Preparation	V_{max} [a] (μmol NADH/min per mg protein)	FMN content [b] (nmol/mg protein)		Turnover number (min^{-1})
Submitochondrial particles (SMP)	18	0.12	[35,36]	$1.5 \cdot 10^5$
Complex I	100	1.2	[4]	$0.8 \cdot 10^5$
FP	1300	12	[16,32]	$1.1 \cdot 10^5$

[a] The initial rates of 1 mM NADH oxidation were measured in the standard assay mixture at several concentrations of hexammineruthenium (HAR) and the activities at infinite electron acceptor concentration were estimated from $1/v$ vs. $1/$[HAR] plots by the extrapolation. The concentration ranges of HAR were 25 μM–1 mM, 0.2–4 mM and 0.5–3 mM for FP, Complex I and SMP respectively. The protein content in the assay mixture was 3 μg/ml, 1 μg/ml and 0.03 μg/ml for SMP, Complex I and FP, respectively. The stock suspensions of SMP and Complex I (1 mg/ml) was prepared by the dilution of particles into the mixture containing: 0.25 M sucrose, 20 mM Hepes (pH 8.0), 0.2 mM EDTA and 10 μM rotenone.
[b] FMN content in SMP, Complex I and FP was taken from the references indicated.

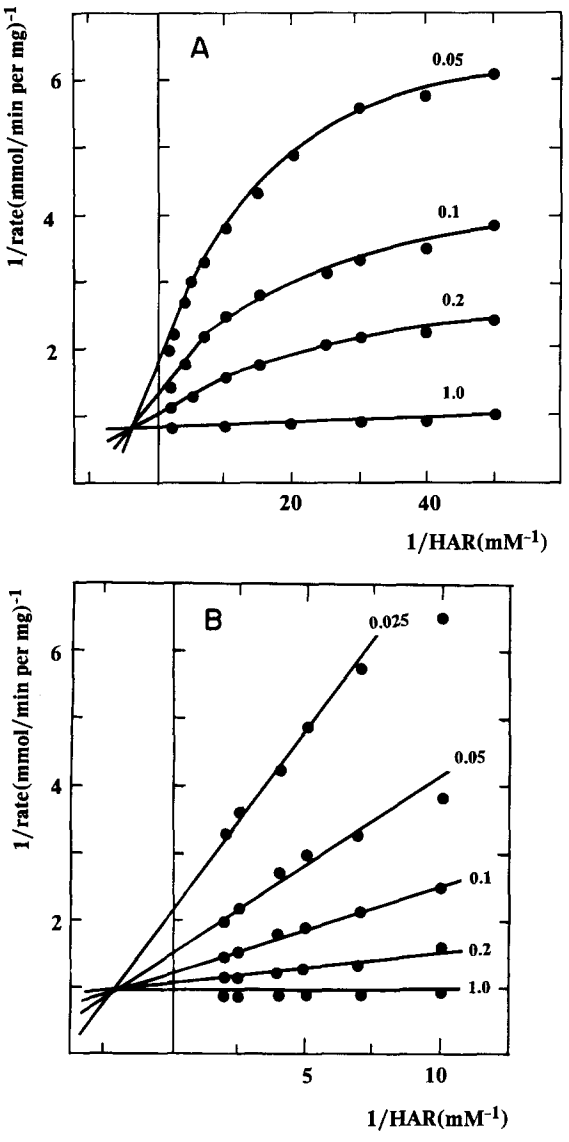


Fig. 3. The double-reciprocal plots for the NADH-HAR oxidoreductase reaction in the presence of different NADH concentrations. The assay conditions were as in Fig. 1. Figures on the curves correspond to the concentrations (mM) of NADH. (A) 20 μM–1 mM range of HAR was employed; (B) 0.2–1 mM range of HAR was employed.

The double-reciprocal plots for the substrate (NADH) at different electron acceptor concentrations are shown in Fig. 4. Somewhat unexpected behavior was observed: extrapolated V_{max} values were the same at any concentration of the electron acceptor, and only the apparent K_m^{NADH} values were increased when concentrations of HAR were lowered. Such a behavior for the bisubstrate reaction is diagnostic for the compulsory ordered mechanism with a formation of ternary complex, where the electron acceptor (HAR) binds to the enzyme first and the second substrate (NADH) binds to the enzyme-acceptor complex, thus shifting the overall equilibrium of the consecutive steps and providing the same level of ternary active complex at any electron acceptor concentration (this is correct if [HAR] ≫

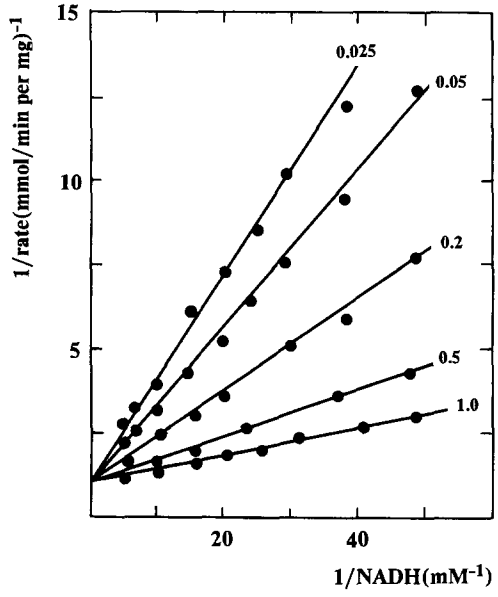


Fig. 4. The double-reciprocal plots for the NADH-HAR oxidoreductase in the presence of different HAR concentrations. Figures on the lines show the concentrations of HAR (mM).

$[E]_{total}$, which is the case for all the reported experiments) [39]. For such a mechanism the dissociation constant of 0.5 mM for the enzyme–HAR interaction was estimated from Fig. 3B. In contrast to the data reported for the NADH-ferricyanide reductase activity of FP [18], we were unable to find any deviation from the simple hyperbolic dependence of HAR-reductase on NADH concentration (Fig. 4). This suggests that a single catalytically competent nucleotide specific binding site is present in FP.

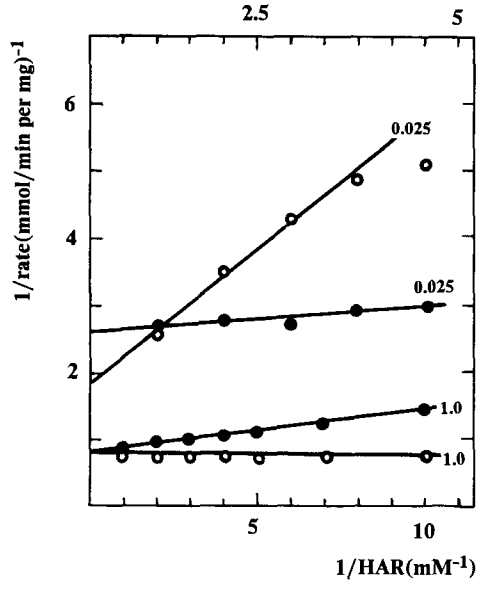


Fig. 5. Effect of guanidine on the kinetics of NADH-HAR oxidoreductase. Figures on the lines show concentrations of NADH (mM). 50 mM guanidine chloride was added to the assay mixture (●). The upper abscissa scale should be read for the upper pair of lines (25 μM NADH).

Table 4
Effect of potassium phosphate on the catalytic activity of NADH dehydrogenase with hexammineruthenium and ferricyanide as electron acceptors (26° C, pH 8.0)

Electron acceptor (100 μM)	Activity [a] (μmol NADH oxidized/min per mg protein)		Stimulation (+) or inhibition (−) (%)
	− potassium phosphate	+ potassium phosphate (0.3 M)	
Ferricyanide	110	160	+40
HAR	1100	550	−50

[a] The assay conditions are described in Table 1 and the Materials and methods section.

3.4. Effect of guanidine and potassium phosphate on the steady-state kinetics of NADH-HAR reductase

We have shown that, in contrast to ferricyanide and menadione reductase, the NADH-HAR reductase activity was inhibited, not stimulated, in the presence of guanidine chloride (Table 1). Since both guanidinium and HAR ions are positively charged and the specific binding of HAR to the enzyme with relatively high affinity was evident from the kinetic data, it seemed conceivable that guanidinium and electron acceptor cations bind to the same site. Indeed, the double-reciprocal plots $1/v$ vs. $1/[HAR]$ at high saturating NADH concentration (1 mM) showed the simple competitive (with HAR) inhibition of the reaction by guanidine (Fig. 5, lower pair of lines). More complex behavior was seen at low (25 μM) concentrations of NADH (Fig. 5, the upper pair of lines). Guanidine activated the reaction at low acceptor concentrations and had no effect at high concentrations of HAR.

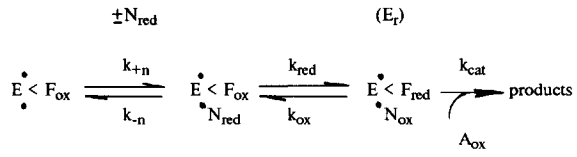
The kinetically evident competition between guanidine and HAR at saturating concentrations of NADH suggests that some negatively charged site on the protein may be involved in their binding. Since the stimulatory or inhibitory effects of guanidine (Refs. [17,30] and Table 1) are seen at rather high concentration of the latter it seemed very difficult if not impossible to obtain the meaningful data on the effect of ionic strength on the enzymatic activity with HAR and its inhibition by guanidine. However, some useful information might be obtained from the studies of the effect of ionic strength on the ferricyanide and HAR reductase activities at nonsaturating concentrations of the electron acceptors. The results presented in Table 4 are expected if free energy of electrostatic interaction between negatively charged 'site' on FP and positively (HAR) or negatively (ferricyanide) charged electron acceptors contributes significantly in the overall reaction rates.

4. Discussion

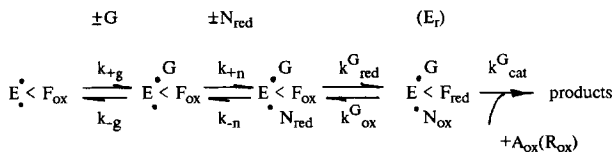
The isolated three-subunit flavo-iron-sulfur protein derived from Complex I shows almost the same catalytic activity in NADH oxidation as seen in submitochondrial particles and Complex I when the artificial one-electron acceptor HAR is used (Table 3). This is in contrast to the data previously reported for other artificial oxidants such as ferricyanide, menadione, DCIP and cytochrome *c* [17,18]. New electron acceptor (HAR) seems to be preferential over those traditionally used for the catalytic assay of NADH dehydrogenase. It neither spectrally interferes with NADH (see [40] for spectral properties of HAR) nor significantly reacts nonenzymatically with the substrate up to 2 mM concentration of each NADH and HAR, whereas significant non-enzymatic reaction between 0.1 mM NADH and 1 mM ferricyanide is observed at pH 7–8. The acceptor may be conveniently used for both direct spectrophotometric assay and acceptor-mediated oxidase assay with an oxygen-sensitive electrode (Figs. 1 and 2). The kinetic mechanism of the NADH-HAR reductase reaction catalyzed by FP significantly differs from that catalyzed by particulate preparations of Complex I [31]. This is expected because FP contains less redox components which may participate in reoxidation of the reduced enzyme by an artificial electron acceptor. Due to the very low E_m values for the iron-sulfur clusters [25], the most conceivable electron donor for HAR in FP is FMN. Thus, FP-catalyzed NADH-HAR reductase is expected to reveal the potential efficiency of the primary events during NADH oxidation. Our data show that the protein structure responsible for the primary NADH oxidation by FP is not altered as compared with intact Complex I. On the other hand, almost identical FMN turnovers found for HAR reduction in submitochondrial particles, Complex I and FP (Table 3) strongly suggest that the primary NADH oxidation is the rate-limiting step in the overall electron transfer reaction catalyzed by uncoupled NADH-ubiquinone reductase.

The kinetics of FP-HAR interaction shed some light on the properties of the primary electron acceptor in FP and provided a mechanistic explanation for activating effect of guanidine on the diaphorase activities that was discovered by Hatefi's group [17,30].

Consider the minimal model of NADH oxidation in the presence of FP and any electron acceptor, for example ferricyanide [18] (Scheme 1), where $E:<F_{ox}$ stands for FP bearing oxidized or reduced FMN (F_{ox} and F_{red}, respectively) and the specific binding sites (•) for NADH (N_{red}) and positively charged guanidinium (G) or HAR (R) cations. Since NADH is strictly two-electron donor and ferricyanide and HAR are strictly one-electron acceptors



Scheme 1.



Scheme 2.

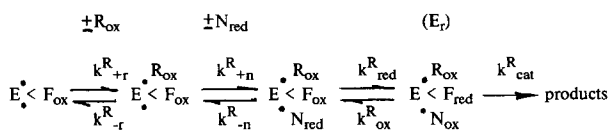
the intermediate flavosemiquinone must participate in the overall reaction. Thus, depending on the particulate reaction mechanism, the final step of Scheme 1 includes reduction of an electron acceptor either by the fully reduced flavin or by the flavosemiquinone or by both. For the sake of simplicity we define k_{cat} as a single rate constant, although it is obvious that depending on the particular mechanism of the reduced enzyme (E_r) oxidation it consists of various combinations of several rate constants and electron acceptor (A_{ox}) concentration. The lack of a deviation from the simple hyperbolic dependence of the reaction rate on HAR concentration (Fig. 3B) suggests that only one kinetically significant mechanism is operating under the steady-state conditions in the presence of 0.2–2 mM HAR. It has been shown that both ordered and ping-pong bi-bi mechanisms are simultaneously operating when NADH is oxidized by ferricyanide in the presence of FP [18].

The apparent K_m^{NADH} value is not a measure of the enzyme-substrate complex dissociation constant ($K_S^N = k_{-n}/k_{+n} \times [N_{red}]$), but under the steady-state conditions it is a combination of at least two equilibrium (K_S^N and $K_{red/ox} = k_{ox}/k_{red}$) and several kinetic constants [18,31,41,42]. When the ping-pong bi-bi mechanism is operating (oxidation of the reduced enzyme after NAD^+ dissociation) the apparent K_m^{NADH} is simply proportional to the electron acceptor concentration when the latter approaches to 0. When the ordered mechanism is operating, K_m^{NADH} approaches $K_{S(app.)} = K_S^N \times K_{red/ox}$ at an electron acceptor concentration extrapolated to zero ($k_{cat} \rightarrow 0$) [31]. It worth noting that any effector which shifts the equilibrium determined by $K_{red/ox}$ towards the acceptor reactive species (E_r) would result in an *increase* of V_{max} (the steady-state rate at saturating NADH) concominantly with a *decrease* of K_m^{NADH} (apparent increase in NADH affinity to the specific binding site). On the other hand, an increase of k_{cat} by any effector would result in an *increase* of V_{max} concominantly with an *increase* of K_m^{NADH}.

The most conceivable mechanistic explanation for the stimulating effect of guanidine on NADH-ferricyanide reductase activity of FP [17,30] is that binding of the guanidinium cation decreases $K_{red/ox}$, thus resulting in an increase of V_{max} and a decrease of K_m^{NADH}. The proposed reaction mechanism in the presence of guanidine is depicted below (Scheme 2), where G binds at the enzyme specific site in equilibrium reaction determined by $K_G = k_{-g}/k_{+g} \times [G]$. In the presence of guanidine $K^G_{red/ox} = k^G_{ox}/k^G_{red}$ is decreased as compared to the corresponding value of $K_{red/ox}$ in Scheme 1. Such an implicit decrease of $K_{red/ox}$ was recently observed as the stabilization of flavosemiquinone during potentiometric titration of FMN in Complex I [44]. No data are yet available on the thermodynamic properties of FMN in FP. The midpoint redox potentials of consecutive one-electron steps and thermodynamic stabilization of flavosemiquinone in FP may significantly differ from those found for Complex I. The thermodynamic analysis of flavin in FP is currently under way in our laboratories. Scheme 2 explains why the stimulatory effect of guanidine depends on both NADH and ferricyanide concentrations (Table 2): this is expected if guanidine increases the steady-state level of the electron acceptor reactive species (E_r).

The kinetic behavior which is characteristic for compulsory ordered sequential mechanisms with a formation of the ternary enzyme-substrate-acceptor complex [39] was observed with HAR as an electron acceptor (Figs. 3 and 4). The hypothetical mechanism of HAR reduction by NADH catalyzed by FP is given in Scheme 3, where R stands for HAR and $K^R_{red/ox} < K^G_{red/ox} < K_{red/ox}$. We do not specify whether the irreversible step of the overall reaction (k_{cat}) is the dissociation of bound reduced HAR, or the latter is oxidized in its bound state in the bimolecular reaction with free oxidized HAR.

Scheme 3 is almost identical to Scheme 2 except for R, which now serves as an activator (similar to guanidine) and as an electron acceptor. The high efficiency of HAR as an electron acceptor is likely due to the shift of the equilibrium for the internal electron redistribution between bound substrate and primary electron acceptor, most probably FMN. In other words, binding of HAR in the vicinity of FMN (the same site at which guanidine binds) makes the redox potential of the prosthetic group more positive. It is pertinent to note that the formation of stable flavin-$Ru(NH_3)_4$ complex with the strong electronic interaction between an isoalloxazine ring and Ru coordinated with N(5) and O(4), has been described [43]. Our interpretation of considerably higher efficiency of HAR as compared with ferricyanide and menadione is in accord with the



Scheme 3.

earlier proposal of Dooijewaard and Slater [18]. They have reached the conclusion that the higher catalytic activity of FP within Complex I (Type-I NADH dehydrogenase) as compared to the isolated three subunit Type-II NADH dehydrogenase was due to a 120 mV decrease in the redox potential of FMN bound to FP. Scheme 3 also explains the difference in the effects of guanidine on HAR-reductase activity in the presence of different NADH concentrations. When the enzyme is saturated by NADH, the only effect of guanidine is a decrease in concentration of the catalytically competent E_r. When the enzyme is not saturated by NADH guanidine, which binds at the same site as HAR, $K_{red/ox}$ decreases, thus decreasing $K_{S(app.)}^{NADH}$ and increasing the concentration of E_r which is reactive with an external acceptor (R).

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