

# Kinetics of transhydrogenase reaction catalyzed by the mitochondrial NADH-ubiquinone oxidoreductase (Complex I) imply more than one catalytic nucleotide-binding sites

N.V. Zakharova, T.V. Zharova, A.D. Vinogradov\*

*Department of Biochemistry, School of Biology, Moscow State University, Moscow 119899, Russian Federation*

Received 28 November 1998; received in revised form 28 December 1998

**Abstract** The steady-state kinetics of the transhydrogenase reaction (the reduction of acetylpyridine adenine dinucleotide (APAD<sup>+</sup>) by NADH, DD transhydrogenase) catalyzed by bovine heart submitochondrial particles (SMP), purified Complex I, and by the soluble three-subunit NADH dehydrogenase (FP) were studied to assess a number of the Complex I-associated nucleotide-binding sites. Under the conditions where the proton-pumping transhydrogenase (EC 1.6.1.1) was not operating, the DD transhydrogenase activities of SMP and Complex I exhibited complex kinetic pattern: the double reciprocal plots of the velocities were not linear when the substrate concentrations were varied in a wide range. No binary complex (ping-pong) mechanism (as expected for a single substrate-binding site enzyme) was operating within any range of the variable substrates. ADP-ribose, a competitive inhibitor of NADH oxidase, was shown to compete more effectively with NADH ( $K_i = 40 \mu\text{M}$ ) than with APAD<sup>+</sup> ( $K_i = 150 \mu\text{M}$ ) in the transhydrogenase reaction. FMN redox cycling-dependent, FP catalyzed DD transhydrogenase reaction was shown to proceed through a ternary complex mechanism. The results suggest that Complex I and the simplest catalytically competent fragment derived therefrom (FP) possess more than one nucleotide-binding sites operating in the transhydrogenase reaction.

© 1999 Federation of European Biochemical Societies.

**Key words:** NADH-ubiquinone reductase; Complex I; Nicotinamide nucleotide; Transhydrogenation; (Bovine heart mitochondria)

## 1. Introduction

The mitochondrial NADH-ubiquinone oxidoreductase (EC 1.6.5.3, Complex I) is the most complex redox-linked proton translocating enzyme of the respiratory chain (see [1] for the recent reviews). Neither the sequence of the intramolecular electron transfer within the enzyme nor the mechanism of the redox-linked proton translocation are known. A number of the speculative  $\Delta\bar{\mu}_{\text{H}^+}$  generation mechanisms has been proposed; all of them are based explicitly or implicitly on the original Mitchell's ideas [2] on a spatial separation of the

electrons and proton derived from NADH on their way to the quinone acceptor. Recently an interesting possibility of the 'conformational' coupling mechanism for Complex I [3,4] similar to those accepted for  $\text{F}_0\text{F}_1$ -ATP synthase [5] or  $\text{H}^+$ -translocating transhydrogenase (EC 1.6.1.1) [6] has been proposed.

Knowledge of the number and nature of the enzyme substrate (nucleotide)-binding site(s) seems to be a prerequisite for any mechanistic model. Several subunits in the catalytic sector of Complex I which contains the three-subunit iron-sulfur flavoprotein FP (51, 24 and 9 kDa) and seven-subunit iron-sulfur subcomplex IP (75, 49, 30, 18, 15, 13 and 11 kDa) were shown to be present in Complex I as single copies per mole of the enzyme (except for 15 kDa) [3]. FP is the minimal catalytically competent fragment of Complex I and its 51 kDa FMN-containing subunit was shown to carry the NADH-binding site [7]. Thus the presence of a single catalytic nucleotide-binding site per mole of the enzyme is usually assumed when the hypothetical coupling mechanisms are considered ([8] for the review).

There are several findings reported in the literature which can hardly be explained by such a single-site model.

(i) Submitochondrial particles [9,10], Complex I [9,11], Type-1 [12] and Type-2 [13] respiratory chain-linked NADH dehydrogenases are capable of oxidation of both NADH and NADPH. Studies on the reduction of the iron-sulfur clusters by NADH or NADPH led Albracht and his associates to propose a heterodimeric model of Complex I in which different protomers react with either nucleotides [14]. The model was recently modified as to propose a monomeric structure with eight iron-sulfur clusters and two FMN groups which serve as primary electron acceptors for NADH and NADPH [15]. Although the latest version of the model is difficult if not impossible to reconcile with the FMN-containing subunit (51 kDa) stoichiometry [3] the observations on different reduction patterns in the presence of NADH or NADPH still need an explanation. Kang et al. demonstrated that both NADH and NADPH-induced superoxide formation by Complex I are bi-phasic in the double reciprocal plots against the nucleotide substrate concentrations [16]. Studies on NAD(P)H-induced lipid peroxidation in SMP led Glinn et al. to conclude that Complex I may distinguish between electron input from NADH and NADPH by differences in the substrate-binding sites [17]. At present, the problem of the site(s) specificity for interaction of NADH and NADPH with Complex I remains unsolved.

(ii) Complex I prepared by the conventional procedure [18,19] and preparations of high molecular mass (Type-1) [19] and low molecular mass (Type-2) [13] NADH dehydrogenases isolated from SMP or Complex I are capable of transhydrogenase reactions. It is not known whether the reactions

\*Corresponding author. Fax: +7 (95) 939 3955.  
E-mail: adv@biochem.bio.msu.su

**Abbreviations:** SMP, submitochondrial particles; FP, three-subunit iron-sulfur flavoprotein; APAD<sup>+</sup>, 3-acetylpyridine adenine dinucleotide; FMN, flavine mononucleotide; DCIP, dichlorophenolindophenol

proceed via a single-site or two-sites mechanism, although two closely associated sites specific for NADH and NADP<sup>+</sup> have been proposed for the three-subunit FP many years ago [13]. Further studies utilizing the pyridine nucleotide photoaffinity analogues suggested that DD transhydrogenation is catalyzed at a site distinct from that responsible for NADH dehydrogenation [20]; 42 kDa subunit of Complex I (which likely corresponds to 39 kDa subunit in more recent nomenclature [21]) has been reported to be associated with the DD transhydrogenase activity [20].

(iii) Oxidation of NADH by SMP and by the preparations derived therefrom is only weakly inhibited by NAD<sup>+</sup> ( $K_i^{\text{NAD}^+}$  is in millimolar range, as compared with micromolar range values of  $K_m^{\text{NADH}}$  [22]). Energized SMP catalyze  $\Delta\bar{\mu}_{\text{H}}$ -dependent ubiquinol-NAD<sup>+</sup> oxidoreduction (reverse electron transfer) with the opposite apparent affinities for the oxidized and reduced nucleotides:  $K_m^{\text{NAD}^+} = 7 \mu\text{M}$  and  $K_i^{\text{NADH}} = 40 \mu\text{M}$

[22]. Recently we have shown that ADP-ribose, a competitive inhibitor of NADH oxidation does not affect and under certain conditions stimulates the reverse electron transfer [23]. These findings were interpreted as to suggest that different binding sites for NADH and NAD<sup>+</sup> operate in the forward and reverse electron transfer [23].

Taken together the data reported in the literature provide no clear-cut information on the number of the nucleotide-binding site(s) in Complex I. In this report we will present the data on the kinetics of DD transhydrogenase activity of SMP, Complex I and FP. The DD transhydrogenation catalyzed by these preparations is shown to proceed by either the simple ternary complex (FP) or by more complex (SMP and Complex I) mechanisms. Such mechanisms can only be explained if two (or more) nucleotide-binding sites are operating. The preliminary account of this work has been published in abstract form elsewhere [24].

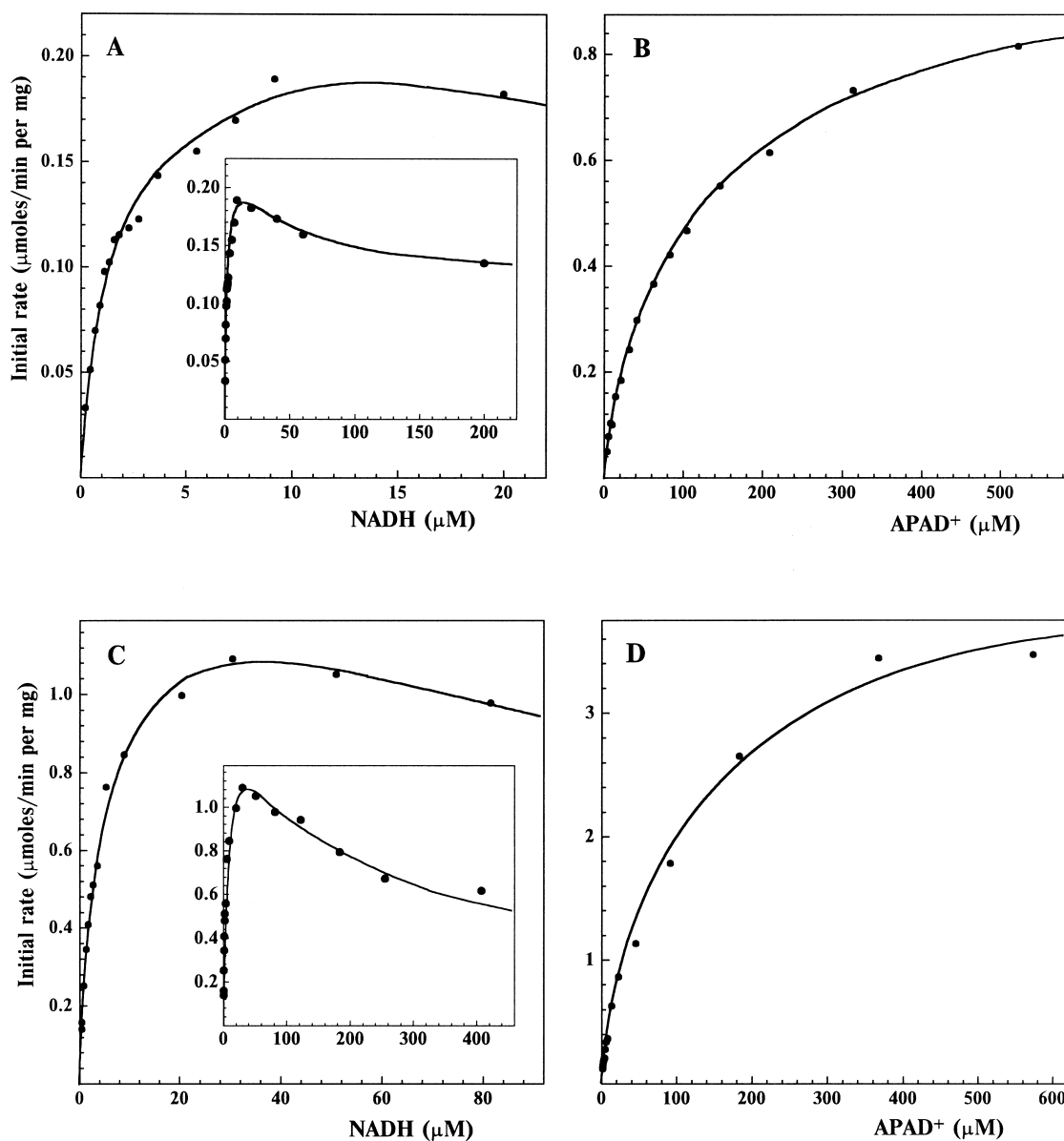


Fig. 1. Dependence of the palmitoyl-CoA-insensitive DD transhydrogenase reaction on the substrate nucleotide concentration. A and B: SMP; C and D: Complex I. Twenty μM APAD<sup>+</sup> (A and C) and 10 μM NADH (B and D) were present as the second substrates.

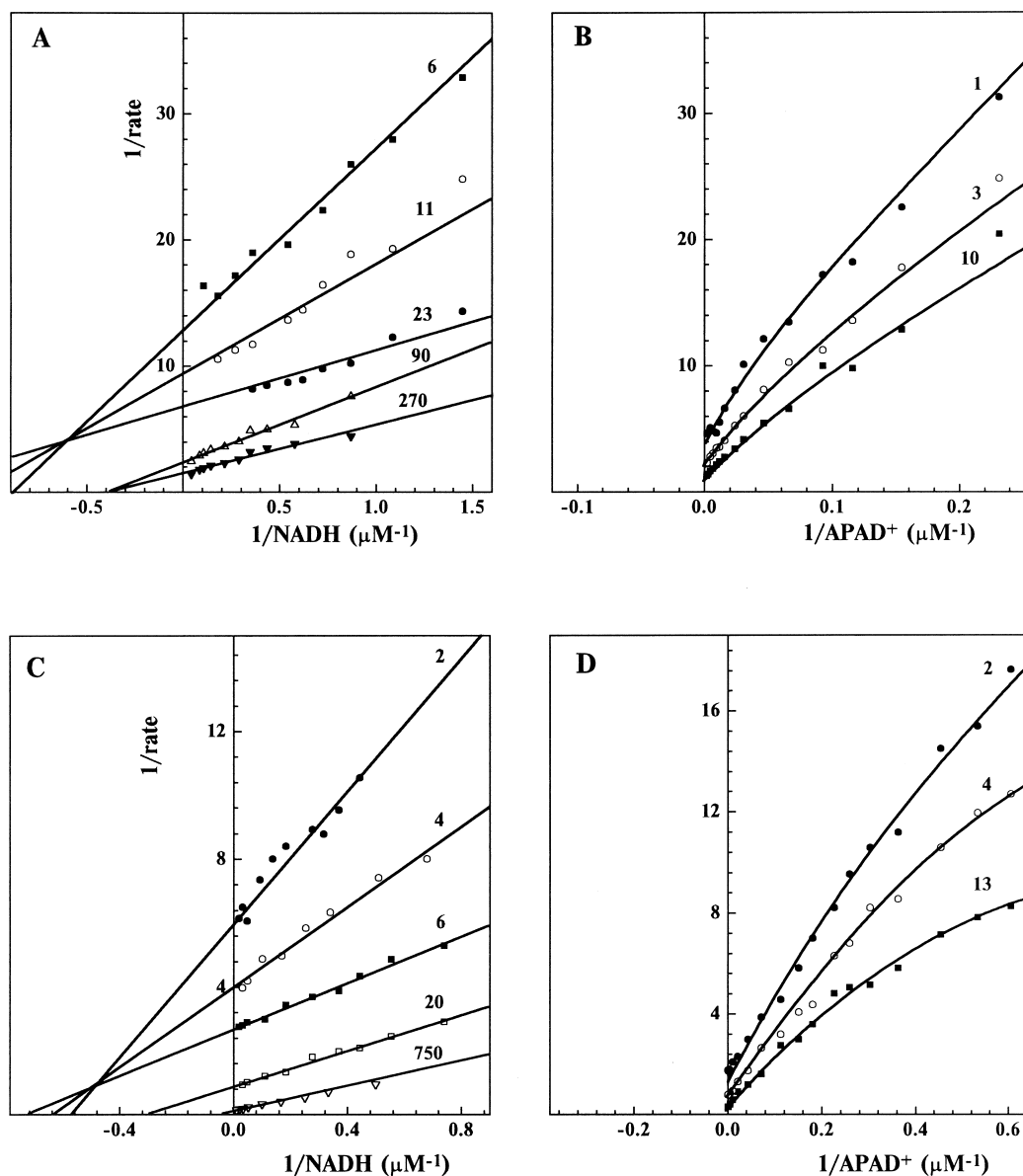


Fig. 2. The double reciprocal plots for DD transhydrogenation catalyzed by SMP (A and B) and Complex I (C and D). The figures on the lines indicate the concentrations of the second substrate ( $\mu\text{M}$ ).

## 2. Materials and methods

SMP [25], Complex I [26] and FP [27] were prepared according to the published procedures. Protein content was determined with biuret reagent.

Transhydrogenase activity was assayed at 25°C in the standard reaction mixture containing 0.25 M sucrose, 20 mM Tris/Cl<sup>-</sup> (or 20 mM HEPES), 0.2 mM EDTA, pH 8.0. When SMP or Complex I were assayed 4  $\mu\text{M}$  rotenone was added to the mixture. The reaction was followed in Hitachi 557 spectrophotometer as an increase in absorption at 375 nm using  $E_{\text{mM}}^{375} = 5.1$  [28]. The other details are indicated in the legends to the figures and table.

NADH (enzymatically prepared, product N 8129), NAD<sup>+</sup>, NADPH, NADP<sup>+</sup>, APAD<sup>+</sup>, palmitoyl-Co A and rotenone were from Sigma (USA). No contaminant NAD<sup>+</sup> in NADH preparations was detected by the alcohol dehydrogenase assay. Tris (base), HEPES and trypsin were from Serva (Germany). Other chemicals were of the highest quality commercially available. The calculation and curve fittings were performed using GIM Version 2 computer program (A. Drachev Copyright).

## 3. Results

### 3.1. Transhydrogenase reaction catalyzed by SMP

The inner mitochondrial membrane contains at least two enzymes capable of transhydrogenase activity: proton-pumping transhydrogenase (EC 1.6.1.1) and Complex I (EC 1.6.5.3). Although transhydrogenase (EC 1.6.1.1) normally catalyzes only the reactions with NADP<sup>+</sup> or NADPH as one of the substrates (TD or DT transhydrogenation), it has been found that under certain conditions the enzyme is capable of a cyclic futile NADH-APAD<sup>+</sup> (DD) transhydrogenation which needs NADP<sup>+</sup> tightly bound at the specific site [6,29,30]. Since SMP may contain some bound nicotinamide dinucleotides it was necessary to exclude possible contribution of transhydrogenase in the overall DD reaction catalyzed by SMP. This was accomplished by a comparison of the inhibitory effects of palmitoyl-Coenzyme A (the specific transhy-

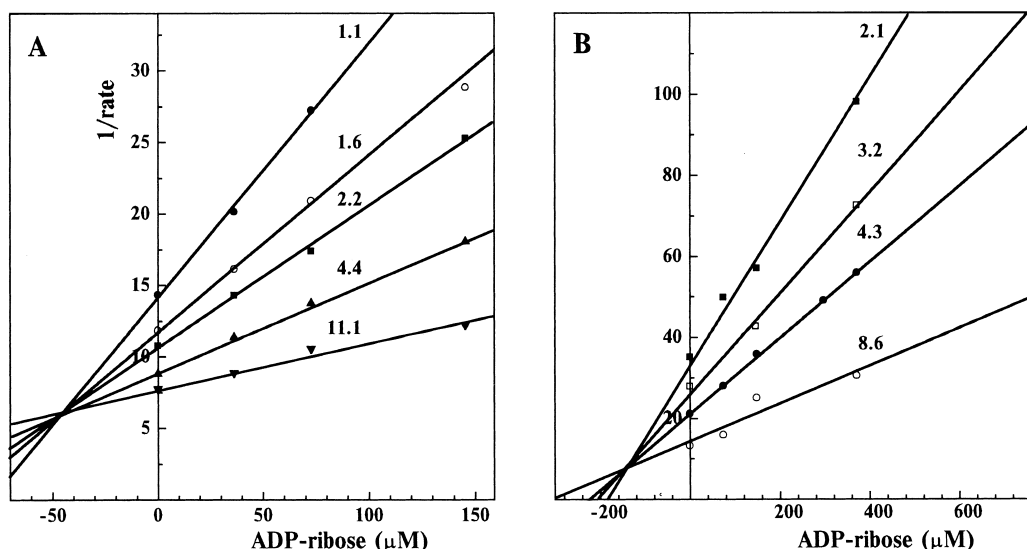


Fig. 3. Inhibition of DD transhydrogenase of SMP by ADP-ribose. A: NADH was the variable substrate; concentrations ( $\mu\text{M}$ ) are indicated by the figures on the lines. The concentration of  $\text{APAD}^+$  was  $18 \mu\text{M}$ . B:  $\text{APAD}^+$  was the variable substrate; concentrations ( $\mu\text{M}$ ) indicated by the figures on the lines. The concentration of NADH was  $8 \mu\text{M}$ .

drogenase (EC 1.6.1.1) inhibitor [31] and trypsin (which is known to destroy EC 1.6.1.1 enzyme [9,32]) on the DD and TD transhydrogenase activities of SMP. As documented in Table 1, at pH 8.0 SMP catalyze the reaction with either NADPH or NADH as the hydride donor with the comparable rates and only the TD reaction was inhibited by palmitoyl-Co A and abolished after tryptic digestion. Another source of other than Complex I DD transhydrogenase activity might be possible contamination of SMP with lipoyl dehydrogenase components of  $\alpha$ -ketoacid dehydrogenases which are known to catalyze the DD transhydrogenase reaction [33]. The control experiments revealed no NADH-lipoate oxidoreductase activity in our SMP preparations. Thus, we concluded that DD transhydrogenase reaction catalyzed by SMP can be quantitatively accounted for the activity of Complex I. This conclusion was further supported when some of the kinetic experiments described below were performed in the presence or absence of palmitoyl-Co A (or before and after tryptic treatment) – the same results were obtained in either cases.

### 3.2. The kinetics of DD transhydrogenase catalyzed by SMP and Complex I

Fig. 1 shows the nucleotides concentrations – initial rate dependencies of the DD transhydrogenation for SMP (A, B) and purified Complex I (C, D). No deviations from the hyper-

bolic dependencies were found for either substrates at their low concentrations ( $< 20 \mu\text{M}$  for NADH). The substrate inhibition was evident when NADH concentration was increased up to the level of about 50 times higher than apparent  $K_m^{\text{NADH}}$ . The patterns of the initial rate-substrate concentration dependencies were essentially the same for SMP and Complex I except for the apparent  $V_{\text{max}}$  values which were about 5 times higher for Complex I.

The kinetic mechanism of the reaction was further analyzed by the mutual variations of the nucleotides within the concentration range where the substrate inhibition could be neglected. Fig. 2 shows the standard double reciprocal plots of the velocities at varying concentrations of both substrates for SMP (A, B) and Complex I (C, D). When NADH concentration was varied ( $1\text{--}15 \mu\text{M}$ ) the plots were linear at any  $\text{APAD}^+$  concentrations; however they did not reveal a simple kinetic behavior. At low  $\text{APAD}^+$  concentrations the lines intercepted in the fourth quadrant whereas at higher  $\text{APAD}^+$  concentrations the lines converged at abscissa (SMP) or at third quadrant (Complex I) as expected for the sequential (ternary complex) mechanisms. At neither  $\text{APAD}^+$  concentrations the reaction proceeded by a binary complex (ping-pong) mechanism, which would have been expected if a single nucleotide-binding site was operating in the reaction. The double reciprocal plots when  $\text{APAD}^+$  was varied were curvilinear.

Table 1  
Transhydrogenase activities of SMP (pH 8.0,  $26^\circ\text{C}$ )

| Reaction                                    | Rate ( $\mu\text{moles/min per mg of protein}$ ) |                                     |                                   |
|---|--|-------------------------------------|-----------------------------------|
|   | Control  | +Palmitoyl-Co A ( $7 \mu\text{M}$ ) | Treated with trypsin <sup>a</sup> |
| $\text{NADH} \rightarrow \text{APAD}^{+b}$  | 0.16   | 0.16                                | 0.15                              |
| $\text{NADPH} \rightarrow \text{APAD}^{+c}$ | 0.09   | 0.00                                | 0.00                              |

<sup>a</sup>Trypsin ( $0.1 \text{ mg per mg of SMP}$ ) was added to the suspension of SMP ( $20 \text{ mg per ml}$ ) in  $0.25 \text{ M}$  sucrose,  $20 \text{ mM}$  Tris/ $\text{Cl}^-$  (pH 8.0) and  $0.2 \text{ mM}$  EDTA. After incubation for 30 min at  $0^\circ\text{C}$  the suspension was diluted 10 times with the same mixture and kept on ice during the experiments (not longer than 1 h).

<sup>b</sup>Concentrations of NADH and  $\text{APAD}^+$  in assay mixture were  $11 \mu\text{M}$  and  $20 \mu\text{M}$  respectively.

<sup>c</sup>Concentrations of NADPH and  $\text{APAD}^+$  in assay mixture were  $340 \mu\text{M}$  and  $200 \mu\text{M}$  respectively.

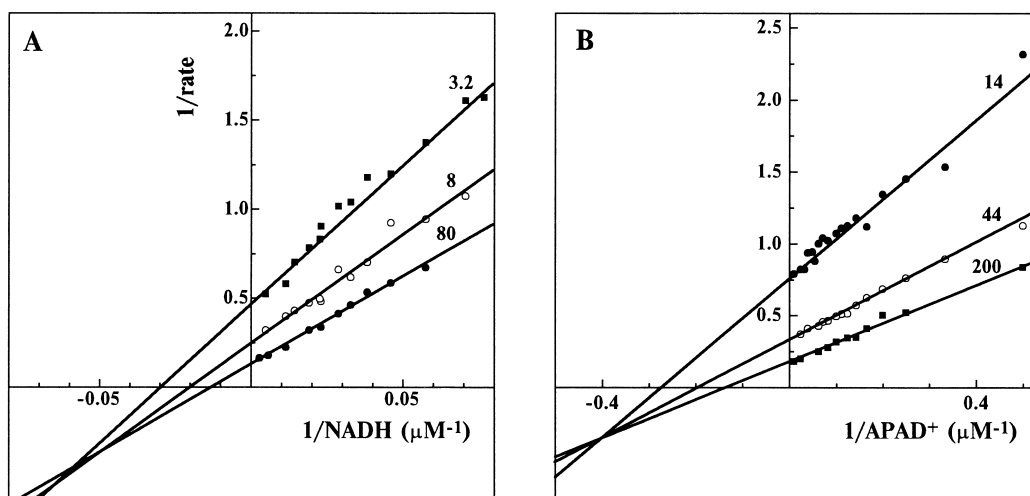


Fig. 4. The double reciprocal plots for DD transhydrogenation catalyzed by FP. Figures on the lines indicate the concentrations of the second substrate ( $\mu\text{M}$ ).

The effect of ADP-ribose, a competitive inhibitor of NADH oxidation [23], on NADH-APAD<sup>+</sup> transhydrogenation catalyzed by SMP was studied. The Dixon plots presented in Fig. 3 showed the inhibition pattern with  $K_i^{\text{ADPR}}$  of 40  $\mu\text{M}$  (competition with NADH, A) the value which is close to that reported for NADH oxidase (26  $\mu\text{M}$ ) [23] and of 150  $\mu\text{M}$  for competition with APAD<sup>+</sup> (B).

### 3.3. Transhydrogenase activity of FP

The simplest catalytically competent fragment of Complex I is the three-subunit monomeric iron-sulfur flavoprotein (FP) which is capable of NADH-artificial acceptor oxidoreductase activities and nicotinamide dinucleotides transhydrogenation [13]. When NADH (50  $\mu\text{M}$ ) was added to FP (80  $\mu\text{g/ml}$ ) an immediate bleaching of the enzyme measured as absorption decrease ( $\Delta A_{445-500}$ ) was observed which was reversed by the addition of 50  $\mu\text{M}$  APAD<sup>+</sup> (not shown). This observation implies, as expected, that FMN participates in the DD transhydrogenase reaction. It was of obvious interest to compare the kinetics of transhydrogenation catalyzed by this preparation with that observed for SMP and Complex I. Fig. 4 shows the mutual dependencies of the reaction rate on the concentrations of the substrate nucleotides. The linear double reciprocal plots for the reactions measured at limited NADH concentrations (where no significant inhibition by NADH occurs) at any concentration of APAD<sup>+</sup> were convergent at third quadrant (A) indicating the ternary complex mechanism (random, ordered or Theorell-Chance mechanism). The same mechanism was evident when APAD<sup>+</sup> concentration was varied at different constant NADH concentrations (B). FP is known to be modified, apparently because of rather drastic treatment during resolution and purification from the parent Complex I, and its catalytic activity with same electron acceptors including APAD<sup>+</sup> (not with positively charged hexammineruthenium III [34]) was shown to be significantly increased in the presence of guanidinium ion [35]. When the experiments depicted in Fig. 4 were performed in the presence of 75 mM guanidine chloride essentially the same kinetic patterns were seen except for quantitative difference in the apparent  $K_m$  and  $V_{\text{max}}$  values (the results are not shown).

## 4. Discussion

The present studies were not aimed to establish the detailed kinetic mechanism of the transhydrogenase reaction catalyzed by Complex I and by its simpler fragment. The question we were attempting to answer was whether a single nucleotide-binding site model fits the kinetic pattern of the simplest diagnostic reaction. The results obtained suggest that more than one substrate/product sites operate in the transhydrogenase activity of Complex I. Because of a complex kinetic pattern (different behavior was evident at low and high concentrations of the second variable nucleotide (Fig. 2) and inhibition of the reaction at high NADH concentrations) further elaborated studies are obviously needed to suggest the detailed kinetic mechanism. The results, however, do eliminate a binary complex (ping-pong) mechanism which is the only possibility for a bireactant reaction catalyzed by a single substrate-binding site-containing enzyme. The data are consistent with the unidirectional competitive inhibition of NADH oxidation by ADP-ribose in SMP [23]. The inhibition of the transhydrogenase activity at high NADH concentration (Fig. 1) can be explained by occupation of the NAD<sup>+</sup>(APAD<sup>+</sup>)-specific site by NADH which results in the dead-end complex formation. Perhaps the most striking observation reported here is the ternary complex mechanism found for the soluble three-subunit monomeric FP (Fig. 4). Such mechanism requires at least two nucleotide-binding sites on the enzyme. One site has been identified on 51 kDa subunit by its specific labelling [7] and also deduced from the single NAD<sup>+</sup>-binding motif in its amino acid sequence [21]. The lack of the additional typical nucleotide-binding motifs in 51 kDa and other smaller (24 and 9 kDa) subunits of FP may not rule out the existence of the second catalytic site. Relevant to the present discussion it is worth noting that low concentrations of NAD<sup>+</sup> were shown to inhibit the NADH-ferricyanide or DCIP reductase activities of FP competitively with the artificial electron acceptors and non-competitively with NADH [36]. We propose that two nucleotide-binding sites are located in FP: one is NADH-specific and another one is NAD<sup>+</sup>(APAD<sup>+</sup>)-specific. It seems very likely that some artificial electron acceptors such as ferricyanide or O<sub>2</sub> (superoxide generation) interact with FP at

the NAD<sup>+</sup>-specific site which is in electronic contact with the NADH-specific site possibly through the 'shuttling' FMN [37]. Whether the postulated NAD<sup>+</sup>-specific site in FP serves as the site which operates in the reverse electron transfer [23] and in DD transhydrogenase catalyzed by SMP and Complex I remains to be established.

**Acknowledgements:** This work was supported by National Institute of Health (USA) Fogarty International Research Collaborative Grant R03 TW00140-01A2, by the Russian Foundation for Fundamental Research (grant 96-04-48185) and by the Program of Advanced Schools in Science (grant 96-15-97822).

## References

- [1] Brandt, U. (Ed.) (1998) *Biochim. Biophys. Acta* 1362 (special issue).
- [2] Mitchell, P. (1966) *Chemiosmotic Coupling in Oxidative and Photosynthetic Phosphorylation*, Glynn Research Ltd., Bodmin.
- [3] Belogradov, G. and Hatefi, Y. (1994) *Biochemistry* 33, 4571–4576.
- [4] Yamaguchi, M., Belogradov, G.I. and Hatefi, Y. (1998) *J. Biol. Chem.* 273, 8094–8098.
- [5] Boyer, P.D. (1998) *Biosci. Rep.* 18, 97–117.
- [6] Hatefi, Y. and Yamaguchi, M. (1996) *FASEB J.* 10, 442–452.
- [7] Deng, P.S.K., Hatefi, Y. and Chen, S. (1990) *Biochemistry* 29, 1094–1098.
- [8] Brandt, U. (1997) *Biochim. Biophys. Acta* 1318, 79–91.
- [9] Djavadi-Ohanian, L. and Hatefi, Y. (1975) *J. Biol. Chem.* 250, 9397–9403.
- [10] Rydström, J., Montelius, J., Bäckström, D. and Ernster, L. (1978) *Biochim. Biophys. Acta* 501, 370–380.
- [11] Van Belzen, R. and Albracht, S.P.J. (1989) *Biochim. Biophys. Acta* 974, 311–320.
- [12] Rossi, C., Cremona, T., Machinist, J. and Singer, T. (1965) *J. Biol. Chem.* 240, 2634–2643.
- [13] Hatefi, Y. and Galante, Y.M. (1977) *Proc. Natl. Acad. Sci. USA* 74, 846–850.
- [14] Van Belzen, R., van Gaalen, M.C.M., Cuypers, P.A. and Albracht, S.P.J. (1990) *Biochim. Biophys. Acta* 1017, 152–159.
- [15] Albracht, S.P.J. and de Jong, A.M.P. (1997) *Biochim. Biophys. Acta* 1318, 92–106.
- [16] Kang, D., Narabayashi, H., Sata, T. and Takeshige, K. (1983) *J. Biochem. (Tokyo)* 94, 1301–1306.
- [17] Glinn, M.A., Lee, C.P. and Ernster, L. (1997) *Biochim. Biophys. Acta* 1318, 246–254.
- [18] Hatefi, Y. and Hanstein, W.G. (1973) *Biochemistry* 12, 3515–3522.
- [19] Ragan, C.I., Widger, W.R. and King, T.E. (1974) *Biochem. Biophys. Res. Commun.* 3, 894–900.
- [20] Chen, S. and Guillory, R.J. (1984) *J. Biol. Chem.* 259, 5124–5131.
- [21] Fearnley, I.M. and Walker, J.F. (1992) *Biochim. Biophys. Acta* 1140, 105–134.
- [22] Vinogradov, A.D. (1993) *J. Bioenerg. Biomembr.* 25, 367–375.
- [23] Zharova, T.V. and Vinogradov, A.D. (1997) *Biochim. Biophys. Acta* 1320, 256–264.
- [24] Zakharova, N.V., Zharova, T.V. and Vinogradov, A.D. (1998) Abstract of 10 European Bioenergetic Conference, Göteborg, Sweden.
- [25] Kotlyar, A.B. and Vinogradov, A.D. (1990) *Biochim. Biophys. Acta* 1019, 151–158.
- [26] Hatefi, Y. (1967) *Methods Enzymol.* 10, 235–239.
- [27] Hatefi, Y. (1978) *Methods Enzymol.* 53, 3–4.
- [28] Stein, A.M., Kaplan, N.O. and Ciotti, M.M. (1959) *J. Biol. Chem.* 234, 979–986.
- [29] Zhang, J., Hu, X., Osmam, A.M. and Rydström, J. (1997) *Biochim. Biophys. Acta* 1319, 331–339.
- [30] Stilwell, S.N., Bizouarn, T. and Jackson, J.B. (1997) *Biochim. Biophys. Acta* 1320, 83–94.
- [31] Rydström, J. (1972) *Eur. J. Biochem.* 31, 496–504.
- [32] Ragan, C.I. (1976) *Biochem. J.* 156, 367–374.
- [33] Koike, M. and Hayakawa, T. (1970) *Methods Enzymol.* 18, 298–307.
- [34] Gavrikova, E.V., Grivennikova, V.G., Sled, V.D., Ohnishi, T. and Vinogradov, A.D. (1995) *Biochim. Biophys. Acta* 1230, 23–30.
- [35] Hatefi, Y., Stempel, K.E. and Hanstein, W.G. (1969) *J. Biol. Chem.* 244, 2358–2365.
- [36] Avraam, R. and Kotlyar, A.B. (1991) *Biokhimiya (USSR)* 56, 2253–2260.
- [37] Sled, V.D. and Vinogradov, A.D. (1993) *Biochim. Biophys. Acta* 1143, 199–203.