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Use of a Reverse Micelle System for Study of Oligomeric Structure of NAD^+-Reducing Hydrogenase from *Ralstonia eutropha* H16

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Abstract—Inclusion of an oligomeric enzyme, NAD^+-dependent hydrogenase from the hydrogen-oxidizing bacterium *Ralstonia eutropha*, into a system of reverse micelles of different sizes resulted in its dissociation into catalytically active heterodimers and subunits, which were characterized in reactions with various substrates. It was found that: 1) the native tetrameric form of this enzyme catalyzes all types of studied reactions; 2) hydrogenase dimer, HoxHY, is a minimal structural unit catalyzing hydrogenase reaction with an artificial electron donor, reduced methyl viologen; 3) all structural fragments containing FMN and NAD^+/NADH-binding sites exhibit catalytic activity in diaphorase reactions with one- and two-electron acceptors; 4) small subunits, HoxY and HoxU also exhibit activity in diaphorase reactions with artificial acceptors. These results can be considered as indirect evidence that the second FMN molecule may be associated with one of the small subunits (HoxY or HoxU) of the hydrogenase from *R. eutropha*.

Key words: *Ralstonia eutropha*, NAD^+-reducing hydrogenase, oligomeric structure, reverse micelles

Hydrogenases catalyze the reaction of reversible cleavage of molecular hydrogen according to the following reaction: $H_2 \leftrightarrow 2\ H^+ + 2\ e^-$. Two main classes of metal-containing hydrogenases exist, Fe-only hydrogenase [1, 2] and NiFe-hydrogenases [3-6].

Soluble NAD^+-dependent hydrogenase from the hydrogen oxidizing bacterium *Ralstonia eutropha* belongs to the class of NiFe-hydrogenases. This enzyme consists of two functional blocks—hydrogenase and diaphorase (Fig. 1). The former catalyzes the reaction $H_2 \leftrightarrow 2\ H^+ + 2\ e^-$, whereas the latter catalyzes the reaction $NAD^+ + 2\ e^- + H^+ \leftrightarrow NADH$ [7-10]. The hydrogenase block is formed by two subunits HoxH and HoxY of molecular masses 56 and 26 kD, respectively. The catalytic site of the hydrogenase dimer is located on the HoxH subunit; this site contains a NiFe-cluster, which is involved in hydrogen molecule cleavage. HoxY subunit contains one [4Fe-4S]-cluster; this cluster is involved in intramolecular transfer of electrons formed during cleavage of a hydrogen molecule [7, 9-13]. Diaphorase (NADH-dehydrogenase) block also consists of two subunits HoxF and HoxU with molecular masses 63 and 30 kD, respectively. HoxF subunit contains FMN, NAD^+(H) binding site, and several Fe-S-clusters. The HoxU subunit also contains Fe-S-clusters, but their composition and localization is not yet precisely established [7, 9, 10, 12-14].

Besides the physiological reaction of NAD^+ reduction, soluble *R. eutropha* hydrogenase catalyzes hydrogenase and diaphorase reactions with artificial electron acceptors (MV, BV, FC, DCP, etc.) [9, 10, 15, 16]. Good evidence now exists that reactions with various substrates involve different structural components of this enzyme [9, 10, 13, 16, 17]. The physiological reaction of NAD^+ reduction by molecular hydrogen is catalyzed only by tetramer; this suggests an important role of integrity of

Abbreviations: Acc) electron acceptor; AOT) aerosol OT, bis(2-ethylhexyl)sulfosuccinate, sodium salt; APAD) 3-acetylpyridine adenine dinucleotide; BV) benzyl viologen; DCP) 2,6-dichlorophenol indophenol; FC) potassium ferricyanide; FMN) flavin mononucleotide; ω_0) surfactant hydration degree; MV) methyl viologen.

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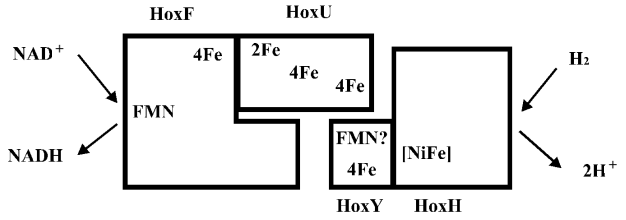

Fig. 1. Schematic presentation of the structure of *R. eutropha* H16 NAD^+-dependent hydrogenase and reactions catalyzed by this enzyme [7, 36].

the electron transport chain connecting the hydrogen cleavage site with the NAD^+(H) binding site [7, 9-11, 17, 18]. The hydrogenase dimer catalyzes reduction of artificial acceptors by molecular hydrogen and diaphorase dimer by NADH [9, 10, 13, 17]. Subsequent separation of dimers into subunits was not successful due to instability of the monomers formed [10, 18]. Preparation of separate subunits by means of genetic engineering methods was not successful due to subunit instability [19-21].

To dissociate hydrogenase molecules and stabilize the resulting functional fragments, we have employed in the present study the method of enzyme incorporation into reverse micelles [22-25]. Earlier this method was successfully employed for dissociation of oligomeric enzymes into catalytically active subunits even when dissociation in aqueous solutions resulted in enzyme inactivation [26-28]. The size of internal micelle cavity is the main parameter regulating oligomeric composition of a protein inserted into a micelle. Experimentally this parameter can be controlled by hydration degree of the surfactant used for micelle formation; the hydration degree ω_0 is determined by the ratio $[H_2O]/[surfactant]$ [29, 30]. The anionic surfactant AOT used in this study is characterized by linear dependence of internal micelle radius (r_m) on the ω_0 parameter; $r_m = 1.5\ \omega_0 + 4$ Å [29]. According to a principle of geometric fit maximum of catalytic activity of the enzyme inserted into the micelle is observed when the size of internal cavity of the micelle is equal to the size of the enzyme or its functional fragment inserted into this micelle [25, 31]. So, in the case of oligomeric enzymes the dependence of catalytic activity on the hydration degree ω_0 is characterized by "wave-like" curve with several maxima. Each maximum is related to formation and functioning of certain oligomeric forms of the protein in micelles of certain size [26-28, 31].

MATERIALS AND METHODS

NAD^+-dependent hydrogenase was isolated from the hydrogen-oxidizing bacterium *Ralstonia eutropha* H16 and purified as described earlier [32]. The specific activity of the enzyme samples was 15-30 U/mg protein at 30°C. According to SDS-PAGE, the purity of the resulting enzyme was 80-95%.

The following chemicals were used in the study: APAD, DCP, NAD^+, MV, and Aerosol OT from Sigma (USA), NADH from Boehringer Mannheim (Germany), and FC and *n*-octane from Reakhim (Russia). Gaseous hydrogen (purity 99.99%) was produced by local suppliers.

All kinetic experiments were carried out at 25°C in 0.05 M Tris-HCl buffer, pH 7.8 (this is the pH optimum for most reactions catalyzed by NAD^+-dependent hydrogenase [33]). At each ω_0 value, all catalytic activities were determined at saturating concentrations of each substrate. In the case of DCP, it was impossible to get saturating concentrations due to low substrate solubility and high Michaelis constant values. In this case, V_{max} values for the reaction of NADH-dependent DCP reduction were calculated by the Michaelis–Menten equation using the method of nonlinear least squares. All values of enzyme activity in reverse micelles were normalized to the enzyme activity assayed (for the same reaction) in the buffer solution.

Hydrogenase activities. The rate of NAD^+ reduction by molecular hydrogen was measured spectrophotometrically by absorbance of NADH formed at $\lambda = 340$ nm using $\varepsilon = 6220$ and 6150 $M^{-1}\cdot cm^{-1}$ for NADH in buffer and reversed micelles, respectively. The reaction was carried out in autocatalytic mode because enzyme pre-activation with NADH or dithionite resulted in enzyme inactivation during its incorporation into micelles.

For catalytic reaction in reverse micelles, 2 ml of 0.1 M AOT in octane was mixed with the calculated volume of buffer solution for achievement of desired hydration degree ω_0. The solution was purged with hydrogen for 10-15 min and the enzyme solution (5 μl) was added and the resulting mixture was shaken up to the state of an optically clear solution. The reaction was initiated by adding 5 μl of 0.4 M NAD^+. For the reaction in the aqueous medium, AOT solution was substituted for buffer.

The rate of hydrogen evolution in the reaction $2\ H^+ + 2\ MV_{red} = H_2 + 2\ MV_{ox}$ was monitored by gas chromatography using Chrom-4 gas chromatograph (Laboratorni Pristroje, Czech Republic) with argon as a gas carrier and molecular sieve type 5A (Serva, Germany) as a stationary phase. The reaction was carried out at 25°C in the argon atmosphere. Before experiment all solutions (buffer, substrates, AOT in octane, and enzyme) were purged with argon for 1 h. Administration of reagents and sampling for gas chromatography were carried out through a rubber septum using a syringe. MV was reduced by administration of excess of sodium dithionite into the reaction medium.

Typical reaction protocol in reverse micelles was: 2 ml of 0.1 M AOT solution in octane was mixed with cal-

Table 1. Conditions of NADH-dehydrogenase activity assay

Acceptor	Reaction in buffer solution			Reaction in micelles		
	ε (λ), $M^{-1}\cdot cm^{-1}$	[NADH], mM	[Acceptor], mM	ε (λ), $M^{-1}\cdot cm^{-1}$	[NADH], mM	[Acceptor], mM
APAD	5980 (365)*	0.75	0.75	5980 (365)*	0.15	0.15
FC	1200 (420)	2.0	2.0	1200 (420)	0.125	0.25
DCP	6700 (520)	0.48	0.3-0.05	6900 (520)	0.48	0.3-0.05

* Differential molar absorbance coefficient, $\varepsilon_{APADred\ -\ NADH}$, was used for the transhydrogenase reaction APAD + NADH.

culated volume of buffer solution; then 10 μl of a solution containing 0.142 M MV and 0.28 M dithionite was added; after purging of the resulting solution with argon for 10-15 min, 5 μl of enzyme solution was added and the reaction mixture was shaken until the solution became optically clear. For the reactions in the aqueous medium, AOT solution was replaced by buffer.

NADH-dehydrogenase activities. Maximal rates of NADH-dehydrogenase reactions in buffer and micelles were determined spectrophotometrically by monitoring a decrease in the concentration of the oxidized forms of the electron acceptors in the reactions with FC and DCP or increase in the concentration of the reduced form in the case of APAD. Assay conditions are listed in Table 1.

Sedimentation analysis. Sedimentation analysis of micelles containing protein was carried out using a Spinco model E analytical ultracentrifuge (Beckman, USA) equipped with photoelectric scanner. Scanning of hydrogenase-containing micelles was carried out at two wavelengths, 280 and 420 nm. Theoretical sedimentation coefficients for protein containing micelles at various hydration degrees of AOT (ω_0) were calculated as described in [34]. Molecular masses of micelle-incorporated protein were calculated according to the following equation [34]: $M_p = M_0((s_p/s_0) - 1)(1 - \rho\upsilon)$, where s is the sedimentation coefficient of a particle with molecular mass M and partial molar volume υ in the solvent with density ρ. Indices p and 0 correspond to micelles containing protein and free micelles, respectively.

RESULTS AND DISCUSSION

For monitoring the activity of spatially separated catalytic sites of the enzyme incorporated into reverse micelles, we selected the following five reactions catalyzed by *R. eutropha* NAD^+-dependent hydrogenase:

hydrogenase reaction with physiological substrate, NAD^+

$$H_2 + NAD^+ \leftrightarrow H^+ + NADH, \quad (1)$$

the reaction of hydrogen formation with artificial electron donor, reduced MV

$$2\ H^+ + 2\ MV_{red} \leftrightarrow H_2 + 2\ MV_{ox}, \quad (2)$$

transhydrogenase reaction with NAD^+ analog APAD

$$NADH + APAD_{ox} \leftrightarrow NAD^+ + APAD_{red}, \quad (3)$$

and two diaphorase reactions with one- and two-electron acceptors, FC and DCP

$$NADH + 2\ FC_{ox} \leftrightarrow NAD^+ + 2\ FC_{red} + H^+, \quad (4)$$

$$NADH + DCP_{ox} + H^+ \leftrightarrow NAD^+ + DCP_{red}. \quad (5)$$

As stated in the introduction, the detergent hydration degree ω_0, determined by the ratio $[H_2O]/[detergent]$, is the main experimentally controllable parameter regulating oligomeric composition of a protein inserted into a micelle. Figures 2-4 show dependencies of maximal rates of the five selected reactions on AOT hydration degree. These results are also summarized in Table 2.

Profiles of all catalytic activities are characterized by maxima at ω_0 = 42.5-44. The theoretically calculated diameter of the inner micelle cavity [29] at the hydration degree of 44 is 139 ± 10 Å. This value well corresponds to the length of the tetramer of *R. eutropha* hydrogenase determined by electron microscopy (127 ± 5 Å [18] and 130 ± 10 Å [35]). According to sedimentation analysis, the molecular mass of protein particles inserted into micelles of such diameter is 200 kD; this also corresponds to the molecular mass of the tetramer (Table 2). The presence of maxima of catalytic activity at ω_0 = 42.5-44 is obviously determined by tetramer functioning. Lack of any other maxima for NAD^+-reducing activity (reaction (1)) on the curve (Fig. 2) is consistent with previous data [7, 9-11, 17, 18], indicating that only native tetrameric form of hydrogenase can catalyze the physiological reaction with NAD^+.

The dependence of hydrogenase activity assayed with the one-electron donor reduced MV (reaction (2))

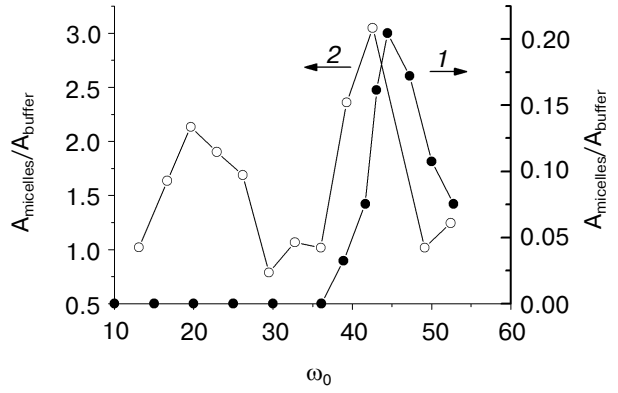

Fig. 2. Dependence of *R. eutropha* hydrogenase activity $A_{micelles}/A_{buffer}$ on surfactant hydration degree (ω_0) for hydrogenase reactions: $H_2 + NAD^+ \leftrightarrow H^+ + NADH$ (curve *1*) and $2\ H^+ + 2\ MV_{red} \leftrightarrow H_2 + 2\ MV_{ox}$ (curve *2*). Experimental conditions: 0.1 M AOT in octane, 50 mM Tris-HCl-buffer, pH 7.8, 0.92 mM NAD^+, 0.71 mM MV, 1.4 mM dithionite, 0.11 μM enzyme.

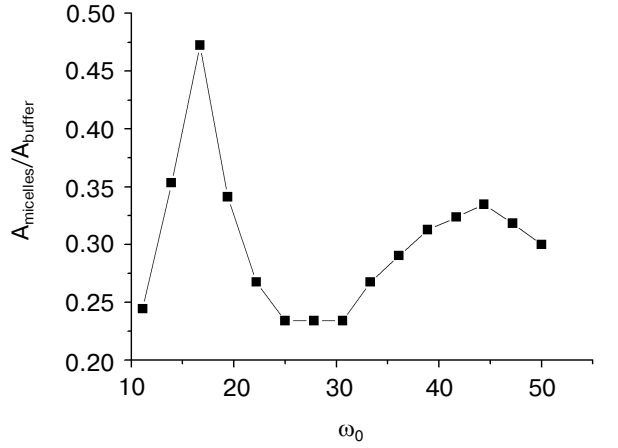

Fig. 3. Dependence of *R. eutropha* hydrogenase activity $A_{micelles}/A_{buffer}$ on AOT hydration degree (ω_0) for the transhydrogenase reaction: $NADH + APAD_{ox} \leftrightarrow NAD^+ + APAD_{red}$. Experimental conditions: 0.1 M AOT in octane, 50 mM Tris-HCl-buffer, pH 7.8; substrate concentrations are listed in Table 1.

on the AOT hydration degree exhibits three optimums (Fig. 2): at $\omega_0 = 42.5$, 33, and 20. The first optimum ($\omega_0 = 42.5$) obviously reflects functioning of tetrameric hydrogenase. According to sedimentation analysis the presence of a small peak at $\omega_0 = 33$ may also be attributed to tetrameric hydrogenase. This protein may be considered as a tetramer deformed in micelles of non-optimal size; it is inactive in the reaction with physiological substrate (NAD^+), but retains activity in the reaction with MV. Low sensitivity of this reaction to tetramer deformation may be explained by: 1) the reaction requires only the minimal part of the electron transfer chain that is stable under tetramer deformation; 2) hydrogenase inactivation in micelles of this size involves FMN and NAD(H)-binding sites on diaphorase subunit which do not participate in reduction of MV by hydrogen.

The third maximum at $\omega_0 = 20$ (Fig. 2) can be assigned to protein particles with molecular mass lower than 90 kD. According to sedimentation analysis data (Table 2) proteins with molecular mass exceeding 90 kD have been inserted into micelles with $\omega_0 = 22.5$. Such functionally active hydrogenase fragment may represent the hydrogenase dimer HoxHY (82 kD). At $\omega_0 = 15$-16 hydrogenase activity was not detected; according to calculations micelles with such hydration degree may include proteins with molecular mass of 50-60 kD. This molecular mass well corresponds to the HoxH subunit containing hydrogen-activating site. This is consistent with previously published data [13] demonstrating that HoxHY dimer (with NiFe-site located on the HoxH subunit and [4Fe-4S]-cluster on the HoxY subunit) is the minimal structural element required for catalysis of the hydrogenase reaction (with all types of substrates) including the reaction of isotope exchange.

The transhydrogenase reaction (reaction (3)) with a structural analog of NAD^+, APAD, should involve the HoxF subunit of diaphorase dimer; this subunit has FMN and NAD(H)-binding sites required for catalysis of this reaction [16]. Theoretically, all protein forms containing the HoxF subunit (tetramer, diaphorase dimer, and HoxF monomer) should exhibit catalytic activity in this reaction. In reality, the profile of dependence of transhydrogenase activity on AOT hydration degree contains only two maxima at $\omega_0 = 44$ and 16.7 (Fig. 3). The first maximum can be evidently assigned to tetramer functioning,

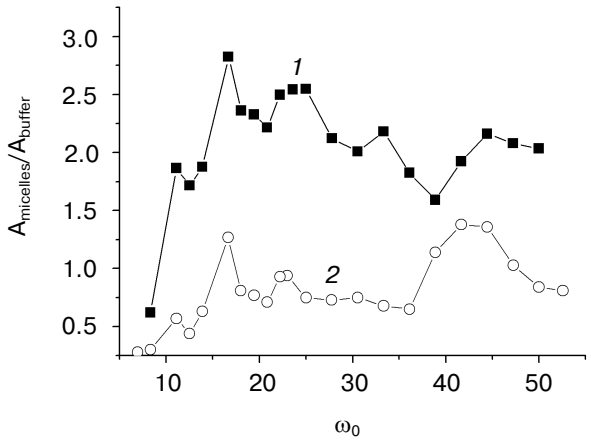

Fig. 4. Dependence of *R. eutropha* hydrogenase activity $A_{micelles}/A_{buffer}$ on AOT hydration degree (ω_0) for the diaphorase reactions: $NADH + 2\ FC_{ox} \leftrightarrow NAD^+ + 2\ FC_{red}$ (curve *1*) and $NADH + DCP_{ox} + H^+ \leftrightarrow NAD^+ + DCP_{red}$ (curve *2*). Experimental conditions: 0.1 M AOT in octane, 50 mM Tris-HCl-buffer, pH 7.8; substrate concentrations are listed in the Table 1.

Table 2. Characteristics of functional fragments of NAD$^+$-dependent hydrogenase inserted into reverse micelles

Reaction	Enzymatic activity at optimal ω_0 (% of activity in the buffer solution)					
	$\omega_0 = 11.2$	$\omega_0 = 16.7$	$\omega_0 = 20$	$\omega_0 = 22.2$	$\omega_0 = 33$	$\omega_0 = 44$
$H_2 + NAD^+$	—	—	—	—	—	20
$2H^+ + 2MV_{red}$	—	—	213	—	106	300
APAD + NADH	—	50	—	—	—	33
$2FC_{ox}$ + NADH	186	282	—	254	218	216
DCP_{ox} + NADH	57	127	—	94	70	140
Molecular mass of protein particles inserted into micelles (sedimentation analysis), kD	30	63	ND	93	200	200
Molecular mass of subunits and hydrogenase dimers (SDS-PAGE data [18]), kD	30; 26	63	82	93	200*	200*
Internal micelle diameter ($r_0 = 1.5\ \omega_0 + 4$ [32]), Å	47	58	64	77	107	139
Putative oligomer composition in micelles	HoxU or HoxY	HoxF	HoxHY	HoxFU	Hox HYFU	Hox HYFU

Note: ND, not determined.

* Molecular mass of native hydrogenase [18].

whereas the second one (at $\omega_0 = 16.7$) is related to protein particles of molecular mass of 63 kD (according to sedimentation analysis data; Table 2). These particles might be the HoxF subunit. We did not find a maximum of catalytic activity at $\omega_0 = 22$-23. According to sedimentation analysis data, micelles with such hydration degree contain proteins with molecular mass (90-93 kD) corresponding to HoxFU-dimer.

All protein particles containing FMN and NAD(H)-sites were catalytically active in diaphorase reactions with artificial one- of two-electron acceptors (FC and DCP, respectively). For these reactions, the dependence of catalytic activities on AOT hydration degree was characterized by five optima (Fig. 4).

Maxima observed at $\omega_0 = 44$ and 33 can apparently be attributed to functioning of native and deformed tetramer as has already been discussed for hydrogenase reactions. In the case of deformed tetramer ($\omega_0 = 33$) marked catalytic activity was observed with one-electron substrates, FC and MV (Figs. 2 and 4). In the case of FC the peaks observed at $\omega_0 = 44$ and 33 were almost equal (216 and 218% of the hydrogenase activity in the buffer, see Table 2). For two-electron acceptor, DCP, the peak was negligibly small.

According to the sedimentation analysis data (Table 2) the diaphorase dimer (of 93 kD [7, 18]) and the HoxF subunit function in micelles with $\omega_0 = 22.2$ and 16.7. Activity of this subunit is somewhat higher than the activity of the HoxFU dimer in both reactions. These data differ from the previously published values of activities of HoxF and HoxFU fragments obtained for the mutant *R. eutropha* strain H424 (pGE15) [13]: activities of the diaphorase dimer and HoxF subunit in the reaction NADH + BV were 56 and 6% of the native enzyme activity. The authors [13] suggest that HoxF subunit is unstable and a putative role of HoxU subunit consists in stabilization of catalytically active conformation of HoxF. In micelles where stabilization of active conformation of HoxF may be achieved by fixation it in micelles of optimal size the structural role of HoxU subunits is not so crucial.

A small maximum of catalytic activity at $\omega_0 = 11.2$ was observed only in the case of diaphorase reactions with artificial acceptors. According to data of sedimentation analysis (Table 2), this maximum can be attributed to functioning of protein particles of 30 kD. Only catalytically inactive HoxY and HoxU subunits have similar molecular masses [7, 18]. The secondary structure of small subunits of Ni-Fe hydrogenases was shown [36] to contain a specific fold similar to that of FMN-binding site of *Clostridium* MP flavodoxin. The authors [36] suggest that the presence of such structural element on the

HoxY subunit of *R. eutropha* hydrogenase may be responsible for the binding of the second FMN molecule. This is consistent with earlier observation [37] that maximal activity of *R. eutropha* hydrogenase is observed when two FMN molecules are bound to tetrameric enzyme. The authors explained the lack of the second FMN molecule in the purified hydrogenase preparation by its loss during purification. It is possible that remaining amount of FMN bound to HoxY may be responsible for diaphorase activity of this subunit in reactions with artificial acceptors in micelles at $\omega_0 = 11.2$. Thus, our results indirectly indicate the possibility of the presence of the second FMN molecule in *R. eutropha* hydrogenase.

Table 2 shows that hydrogenase activity in micelles is 2-5 times lower in reactions with specific substrates, NAD^+ and its analog, APAD, but in reactions with artificial substrates (FC, DCP, MV) the enzyme activity increases by 1.5-3-fold compared with corresponding reactions in the buffer solution. Deformation of FMN and NAD^+(H) binding sites is the most probable reasons for diminished reaction rates with specific substrates because the rate-limiting step of these reactions is related to functioning of these particular sites [16]. For artificial electron acceptors the rate-limiting step is electron transfer from the terminal element of the enzyme electron transport chain to the acceptor molecule [16, 33]. If enzyme functioning in micelles is not accompanied by change in rate-limiting step, impairment of structure of FMN and NAD(H)-binding sites should not considerably affect the rates of reactions with artificial acceptors. It is possible that dissociation of tetramer into dimers and subunits result in increased substrate susceptibility of tetramer-shielded sites interacting with electron acceptors. This may increase reaction rates. Similar data were obtained for *R. opacus* NAD^+-dependent hydrogenase [9]. Dissociation of tetramer into dimers was accompanied by 3-6-fold increase in the rates of hydrogenase and diaphorase reactions with MV, BV, FMN, and methylene blue as substrates. Increase in partial hydrogenase activities during dissociation of tetramer was also observed in the case of *A. eutrophus* Z1 hydrogenase [32].

Thus, employment of the method of enzyme incorporation into reversed micelles for dissociation and stabilization of functional fragments of *R. eutropha* NAD^+-dependent hydrogenase allowed us to characterize catalytic properties of structural elements of this enzyme and underline the role of oligomeric structure of this enzyme in catalysis.

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