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Activity of NADH oxidase from *Thermus thermophilus* in water/alcohol binary mixtures is limited by the stability of quaternary structure

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ABSTRACT

NADH oxidase from *Thermus thermophilus* (NOX) is a homodimeric flavoenzyme; it belongs to the nitroreductase and flavin reductase families. In this work we investigate the effect of water miscible alcohols (methanol, ethanol, propanol and isopropanol) on activity and quaternary structure of this thermophilic enzyme. The enzyme activity of NOX was studied in a broad range (0–60%) of alcohol concentrations. In all studied alcohols, NOX displays optimum activity at low alcohol concentration (<20%). Under these conditions, the enzyme catalytic efficiency ($k_{cat,app}/K_{M,app}$) significantly increases mainly due to ~10-fold increase of the catalytic constant. At high alcohol concentrations (>30%), NOX activity is time dependent and slowly diminished with an apparent time constant of several hours. We found that prolonged incubation in the presence of alcohols results in: (i) dissociation of the homodimeric native structure of the enzyme and (ii) release of catalytically essential flavin cofactor from the intermonomer interface. The inactivation is fully reversible on increasing enzyme concentration and NOX activity is completely recovered even in the presence of high alcohol concentrations. Moreover, exogenous addition of the flavin cofactor dramatically stabilizes dimeric form of the enzyme and results in shift of apparent K_d to the lower values. Theoretical simulations showed that flavin dissociation and monomer/dimer equilibria are strongly coupled.

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1. Introduction

Enzymes function as highly efficient catalysts of certain reactions at specific conditions. Their properties may be further tuned by modifications of the reacting protein, by methods of protein engineering or by changing properties of the solvent, i.e. by solvent/medium engineering. There is a significant experimental and theoretical effort put in studies of the enzyme properties in the non-aqueous solvents [1–4]. Some non-aqueous solvents such as common organic solvents (octanol, DMSO, DMF), detergents, polymers and ionic liquids [5–8] have been used as medium engineering tools to explore a diversity of the enzyme catalyzed reactions. Such solvents may enable catalytic reactions not otherwise feasible in water and they can be utilized for industrial and pharmaceutical purposes [9–11]. One of the main advantages of the non-polar solvents, i.e. increased solubility of the hydrophobic substrates, may have significant impact on the action of enzymes. Non-aqueous solvents perturb the hydration of enzyme surface, often by having a deleterious effect on its catalytic efficiency. In fact, the hydration layer surrounding enzymes is critical for their catalytic performance in both aqueous and non-aqueous solvents [9]. In some cases, the water molecules in the active site play a structural role; the water molecules stabilize open and active conformations and thus affect the catalytic efficiency of the enzyme as we have shown to be the case in NADH oxidase from *Thermus thermophilus* (NOX) [12].

Water/organic solvent binary mixtures affect a number of physicochemical properties of all components of the enzymatic reaction mixture. Therefore, the effects of organic solvents on enzyme activity are rather complex and specific to each case, e.g. enzyme activity as well as enantioselectivity can be increased, decreased or unaffected [13]. In general, the enzyme activity decreases in the presence of organic solvents. Several explanations for the low catalytic power of enzymes have been suggested [9,14]: (1) diffusional limitations of the substrate and enzyme; (2) blockage of the active centre; (3) conformational change; (4) unfavorable energetics of the substrate desolvation; (5) transition state destabilization; (6) reduced conformational mobility; and (7) suboptimal pH situation. Moreover, the activities of enzymes depend on the

Abbreviations: NOX, NADH oxidase; $K_{M,app,NADH}$ and $K_{M,app,FMN}$, Michaelis–Menten constant for NADH and FMN respectively.

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method of their introduction into organic solvents. Preparation becomes critical particularly at low water concentrations [15].

Alcohols bind to proteins with low affinities with dissociation constants in the high micromolar to milimolar range. These binding affinities and competition with water molecules, due to extensive hydrogen bonding, present enormous challenges in understanding the nature of alcohol specific and nonspecific effects on protein properties. Several examples of enzyme activation at low concentrations of alcohols have been reported [8,16]. We have shown that thermophilic NOX can be activated in the presence of destabilizing reagents such as chaotropic salts and common denaturants such as urea, guanidinium chloride [17,18]. The well-known denaturing properties of alcohols indicate analogous mechanism of alcohol-induced enzyme activation [19–21].

NOX is a homodimeric flavoenzyme belonging to the nitroreductase and flavin reductase families. Three-dimensional structures of NOX and homologous proteins of this superfamily are known [22]. Enzymes of this family are oligomeric flavin containing proteins. Monomeric subunits interact *via* large interface formed by the long α-helices. The flavin moiety is placed between the subunits, which is quite rare among other flavoproteins (Tóth, Žoldák unpublished results). Biological function and interaction partners of this thermophilic enzyme *in vivo* are not known. The catalysis of NADH oxidation *in vitro* by NOX consists of two steps. The first step comprises oxidation of NADH by using the internal flavin cofactor. In the second step, the reduced flavin cofactor transfers two electrons and hydrogens to other electron acceptor (cosubstrate). The specificity for the second step is quite low and NOX may use several cosubstrates of different chemical structure (e.g. molecular oxygen, cytochrome c, external FAD, FMN, ferricyanide) [18,23–27].

High stability, low specificity and capacity to reduce large number of artificial aromatic molecules make NOX an attractive enzyme for applications in non-conventional media, especially in biotechnological applications [28–31]. Therefore, we have characterized in details NOX activity under different conditions, e.g. large ranges of pH, salt concentrations and temperatures [17,18,26,27,32]. In the present work, enzyme activity is studied in water miscible short-chain alcohols: methanol, ethanol, propanol and isopropanol, i.e. solvents which can be classified as hydrophilic solvents [16]. Our results indicate that alcohol-induced modulation of NOX activity (activation at low concentrations and inhibition at high concentrations of alcohols) is a result of the alcohols' denaturation effect on proteins.

2. Materials and methods

Sodium cacodylate, Tris/HCl, KBr were purchased from Fluka; methanol, ethanol, propanol, isopropanol, NaI, NaCl, LiCl, FMN and NADH from Sigma–Aldrich; NH_4Cl, KCl from Lachema.

2.1. Protein expression and purification

NOX was overproduced in *Escherichia coli* JM 108. The purification procedure for overproduced NOX was described previously [23–25]. The purity of isolated enzyme was confirmed by SDS/polyacrylamide gel stained with Coomassie Brilliant Blue. Apoform of the protein was prepared as described earlier [17]. All measurements were conducted with freshly prepared FMN isoform of the enzyme. Before use, the protein was dialyzed against corresponding buffer for at least 10 h.

2.2. Steady-state kinetics

Kinetics measurements were performed on a Varian Carry Bio100 spectrophotometer. The kinetic parameters were determined from the initial decrease in NADH absorbance at 340 nm ($\varepsilon_{340\,nm} = 6220\,M^{-1}\,cm^{-1}$) at 20 °C as described earlier [27]. Measurements were performed after appropriate incubation time in the dark. The enzyme concentrations were varied between 27 and 2500 nM in the presence of different alcohol concentrations (methanol, ethanol, propanol, isopropanol). Alcohols used in these experiments have low boiling points (for pure alcohols in the range of 65–97 °C) [33]. Samples were therefore placed in sealed tubes.

Standard conditions for measurements in the absence of alcohols: 50 mM sodium cacodylate, pH 7.5 containing 60 μM FMN as a cosubstrate ($\varepsilon_{445\,nm} = 12{,}500\,M^{-1}\,cm^{-1}$), and 110 μM NADH as a substrate. The enzymatic reaction was initiated by the addition of NADH. The stock solution of NADH was freshly prepared before each set of experiments. Michaelis–Menten constant was calculated from initial velocity of NADH oxidation at several substrate/cosubstrate concentrations. Spectral measurements were carried out under conditions where the entire absorbance of solution was lower than 1.5. Initial rates as a function of [NADH] substrate and [FMN] cosubstrate concentrations were fitted globally using equation for ping–pong bisubstrate mechanism:

$$v = \frac{V_{max} \cdot [NADH] \cdot [FMN]}{K_{M,NADH} \cdot [FMN] + K_{M,FMN} \cdot [NADH] + [FMN] \cdot [NADH]},$$

where $K_{M,NADH}$ and $K_{M,FMN}$ are Michaelis constants for NADH and FMN respectively. V_{max} is the maximum velocity. Chi-squared values were <0.02.

2.3. Circular dichroism

The circular dichroism spectra in the far-UV (peptide) and in the near-UV (aromatic) regions were measured by Jasco J-600 spectropolarimeter equipped with a PTC-348 WI Peltier element. Protein concentration was 3.2 μM in 50 mM sodium cacodylate buffer, pH 7.5 in the presence of various alcohol concentrations in the reaction. All samples were incubated for 2 h in the dark at 20 °C. CD spectra were accumulated 10 times.

2.4. Analytical ultracentrifugation

Sedimentation velocity experiments were performed in the Center for Analytical Ultracentrifugation of Macromolecular Assemblies located in the Department of Biochemistry at The University of Texas Health Science Center at San Antonio (USA). Sedimentation velocity studies were carried out in an XL-A Beckman analytical ultracentrifuge in a four-hole AnTi-60 rotor. The rotor speed was 50,000 rpm. Data were collected at 20 °C using optical absorption detection at 220 nm. The scans were analyzed using the UltraScan II version 9.8 data analysis software developed by Dr. Borries Demeler (http://ultrascan.uthscsa.edu/). The data were corrected for buffer density and viscosity. Value for the partial specific volume of NOX was 0.7602 cm^3/g (as calculated from the known amino acid sequence). Protein samples (6.7 μM) with or without 60% alcohol were incubated at room temperature for 24 h before sedimentation velocity experiments.

2.5. Flavin dissociation in the presence of alcohols as followed by dialysis

Dialysis experiment was performed at 20 °C. Freshly prepared FMN isoform of NOX was extensively dialyzed against the buffer or in the presence of 60% propanol using the Amicon Ultra-15 Centrifugal Filter Unit with Ultracel-10 kDa membrane. Briefly, 3.1 μM NOX was dialyzed in the presence of 60% propanol in the dark. Before and during dialysis, fluorescence intensity of the sample was measured at 521 nm after the excitation at 445 nm. Several control experiments including measurements of undialyzed samples in the

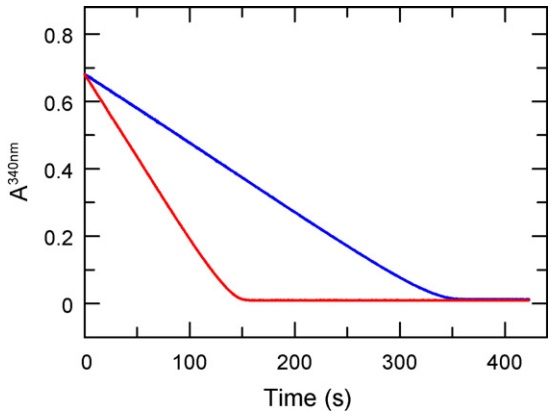

Fig. 1. NADH oxidation by NOX in the absence and presence of ethanol. Time course of the oxidation of 110 μM NADH by 27 nM NOX as monitored at 340 nm in the absence (blue curve) and presence of 20% ethanol (red curve) in 50 mM sodium cacodylate buffer, pH 7.5 and 60 μM FMN at 20 °C. Initial rates of NADH oxidation by NOX were obtained using linear regression of the experimental data in the time domain of 10–50 s. In the absence of ethanol, the slope is equal $-2.03 \pm 0.001 \times 10^{-3}$ s^{-1} (coefficient of linearity $r = -0.9999$); and in the presence of 20% ethanol, the slope is equal $-4.9 \pm 0.4 \times 10^{-3}$ s^{-1} (coefficient of linearity $r = -0.9999$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

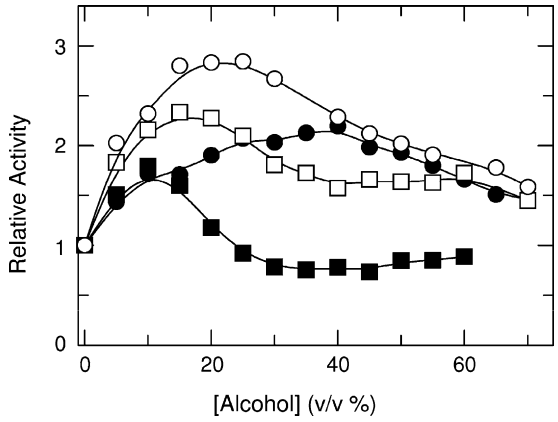

Fig. 2. Dependence of NOX activity on alcohol concentrations. The data are shown in relative values where relative activity is calculated using the value in the absence of alcohol. NOX concentration was 27 nM and FMN cosubstrate concentration 60 μM. The starting concentration of NADH substrate was 120 μM. Measurements were performed in 50 mM Na-cacodylate buffer, pH 7.5 at 20 °C after 2 h of incubation in the dark in the presence of: methanol (closed circles), ethanol (open circles), isopropanol (open squares) and propanol (closed squares). The solid curves are guide for eyes. The standard error of the measurements is ≤20% based on triplicate experiments. Normalized value represents specific activity of 11 U/mg.

absence and in the presence of 60% propanol were also performed and they were run as parallel experiments. The chemical stability of FMN and differences in lamp intensity and photomultiplier sensitivity were also checked and corrected.

2.6. Analysis of the inactivating kinetics

Experimental data were fitted into the following function:

$$A(t) = A \cdot \exp(-\lambda \cdot t) + B,$$

where $A(t)$ is a measured activity at a given time t, A is the amplitude of the reaction, λ is the apparent rate constant and B is the final activity. Time constant τ is calculated as $1/\lambda$.

3. Results

3.1. Activity of NOX is increased by alcohols

In the present study, activity of NOX was assayed using NADH as the electron donor and external FMN as the electron acceptor. Initial rate of the enzyme mediated NADH oxidation was followed by a decrease in absorbance at 340 nm (Fig. 1). The rate was increased 2.4-fold in the presence of 20% ethanol. The rate of the non-enzymatic (background) NADH oxidation was not influenced by the presence or absence of ethanol. The higher oxidation rate was therefore the result of an increased enzyme activity. The increase of the enzyme activity in the presence of ethanol was investigated further at variable solvent compositions: buffer concentration, pH and ionic strength. The concentration of cacodylate buffer was varied over the range 5–100 mM. The effect of the buffer concentration on this activation process was minor; however, the effect of pH plays an important role in NOX activity [26]. Upon decreasing the pH from 7.5 to 6.0, the enzyme activity increases 2-fold in the absence of ethanol. At pH 6.0, enzyme activity increased only 1.2-fold in 20% ethanol (data not shown), possibly as a result of coupling between pH and alcohol dependent activation. In addition, the shift of effective pH may also contribute to this effect. Salts are also very effective modulators of the NOX activity [17,18]. The effect of ionic strength induced by different cations (Na^+, K^+, Cs^+, NH_4^+) on the activity of the enzyme in ethanol at pH 7.5 was investigated in a broad range of concentrations, 0–1000 mM, of their chloride salts. The maximum activity was achieved at 0.2–0.3 M salts. Combination of the 200 mM salts and 20% of ethanol induced about 5-fold increase in the enzyme activity (data not shown). These experiments demonstrate that increasing of the enzyme activity by ethanol is a reproducible behavior observed under variable solvent compositions.

The increased enzyme activity observed in the ethanol/buffer solvent mixture prompted us to explore a broader range of alcohol concentrations (0–70%) as well as different alcohols (methanol, ethanol, propanol, isopropanol). Higher alcohol concentrations than 70% were not possible to use because of a limited enzyme solubility and phase separation problem in the case of isopropanol and propanol. Alcohol-dependent enzyme activity followed a similar pattern for all investigated alcohols (Fig. 2). Except for propanol, NOX activity was higher in all studied alcohol/buffer mixtures than in buffer only. Maximum NOX activity was achieved at different concentrations for different alcohols. These values roughly correlate with the size of the aliphatic chain, e.g. maximum activity of NOX was achieved at ~40% of methanol, ~30% ethanol, ~17% isopropanol and ~10% of propanol.

3.2. Michaelis–Menten parameters in the presence of alcohols

Increased enzyme activity in the presence of alcohol may originate from increased catalytic constant or improved substrate affinity—Michaelis constant. To establish which kinetic process is affected by alcohols, initial velocity measurements were carried out at different substrate (NADH) and cosubstrate (FMN) concentrations. There are two half-reductive/oxidative reactions, whose rates depend on NADH and FMN concentrations. From NADH and FMN, initial velocity dependencies – maximum velocity V_{max} and two Michaelis constants $K_{M,app}$ – could be derived. These parameters were obtained from global fitting procedure at 0, 20 and 60% alcohols (Fig. 3 and Table 1).

The apparent rate constant increased in the presence of low concentrations of alcohols. For example, in the presence of 20% ethanol the increase is 14-fold when compared to the value in the buffer. Michaelis constant for NADH slightly decreased at low alcohol concentration (except propanol) whereas Michaelis constant for FMN is not affected. In the presence of 20% propanol, the Michaelis con-

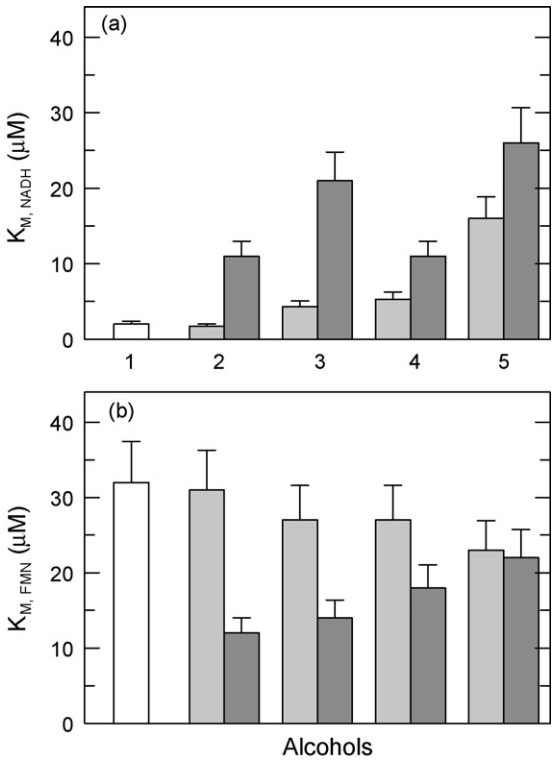

Fig. 3. Apparent Michaelis constants for (a) NADH and (b) FMN in the absence and presence of alcohols. Apparent Michaelis constants for (a) NADH and (b) FMN in the absence of alcohols (1) and in the presence of methanol (2), ethanol (3), isopropanol (4), and propanol (5). (a) Michaelis constants, $K_{M,app}$ for NADH in the presence of 20% alcohol concentration (light gray histogram) and 60% alcohol concentration (dark gray histogram). White bar represents $K_{M,app}$ in the absence of alcohols. NADH substrate concentration was within the range 5–120 μM in the presence of constant 60 μM FMN concentration. (b) Michaelis constants, $K_{M,app}$, for FMN in the presence of 20% alcohol concentration (light gray histogram) and 60% alcohol concentration (dark gray histogram). White bar represents $K_{M,app}$ in the absence of the alcohol. FMN concentration used was in the range 5–140 μM in the presence of constant 60 μM NADH concentration. In all cases, measurements were performed in 50 mM Na-cacodylate buffer, pH 7.5 at 20 °C after 2 h of incubation in the dark. Michaelis constants were calculated from the equation for ping–pong mechanism using global fitting procedure. Enzyme concentration was 27 nM.

stant for NADH slightly increased. Accordingly, it may be concluded that higher activity of NOX at low alcohol concentrations originates from improved k_{cat}.

At 60% alcohols, the catalytic constant had the values of 1.6 s^{-1}. K_M values for NADH dramatically increased, e.g. in the presence of ethanol, the Michaelis constant increased about 10-fold to 21 μM. Surprisingly, at 60% alcohol concentrations $K_{M,FMN}$ slightly decreased. Thus, at high alcohol concentration, decrease in activity is due to higher $K_{M,NADH}$ values.

In summary, the first redox half-reaction of the enzyme with NADH is much more sensitive to the presence of alcohol. Thus, the dependences of the NOX activity in the presence of alcohols are due to trade off between the increase of the catalytic constants and the decrease in NADH substrate affinity.

Table 1
Catalytic constant ($k_{cat,app}$), Michaelis constants for FMN cosubstrate ($K_{M,app}$), Michaelis constants for NADH substrate ($K_{M,app}$) and catalytic efficiency, $r = k_{cat,app}/K_{M,app}$. Experiments were performed using conditions as described in Fig. 4. Data were fitted globally using equation for ping–pong bisubstrate mechanism (c.f. Section 2). In the absence of alcohol, apparent Michaelis constants for FMN and NADH are 32 ± 2 μM and 2 ± 0.8 μM, respectively and $k_{cat,app}$ = 1.8 ± 0.1 s^{-1}.

Type of alcohol	$k_{cat,app}$ (s^{-1})	FMN $K_{M,app}$ (μM)	NADH $K_{M,app}$ (μM)
20% alcohol			
Methanol	15 ± 0.5	31 ± 2	1.7 ± 0.5
Ethanol	19 ± 1	27 ± 2	4.3 ± 0.7
Isopropanol	13 ± 0.4	27 ± 2	5.3 ± 0.6
Propanol	8.7 ± 2	23 ± 3	16 ± 2
60% alcohol			
Methanol	1.7 ± 0.1	12 ± 3	11 ± 2
Ethanol	1.5 ± 0.1	14 ± 3	21 ± 4
Isopropanol	1.6 ± 0.1	18 ± 4	11 ± 2
Propanol	1.6 ± 0.16	22 ± 5	26 ± 6

3.3. Slow inactivation of NOX is dependent on its concentration

Results of ethanol and propanol, i.e. the alcohols with the extreme effects on NOX activity, are presented. Time dependence of NOX activity was analyzed at different concentrations of the enzyme. Time courses of the relative activities of 27 and 270 nM enzyme at 20% ethanol (Fig. 4a) were measured over 24 h. At high enzyme concentration, there was no change in the enzyme activity over 24 h. In contrast, activity of 27 nM enzyme dramatically decreased to 50% of the original value within 24 h. Analysis of the reaction progress using single exponential equation showed an apparent time constant about 1 h. In the presence of 20% propanol, even 270 nM enzyme showed small (<10%) degree of the inactivation over the time (Fig. 4b). At 27 nM enzyme, its inactivation was very dramatic and the initial value of the activity dropped by more than 80%. Analysis of the activity decay led to an apparent time constant of ~8 h for this inactivation process. Time dependent inactivation of the enzyme was found also in 20% methanol (about 15% inactivation) but not in 20% isopropanol. There was no inactivation of the enzyme at high protein concentrations in the presence of 20% alcohols (methanol, ethanol, and isopropanol).

The only condition at which significant inactivation of NOX at 270 nM concentration occurred was in the presence of 60% propanol (Fig. 4c). This inactivation was very slow and was not finished within 25 h. According to monoexponential fit, an apparent time constant was longer than 30 h. Upon 1 h and 25 h of incubation in 60% propanol, 27 nM enzyme displayed similar $K_{M,app}$ constants for NADH of 13.0 and 14.5 μM (data not shown). The alcohol-induced inactivation of NOX that depends on the enzyme concentration indicates an effect of alcohols on the quaternary structure of NOX.

3.4. Inactivation of NOX in the presence of 20% propanol is a reversible process

To demonstrate that inactivation is a result of the reversible monomer/dimer equilibrium, where the monomer is inactive and the dimer active form of NOX, we have developed an assay for it. In this assay, the protein concentration is stepwise increased and thus the equilibrium is shifted towards dimeric active species. In the case of irreversible and/or monomolecular inactivation, the inactivated enzyme cannot be re-activated by high enzyme concentration. Re-association of the protein monomer into the active dimer was assessed from measurements of the initial rates. Such assay might be useful also for other multimeric proteins where the enzyme function is strongly related to its oligomeric state.

NOX was analyzed after incubation in 20% propanol. Due to extremely slow inactivation, the total incubation time of the enzyme with propanol was 240 h in the dark at 20 °C. In this assay, three independent and parallel sets of experiments were performed. Measurements of the initial velocities were performed every 24 h. In the first set of experiments, enzyme concentration was held constant—27 nM. The enzymatic activity was decreased exponentially, and after 240 h, the remaining activity was of ~20% of its original level. In the second set of experiments, the enzyme concentration was 270 nM. At this protein concentration, there was observed only a negligible decrease in the enzymatic activ-

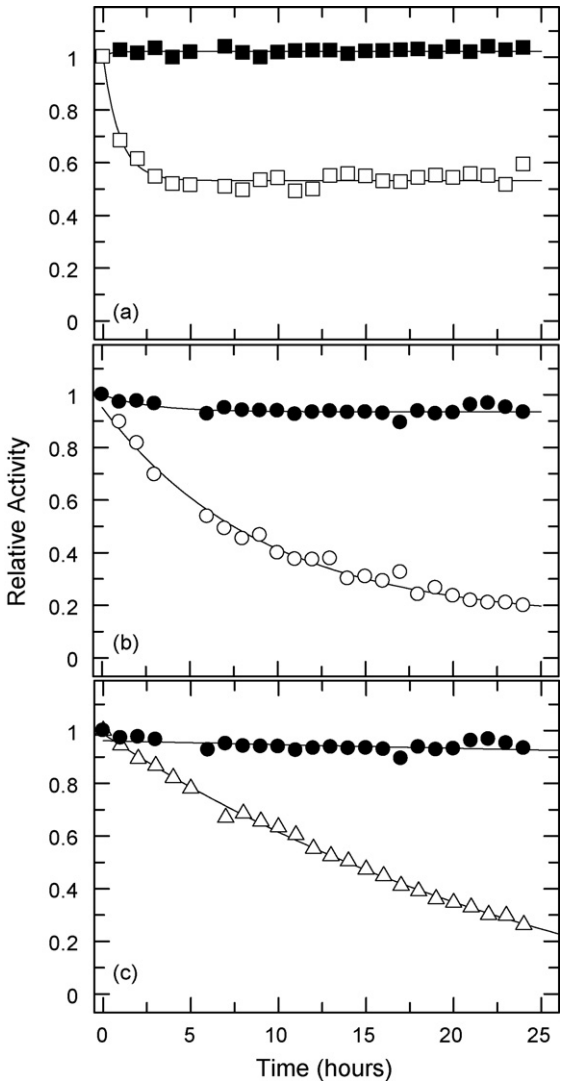

Fig. 4. Time dependent relative activity of NOX in the presence of alcohols. Time course of relative NOX activity in the presence of (a) 20% ethanol, (b) 20% propanol at 27 nM (white symbols) and 270 nM (black symbols) of NOX; and (c) in the presence of 20% (black circles) and 60% (white triangles) propanol at 270 nM NOX. Here, the relative activity was calculated according activity at time $t = 0$ in the presence of alcohols. Protein samples were prepared and equilibrated at 20 °C in the dark. After selected time, 1 mL aliquot was taken and activity was measured in the presence of 60 μM FMN. The reaction was started after addition of 110 μM NADH. Measurements were performed in 50 mM Na-cacodylate buffer, pH 7.5 at 20 °C. Each point represents independent measurement of the initial velocity NADH oxidation (c.f. Fig. 2). Solid curves passing the black symbols are only guide for eyes. Solid curves passing the white symbols are curve fits of the experimental data using single exponential equation. The apparent time constants, amplitudes and offset of the reaction are (a) $\tau \sim 1$ h, $A = 0.46$ and $o = 0.54$ for inactivation of 27 nM NOX in 20% ethanol; (b) $\tau \sim 10$ h, $A = 0.86$ and $o = 0.14$ for inactivating of 27 nM NOX in 20% ethanol, and (c) $\tau > 30$ h, $A \sim 1$ and $o = 0$ for 270 nM NOX in 60% propanol.

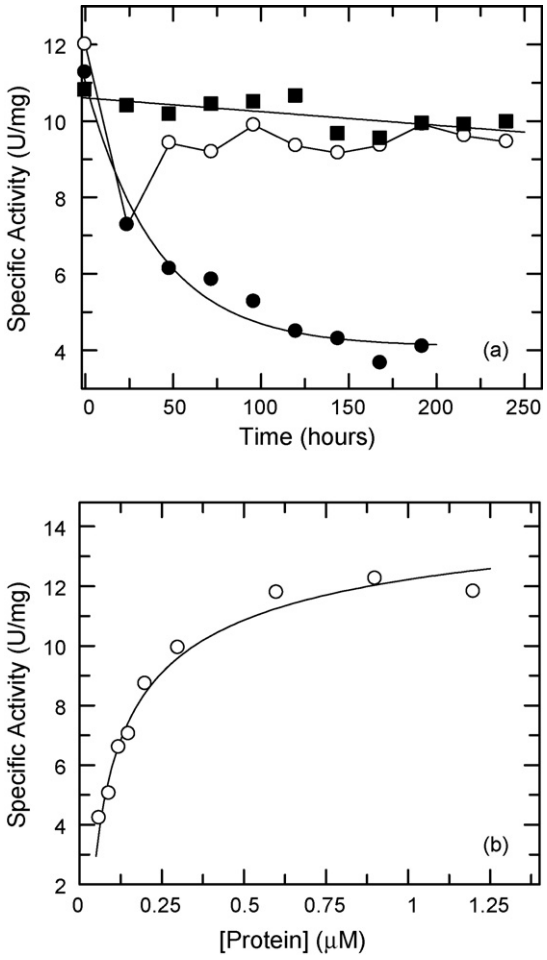

Fig. 5. Reactivation of the enzyme in the presence of 20% propanol. (a) Re/activation of NOX in 20% propanol after sequential addition of the enzyme. The enzyme activities at concentration of 270 nM (black squares) and 27 nM (black circles) were assayed over 10 days. Every 24 h, NOX concentration was stepwise increased (white circles) and the activity of the mixture was measured. After 10 days, concentration of NOX reached the value of 270 nM. Specific activity was calculated to take into account total amount of the enzyme. The control experiments using stock enzyme solution were performed in a parallel fashion and displayed no changes. Solid curves are only guide for eyes. (b) Dependence of the specific activity on enzyme concentration. The samples were equilibrated 20 days at room temperature. The control experiments using stock enzyme solution were performed in a parallel fashion. Solid curve is obtained from non-linear regression fit of the monomer/dimer equilibrium with K_d of 100 nM. Measurements were performed in 20% propanol, 50 mM sodium cacodylate buffer, pH 7.5 and 45 μM FMN at 20 °C. The reaction was started after addition of 110 μM NADH.

ity after 240 h of incubation. In the third set of experiments, the starting concentration of the enzyme was 27 nM and concentration was increased by 27 nM (from the high concentrated stock solution) every 24 h after initial velocity measurement. The activity of highly concentrated enzyme stock solution (necessary for sequential aliquots in the third experiment) measured at beginning and at the end of the assay showed no inactivation of NOX at given conditions. After 240 h, the final concentration of the enzyme was 270 nM. Specific activities (normalized on amount of the protein) in the second and third experiments were very similar (Fig. 5). In the first set of measurements, the enzyme activity was about 20%. This indicates that the low activity of the 27 nM enzyme in the presence of propanol could be regenerated by increased concentration of the protein. This strongly indicates that alcohol-induced inactivation is due to reversible dissociation of the active homodimeric form of NOX.

3.5. Secondary structure of NOX is unaffected by alcohols

Structural properties of the enzyme in the presence of alcohols were investigated by circular dichroism (CD) in the far-UV region. The CD spectrum of NOX in buffer shows two minima, typical for a protein with a high α-helical content [27,32]. CD spectra were measured in the presence of all investigated alcohols (data not shown). The effect of propanol on the secondary structure of NOX in the presence of both 20% and 60% propanol after 1 h of incubation was evaluated. We observed a small increase in the intensity of the CD band indicating minor changes of the enzyme conformation after immediate addition of propanol (Fig. 6). Further incubation of NOX

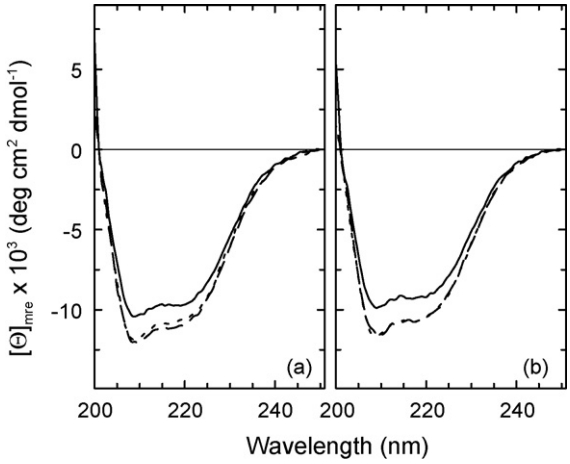

Fig. 6. Circular dichroism spectra of NOX in the presence and absence of alcohols. Circular dichroism spectra of NOX in the far-UV region in the absence of alcohols (solid curve) and in the presence of 20% (dashed curve), 60% propanol (dotted curve). Time of incubation was (a) 1 h and (b) 24 h. NOX concentration was 3.2 μM in 50 mM sodium cacodylate buffer, pH 7.5 and absence or presence of alcohol at 20 °C.

in 60% propanol showed no changes in the secondary structure of NOX.

3.6. Quaternary structure of NOX is perturbed in the presence of alcohol

Sedimentation velocity analysis was performed in order to determine the quaternary structure of NOX in the presence of 60% alcohols after ~24 h incubation (Fig. 7). Due to technical limitations, analytical centrifugation experiments were possible only at relatively high protein concentration, ~4 μM. NOX in the reference buffer (50 mM Na-cacodylate buffer, pH 7.5 at 20 °C) was found to be homogeneous as analyzed by sedimentation velocity. The sedimentation coefficient of NOX had the expected value of about 3.3 S (Fig. 7). This sedimentation coefficient was almost identical to the one obtained previously and assigned to dimeric NOX [26]. The incubation of NOX in the presence of 60% alcohol induced an increased heterogeneity of NOX population in the following order: methanol < ethanol < isopropanol < propanol. While in methanol the protein is homogeneous with the sedimentation coefficient 3.3 S, in the presence of propanol the protein is quite

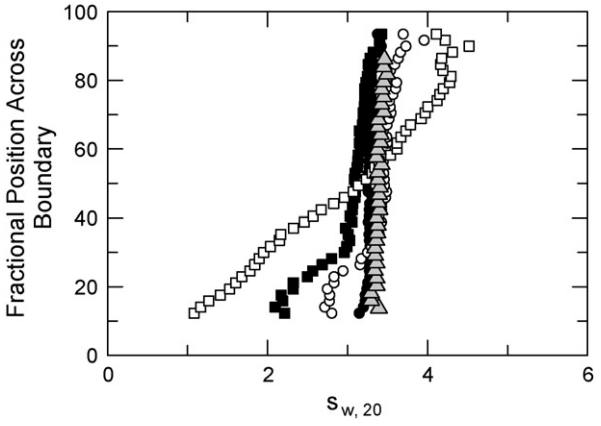

Fig. 7. Analytical ultracentrifugation of NOX in the absence and presence of 60% propanol. Sedimentation constants s_{20w} obtained from analytical ultracentrifugation of NOX in 50 mM Na-cacodylate buffer, pH 7.5 without (gray triangles) and in the presence of 60% methanol (open circles), ethanol (closed circles), isopropanol (closed squares) and propanol (open squares) propanol. Sedimentation velocity experiments were performed as described in Section 2.

heterogeneous with sedimentation coefficients in the range from 0.8 to 4.0 S. It appears that high (60%) concentration of propanol induced dissociation of the dimeric native structure of NOX and high heterogeneity of the sample.

4. Discussion

Catalytic activities of enzymes in water/organic solvents are usually dramatically decreased; several responsible factors were suggested [9,14]. Activity of NOX in the four different water/alcohol binary mixtures, however, displayed optimum at low alcohol concentrations. Enzyme activity increased at low (<20%) alcohol concentration and decreased at high (>40%) alcohol concentration. The mechanism of the increased enzyme activity in the presence of alcohols is not clear. The activation of NOX is a fast process as no evidence for a time-dependent activation could be obtained. Interestingly, the activity of α-chymotrypsin and other enzymes are slightly increased in several binary mixtures of water and organic solvents [16,34–38]. In the case of NOX, low concentrations of the alcohols improve catalytic efficiency of the enzyme.

This rapid activation is limited by the slow inactivation. NOX activity in the presence of alcohols likely reflects changes in thermodynamic activity of substrate and enzyme and consequent differences in the substrate solvation and enzyme hydration. A simple theoretical model, taking into account hydration of enzyme and variation of thermodynamic activities, predicts that mainly the Michaelis constant, $K_{M,app}$, is affected whereas k_{cat} should remain unaffected in the presence of organic solvent [39]. In the case of NOX, apparent $K_{M,app}$ constant for FMN decreased in the presence of 60% alcohol; this is consistent with the model. On the other hand, the apparent $K_{M,app}$ constant for NADH is increased at high alcohol content. This indicates that binding of NADH is much weaker in the presence of alcohol than in the aqueous solution and that substrate solvation is affected by additional factors, e.g. interactions between NADH and protein moiety can be affected by decreased hydrophobicity and electrostatic effects. In contrast with the prediction of the above-mentioned model, in the case of NOX $k_{cat,app}$ increases 14-fold and overcomes the decrease in the Michaelis constant. Thus, activation of NOX is governed by the increase in the apparent $k_{cat,app}$ value; this is not expected from the changes in the thermodynamic activity coefficient and thus strongly implies additional factor(s), e.g. local structural change of the enzyme active site.

4.1. On the prediction of enzyme denaturation in the presence of alcohols

In the presence of water miscible organic solvents, proteins tend to denature due to weakening of hydrophobic interactions and modulation of electrostatic interactions. In fact, organic solvents decrease the dielectric constant of the solvent and therefore, unfavorable long-ranged electrostatic interactions are inefficiently screened. This might be the mode of action of alcohols in the case of NOX that contains large excess of the positive charges at the dimeric interface region. There are several models predicting the denaturing tendency of organic solvents [40–43]. Some of the models are based on partitioning between water/organic solvents; on solubility studies and on free energy micellisation [42,43]. Rosell et al. used naphatalene solubility to predict denaturing tendency based on simple hydrophobic effect [44]. Solubilisation coefficients for naphatalene are known for all investigated alcohols: methanol (0.37 M^{-1}), ethanol (0.62 M^{-1}), isopropanol (1.04 M^{-1}), and propanol (1.33 M^{-1}). These coefficients correlate with reciprocal value of the critical concentration, which is defined as concentration of the organic solvent causing 50% denaturation [41]. Based on this scale, propanol is the most powerful dena-

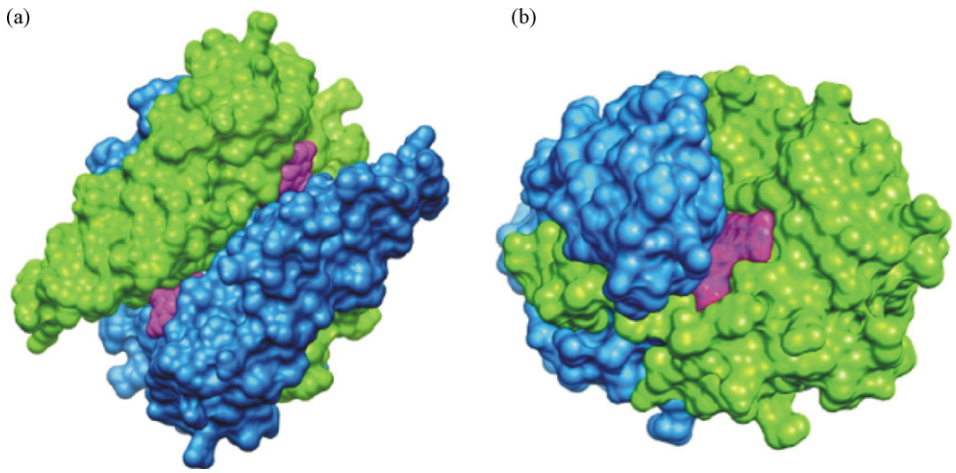

Fig. 8. Three-dimensional structure of homodimeric NADH oxidase from *Thermus thermophilus* (pdb code 1nox). Shown is the structure as molecular van der Waals surface from (a) top view and (b) side view. Subunits are colored as green and blue, respectively. FMN cofactor is shown as magenta colored molecular surface. The figure was prepared using UCSF Chimera [41]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

turing solvent for proteins. Methanol is the weakest and high concentrations are necessary for protein denaturation. A similar conclusion can be also drawn from effect of alcohols on NOX. The binary mixture propanol/water is the strongest denaturating binary mixture and the enzyme activity is dramatically decreased in contrast with the other alcohols, even structurally similar isopropanol. For weakly denaturing alcohols such as ethanol, methanol, the activity versus alcohol concentration shows wide distribution in contrast with isopropanol and propanol that show relatively narrow profiles. The width of the dependence of the enzyme activity on concentration of organic alcohols likely reflects the alcohols' ability to reduce hydrophobic interactions. Taking into account denaturing properties of alcohol mixtures, we suggest that the destabilization of NOX dimeric interface is the reason for the observed increase in its activity. This is in accordance with our previous observations that mild concentrations of the denaturing reagents urea or guanidinium chloride significantly enhance NOX activity [17,18].

4.2. Non-first-order kinetics of enzyme inactivation indicate a role of protein–protein interaction

Our experiments indicate that irreversible inactivation, aggregation and presence of less active forms of the enzyme can be ruled out as the result of the following observations: (i) inactivation process is absent at high enzyme concentrations; (ii) in the presence of 60% propanol, the apparent rate constant is dependent on the protein concentration; (iii) the enzyme activity is recovered after addition of high enzyme concentration; and (iv) the Michaelis constant for NADH did not change during the process of the inactivation. In the case of irreversible reaction, the enzyme should be completely inactivated with no residual activity after sufficient time. However, both in ethanol and propanol, there were significant residual activities specific to NOX that remained constant in the time but depend on the enzyme concentration. This points to a reversible process. Apparently no inactivation occurred at high enzyme concentrations; this indicates the presence of limiting second-order kinetic process, i.e. association of the monomers at low protein concentrations. To reconcile the above observations, we suggest that NOX undergoes reversible dissociation of its homodimeric structure:

$$M_2\,(\text{active}) \rightleftarrows 2M\,(\text{inactive})$$

where M_2 is the native dimeric protein and M is the monomer. At low enzyme concentrations association is slower, rate limiting. Due to slow association, a significant population of inactive monomeric species is presented. At high enzyme concentration, association is faster; population of active dimeric species increases. Such a mechanism is supported by the three-dimensional structure of the enzyme. The active site of the enzyme is placed in a cleft between subunits and the cofactor is also stabilized by the interaction with both monomers (Fig. 8). In the monomer form at acidic pH, NOX is not able to bind flavin cofactor and is inactive [26]. Dissociation and association of flavin cofactor in the presence of propanol was experimentally observed using equilibrium dialysis method (c.f. Section 2).

4.3. Contribution of the flavin binding to monomer/dimer equilibrium

In the next step, we analyzed reversible subunit and flavin dissociation and performed simulation of the model which mechanistically proceeds in the two steps: (i) FMN binding reaction to dimeric apoform of the enzyme and (ii) monomer/dimer equilibrium. Overall mechanism is described by the two dissociation constants:

$$M_2 \leftrightarrows 2M$$

$$M_2 + 2\text{FMN} \leftrightarrows M_2{\cdot}\text{FMN}_2$$

The monomer/dimer equilibrium is calculated from the dependence of specific activity on total enzyme concentration. We found that K_d of FMN binding is ~100 nM in the presence of 60% propanol. The flavin affinity is K_d 90 nM in the absence of alcohols [22]. For simplicity, K_d for FMN binding can be considered as independent of alcohol concentration. These parameters were used for simulation of the overall reaction and for determination of the relative population of the monomeric and dimeric species. Simulation showed that under this set of conditions, the presence of FMN largely stabilizes dimeric flavin bound species (blue dashed curve) and shifts K_d towards lower values by several order of magnitudes. Dimerisation and flavin binding are strongly coupled reactions and as indicated from overlapping dependences of flavin binding and dimeric fraction of NOX on the protein concentration (Fig. 9). In absence of FMN, dimer is destabilized and dissociation occurs at higher concentrations of the protein. These dependences strongly indicate that both reactions (flavin and dimer dissociations) are energetically coupled, which results in apparent single step dissociation mechanism.

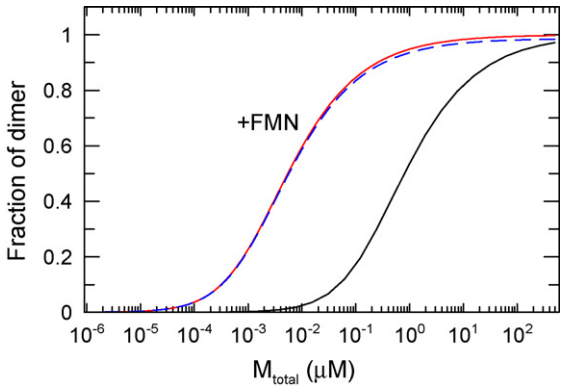

Fig. 9. Fraction of dimer as function of the total protein concentration. Fraction of dimer was calculated assuming two-step mechanism, i.e. the monomer/dimer equilibrium and flavin binding in the dimeric form. Solid curves show dimeric species, i.e. $M_2FMN_2 + M_2$ in the absence (black curve) or presence 45 μM FMN (red curve). Blue dashed curve shows active enzyme molecules M_2FMN_2. For simulation following parameters were used [FMN] = 45 μM (blue and red curves) and [FMN] = 0 μM (black curve), K_d for flavin binding and monomer/dimer equilibrium was 90 nM and 100 nM, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

4.4. Reversible dissociation of NOX in the presence of water/alcohol mixtures

The activity assay showed that the inactivation of NOX is a reversible enzyme concentration-dependent process (Fig. 5). NOX quaternary structure is strongly related to its function because its active site crevice is placed directly in the intermonomeric interface and the cofactors are stabilized by the both monomers (c.f. Fig. 8). The dependence of NOX activity on alcohol concentration is likely the result of two consecutive processes: (i) activation of NOX in low alcohol concentrations due to perturbation/increased dynamics of its active site and (ii) perturbation of the quaternary structure at high alcohol concentrations (the most pronounced in propanol) accompanying the loss of NOX activity.

During the catalytic cycle, enzyme's active site proceeds through conformational changes. Our previous data showed that weakening and slight destabilizing of the protein structure (by chaotropic salts and denaturants) is accompanied by an increase in activity of NOX [17,18,27]. Molecular dynamic simulations of NOX indicated how protein dynamic can regulate enzyme activity [12]. The key event is cofactor assisted gating mechanism, where the flavin cofactor actively contributes to the opening and closing of the active site by tryptophan 47. Slight destabilization of the local active site structure by alcohols may be responsible for activation of enzyme.

Dissociated monomer with native-like secondary structure is completely inactive. Apparently, the active site composed by amino acids of both monomers and flavin cofactor stabilization achieved by the contribution of interaction of both monomers are important factors for activity and stability of the active conformer of NOX. Indeed, unusually placed cofactor/active site within this family of enzymes into interface might reflect the evolution towards achieving a high degree of stability at the physiological temperatures of thermophiles with relatively short polypeptide chains [45–47] and sufficient flexibility of the active site [18,27].

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