ISSN 0003-6838, Applied Biochemistry and Microbiology, 2017, Vol. 53, No. 2, pp. 157–164. © Pleiades Publishing, Inc., 2017.
Original Russian Text © T.V. Tikhonova, E.S. Slutskaya, V.O. Popov, 2017, published in Prikladnaya Biokhimiya i Mikrobiologiya, 2017, Vol. 53, No. 2, pp. 155–163.

Peroxidase Activity of Octaheme Nitrite Reductases from Bacteria of the *Thioalkalivibrio* Genus

T. V. Tikhonova*, E. S. Slutskaya, and V. O. Popov

Bach Institute of Biochemistry, Research Center of Biotechnology, Russian Academy of Sciences, Moscow, 117091 Russia
**e-mail: ttikh2008@yandex.ru*

Received September 30, 2016

Abstract—Closely related penta- and octaheme nitrite reductases catalyze the reduction of nitrite, nitric oxide, and hydroxylamine to ammonium and of sulfite to sulfide. NrfA pentaheme nitrite reductase plays the key role in anaerobic nitrate respiration and the protection of bacterial cells from stresses caused by nitrogen oxides and hydrogen peroxide. Octaheme nitrite reductases from bacteria of the *Thioalkalivibrio* genus are less studied, and their function in the cell is unknown. In order to estimate the possible role of octaheme nitrite reductases in the cell resistance to oxidative stress, the peroxidase activity of the enzyme from *T. nitratireducens* (TvNiR) has been studied in detail. Comparative analysis of the active site structure of TvNiR and cytochrome c peroxidases has shown some common features, such as a five-coordinated catalytic heme and identical catalytic residues in active sites. A model of the possible productive binding of peroxide at the active site of TvNiR has been proposed. The peroxidase activity has been measured for TvNiR hexamers and trimers under different conditions (pH, buffers, the addition of $CaCl_2$ and EDTA). The maximum peroxidase activity of TvNiR with ABTS as a substrate (k_{cat} = 17 s^{-1}; k_{cat}/K_m = 855 mM^{-1} s^{-1}) has been 100–300 times lower than the activity of natural peroxidases. The different activities of TvNiR trimers and hexamers indicate that the rate-limiting stage of the reaction is not the catalytic event at the active site but the electron transfer along the heme c electron-transport chain.

Keywords: octaheme nitrite reductase, peroxidase activity, oxidative stress

DOI: 10.1134/S0003683817020168

INTRODUCTION

Two families of multiheme nitrite reductases containing five or eight hemes C bound to a single polypeptide chain have been described at the present time [1–6]. Enzymes of both families catalyze six-electron nitrite reduction to ammonia. Pentaheme cytochrome *c* nitrite reductases (NrfAs) have been isolated from different classes of microorganisms and characterized in detail [1–4, 7–12]. The main role of NrfAs in the cell is considered to be related to its participation in respiration in bacteria that use nitrate as a final electron acceptor. In addition to nitrite reduction, NrfAs catalyze the reduction of nitrogen oxides (NO, N_2O), hydroxylamine, and sulfite [13–17]. Wide substrate specificity provides the key role of NrfAs in protective processes of the bacterial cell against stress caused by the presence of nitrite, nitrogen oxides, and hydroxylamine [18–20]. In the ε-proteobacterium *Wolinella succinogenes,* NrfA makes the defining contribution to cell resistance to oxidative stress caused by the presence of hydrogen peroxide [18].

Octaheme nitrite reductases have been less characterized. Two representatives of octaheme nitrite reductases have been isolated from closely related bacterial species of the *Thioalkalivibrio* genus at the present time: *T. nitratireducens* and *T. paradoxus* [5, 6, 21, 22]. In addition to nitrite reduction to ammonium, both enzymes catalyze two-electron reduction of hydroxylamine to ammonium and six-electron reduction of sulfite ion to sulfide. The enzymes have highly homologous amino acid sequences and similar spatial structures [6, 22]. Both proteins contain seven hemes *C*, which are coordinated by two histidine residues in axial positions. The eighth heme, the catalytic one, is coordinated by the lysine residue in the proximal position; the distal position is occupied by either a water molecule or hydroxide ion. The active site localizes at the distal side of the catalytic heme and includes tyrosine, histidine, and arginine residues. The structure of the active site and the folding of five out of eight hemes coincide in penta- and octaheme nitrite reductases [22]. Special features of octaheme nitrite reductases include the presence of an additional covalent bond between the catalytically important Tyr303 residue at the active site and the neighboring Cys305 residue, the presence of a second domain containing three additional hemes C, and the formation of a stable hexameric structure in solution [22].

The role of octaheme nitrite reductases in *T. nitratireducens* and *T. paradoxus* cells has not been revealed. It is known that *T. nitratireducens* is not able to use

nitrite as an electron acceptor upon culturing under anaerobic conditions [23, 24]. Nitrate growth results in nitrite accumulation in medium. *T. paradoxus*, in turn, does not use compounds of nitrogen with oxygen in respiration upon anaerobic growth; moreover, assimilatory nitrate and nitrite reduction is absent in this bacterium [25]. However, octaheme nitrite reductases are synthesized by cells of both bacteria, and high nitrite reductase activity has been found in cell homogenates with reduced methyl viologen as an electron donor.

We can suppose that, similar to NrfAs, octaheme nitrite reductases can participate in the protection of the bacterial cell from stress caused by the presence of such toxic agents as hydrogen peroxide and nitrogen oxide. We have previously shown that nitrite reductase from *T. nitratireducens* (TvNiR) has remarkable peroxidase activity [21].

The purpose of the work is to study the effect of reaction conditions on hydrogen peroxide reduction catalyzed by TvNiR, to compare the peroxidase activities of TvNiR and natural cytochrome c peroxidases, and to analyze structural peculiarities defining TvNiR peroxidase activity.

METHODS

Reagents used in the work. The work was carried out with the following reagents: **ABTS** (sodium 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonate), Sigma, United States); methyl viologen (Sigma, United States); sodium dithionite (BDH chemicals, Great Britain); N-1-(naphthyl)ethylenediamine (Sigma, United States); $CaCl_2$ (Merck, Germany); and EDTA (Sigma, United States). All other reagents were produced in Russia and were of analytical grade. All solutions were prepared with water purified in a Milli Q instrument (Millipore, United States).

T. nitratireducens ALEN 2 cells were maintained and cultured as described in [21, 26].

A TvNiR homogeneous preparation from the soluble fraction of *T. nitratireducens* homogenate was obtained by the two-stage method [26], including low-pressure anion exchange chromatography with DEAE-sepharose and gel filtration chromatography in a Superdex^{TM} 200 column (GE Healthcare, United States) in an AKTA FPLC chromatograph (GE Healthcare). The homogeneity of TvNiR preparations was controlled by denaturing electrophoresis (SDS-PAGE).

The protein concentration in TvNiR preparations was determined by Bradford protein assay.

Determination of TvNiR activities. The nitrite reductase activity of TvNiR preparations was determined by the rate of a decrease in the nitrite concentration as described in [26] with methyl viologen reduced with dithionite as an electron donor. The amount of the enzyme that catalyzes the diminution of 1 μmol of NO_2^- per 1 min was taken as a nitrite reductase activity unit.

Peroxidase activity was determined spectrophotometrically by the rate of ABTS oxidation (ε_{414} = 31.1 mM^{-1} cm^{-1}) in the presence of hydrogen peroxide. The reaction mixture contained 0.1 M HEPES buffer, pH 7.0 (or 0.1 M potassium phosphate buffer, PPB, pH 7.0), 0.2 mM ABTS, 1.0 mM hydrogen peroxide, and the enzyme in a concentration of 1.2–8 μg/mL. All measurements were performed at 30°C. The amount of the enzyme that catalyzes the diminution of 1 μmol H_2O_2 per 1 min was taken as a TvNiR peroxidase activity unit.

All kinetic experiments were performed under conditions of the linear dependence of the reaction rate on the TvNiR concentration. Michaelis–Menten constants by ABTS and hydrogen peroxide were determined at a fixed concentration of the second substrate, which was close to saturation.

The following buffer systems were used in order to determine the pH optima of TvNiR peroxidase and nitrite reductase activities: 0.2 M CH_3COONa–CH_3COOH (pH 4.0–6.0), 0.1 M HEPES buffer (pH 6.0–8.0). A buffer change at a pH of 6.0 did not affect the reaction rate.

In order to test the effect of Ca^{2+} ions on activity, the enzyme was transferred from 0.1 M PPB to 0.1 M HEPES buffer, pH 7.0, supplemented with a $CaCl_2$ solution (1.0–5.0 mM), and incubated for 15 min; after that the peroxidase and nitrite reductase activities were measured. Upon the study of the EDTA effect, the EDTA solution (100 mM) was preliminary titrated to pH 7.0.

In order to study the effect of nitrite on TvNiR peroxidase activity, concentrations of nitrite and peroxide varied in an interval of 0–5 mM and 0.5–1 mM, respectively.

Modeling and visualization of the structure of TvNiR complex with peroxide was performed with PyMOL software. The structure of TvNiR complex with nitrite (PDB ID 3RKH) was used as the initial model.

RESULTS AND DISCUSSION

Structural bases for the peroxidase activity of multiheme nitrite reductases. It is known that cytochrome *c* [27], its derivatives [28], and microperoxidases with various lengths of the peptide chain based on it [29] are able to catalyze both nitrite reduction (nitrite reductase activity) and oxidation of a number of substrates in the presence of hydrogen peroxide (peroxidase activity). In both cases the reaction occurs on heme C; moreover, the reaction rate increases with a transfer from a six-coordinated bis-histidine heme as in the native cytochrome *c* to a five-coordinated heme as in carboxymethylated cytochrome *c* (CM-cyt *c*) and microperoxidases 11 and 99 (table).

Active sites of specific heme-containing peroxidases (peroxidases of plants, fungi, and bacteria [30–35]) have been found to contain a number of catalytically important conservative amino acid residues (Fig. 1) causing remarkable acceleration of the reaction as compared to cytochrome c. Their location, orientation in relation to each other and the iron ion, heme environment in general, and redox properties define biochemistry of the whole process. Conservative amino acid residues in the active site of nearly all known peroxidases are represented by histidine and arginine, which are localized on the distal side of the catalytic heme, and histidine and aspartic acid, which are localized at the proximal position. The most catalytically active cytochrome c peroxidases (CCP, table) contain tryptophane, which is located on the proximal side of the catalytic heme and bound to it by van der Waals interactions, in addition to the mentioned residues (Fig. 1) [36, 37].

According to the commonly accepted mechanism of peroxidase catalysis [36–38], the histidine residue (His52 at Fig. 1) situated at the distal side of the catalytic heme performs acid-base catalysis, promoting the detachment of a proton from a peroxide molecule at the first stage of the reaction, which further binds with the catalytic heme in the form of peroxide anion. A proton from the histidine imidazole is then transferred to the second atom of peroxide's oxygen with the subsequent detachment of a water molecule and the formation of ferryl cation radical (Fe(IV)=O). Distal arginine (Arg48, Fig. 1) promotes both the right orientation of hydrogen peroxide at the enzyme active site and its heterolytic cleavage.

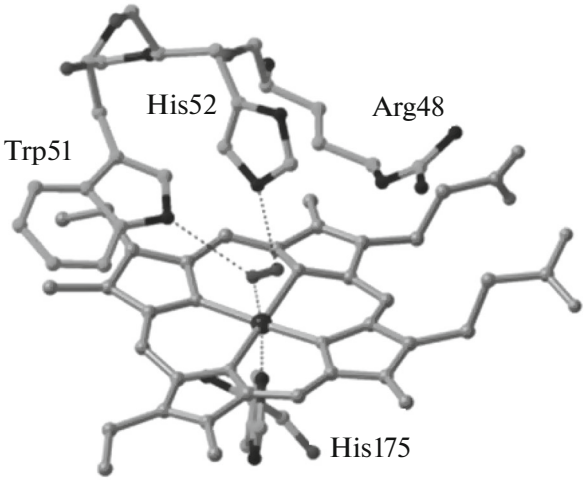

Fig. 1. Structure of the active site of cytochrome c peroxidase from *Saccharomyces cerevisiae* [36]. Catalytically important amino acid residues are shown. A peroxide molecule is bound at the active site with the formation of a coordination bond with Fe(III) ion and hydrogen bonds with Trp51 Nε and His52 Nε. Numeration of amino acid residues is presented in accordance with PDB (2CYP) [38].

As has been already noted, the active site of TvNiR is a heme C coordinated by a lysine residue (Lys188) in the proximal position [22]. The distal position of the catalytic heme in the crystal structure of the free enzyme is occupied by a water molecule, which forms hydrogen bonds with Arg131, Tyr303, and His361 residues that localize in close proximity to the catalytic heme (Fig. 2). When the water molecule is substituted with nitrite, it forms a coordinate bond with the

Kinetic parameters of the peroxidase reaction catalyzed by various cytochrome *c* containing proteins

Enzyme	Peroxidase activity					
	pH	substrate	A_{sp}, μmol/min mg	k_{cat}, s^{-1}	$K_м$, mM	$k_{cat}/K_м$, $mM^{-1}\ s^{-1}$
TvNiR hexamer (380 kDa)	7.0	ABTS	0.17	0.17	0.02	8.5
	5.0	ABTS	1.41	1.38	0.02	70
TvNiR trimer (190 kDa)	7.0	ABTS	6.60	6.50		
	5.0	ABTS	17.45	17.1		(855)
Peroxidase from horseradish roots [41]	6.8	ABTS	1045	750	0.18	4.1×10^3
	5.0	ABTS	1150	810	0.27	3.0×10^3
Peroxidase from soy beans [41]	6.8	ABTS	2110	1230	173	7.1
	5.0	ABTS	4565	2660	45	59.2
CCP from *S. cerevisiae* [36]	5.0	ABTS				4.5×10^4
CM-cyt *c* [28]	7.0	ABTS				0.017
	5.0	ABTS				0.072
	3.5	ABTS				0.21
Cyt *c* [28]	6.0	H_2O_2				0.24×10^{-3}

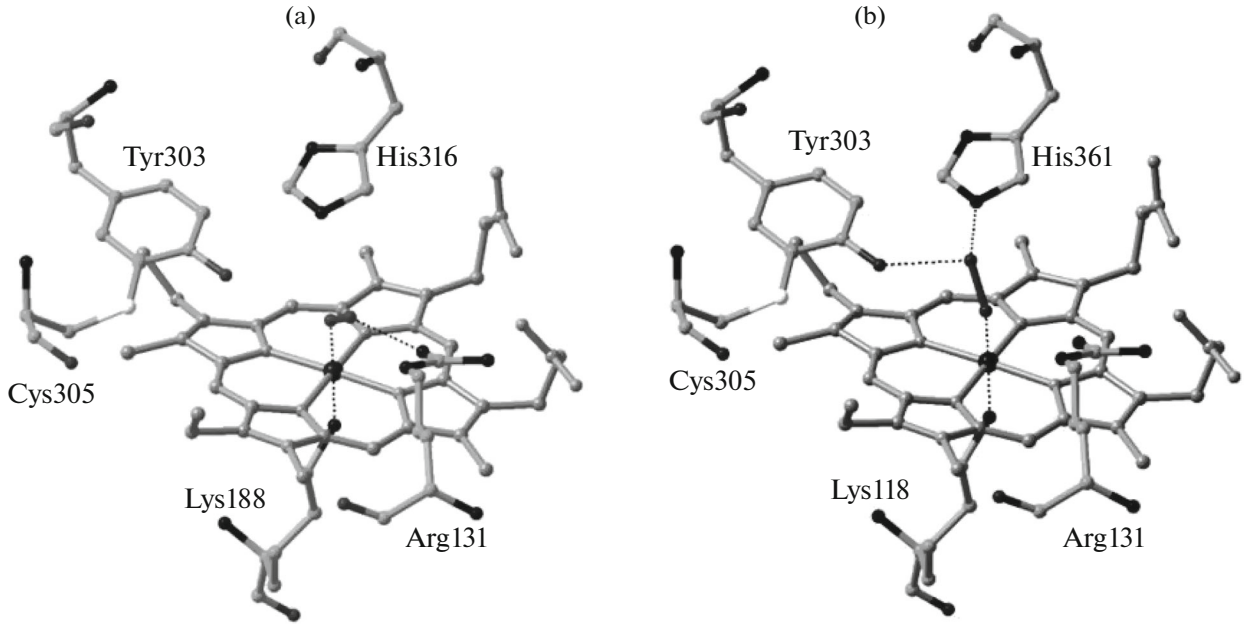

Fig. 2. Structure of the active site of the TvNiR complex with nitrite (PDB ID 3RKH) with an inserted peroxide molecule coordinated by O1 atom with Fe(III) atom of the catalytic heme. Two orientations of peroxide at the active site by bonds O1–N and O2–N of a nitrite molecule are shown: a—peroxide binding is accompanied by the formation of hydrogen bonds between O2 and Tyr303 and His361 residues, b—between O2 and the guanidine group of Arg131. The structure of the active site and the peroxide molecule are represented in the form of the ball-and-stick model.

Fe(III)/Fe(II) ion of the catalytic heme via the nitrogen atom; one nitrite oxygen atom (O1) forms a hydrogen bond with Arg131, and the second one (O2) bonds with His361 and Tyr303. It has been supposed that Arg131 participates in the orientation of a substrate molecule and stabilization of reaction intermediates [22], while His361 and Tyr303 participate in catalysis, activating the substrate via additional polarization of bonds and playing the role of proton donors. Modeling of the possible binding of a peroxide molecule at the TvNiR active site showed that two localizations of a HO–OH molecule are possible: by bonds O1–N and O2–N of a nitrite molecule (Fig. 2a, 2b). In both cases a coordination bond between an iron ion in the active site and one of peroxide oxygen atoms (O1) is formed. The oxygen atom O2 in one case forms hydrogen bonds with Tyr303 and His361 residues upon the binding of peroxide at the CCP active site; in another case, it bonds with NH of the Arg131 side chain.

Consequently, the five-coordinated state of the catalytic heme *C* and similarity of catalytic residues at the active sites of TvNiR and heme peroxidases is the structural base for the revelation of TvNiR peroxidase activity.

Electron transport to the active site in cases of both nitrite reductase and peroxidase reactions can be performed via a chain of closely located hemes, which form two branches including hemes 1, 2, 3, and 5 and hemes 7 and 8. Both branches converge to heme 6, which passes electrons to catalytic heme 4 (Fig. 3).

Kinetic characteristics of TvNir peroxidase activity and factors affecting it. In order to optimize conditions for the determination of the peroxidase activity of the TvNiR preparation, we varied substrate concentrations, ABTS in a range of 0.2–2.0 mM, and H_2O_2 in a range of 0.5–1 mM, with a constant concentration of the second substrate. In both cases the dependences were described by the Michaelis–Menten equation. The Michaelis constant for hydrogen peroxide was 0.96 ± 0.14 mM at a constant ABTS concentration close to saturation (0.2 mM). The Michaelis constant for ABTS at a saturating concentration of hydrogen peroxide of 5.0 mM was 0.025 ± 0.005 mM. The maximum specific peroxidase activity of the TvNiR hexameric form was 0.17 ± 0.01 μmol of ABTS/min per mg of protein (0.05M HEPES buffer, pH 7.0, 30°C). The observed catalytic constant (k_{cat}) of the transformation of the enzyme-substrate complex for the hexamer calculated per monomer concentration of 0.17 s^{-1} (table). TvNiR peroxidase activity was higher in 0.1 M HEPES buffer (and also in another organic buffer—MES) as compared to 0.1 M PPB; it can be possibly explained by TvNiR inhibition with large multicharged anions such as phosphate. It has been previously shown [39] that phosphate is tightly bound at the active site of the enzyme oxidized form; it coordinates iron (III) atom of the catalytic heme by an oxygen atom, while three other oxygen atoms form hydrogen bonds with catalytic residues His361 and Tyr303. Consequently, phosphate is an efficient inhibitor of

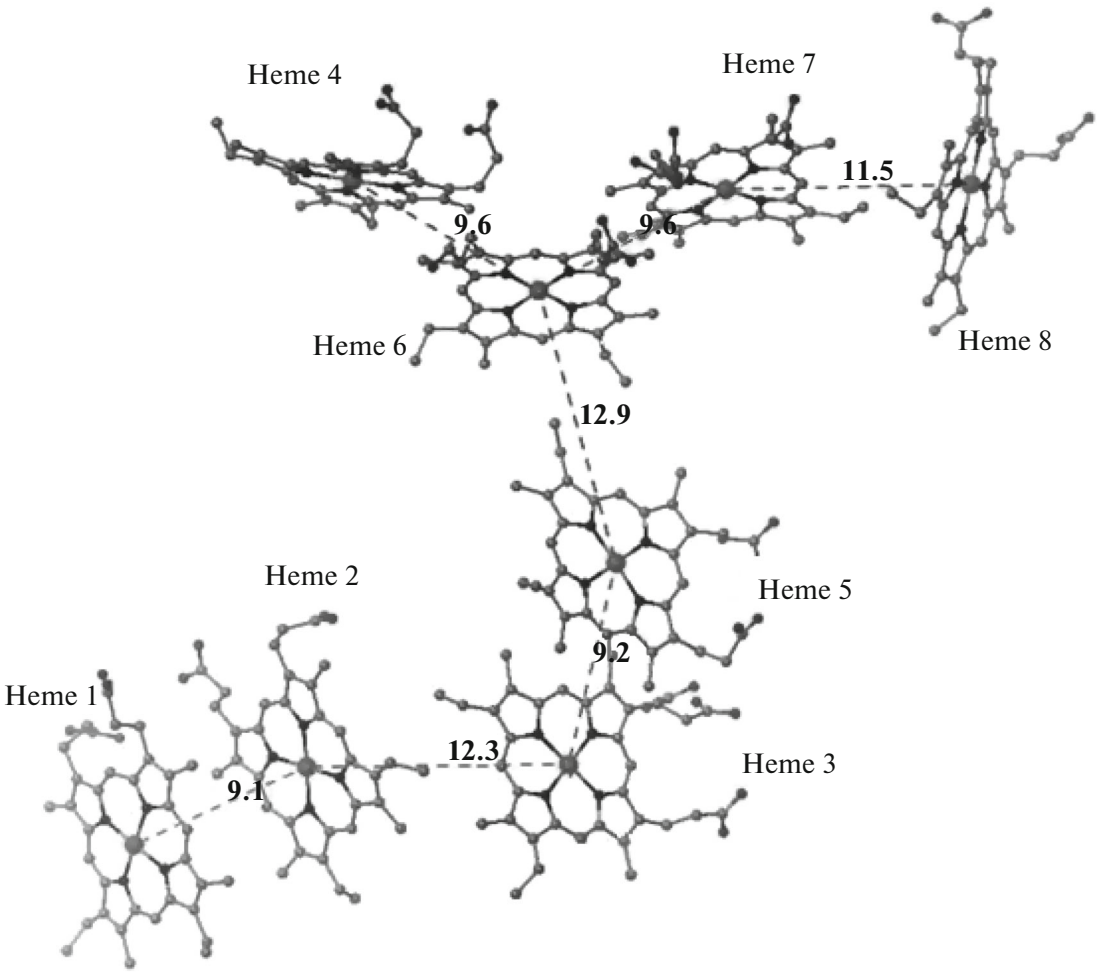

Fig. 3. Structure of the TvNiR electron transport chain [22]. Hemes are numerated in order of the position of heme-binding motifs from the N-terminus of the amino acid chain. Hemes 1, 2, 3, and 5 form branch (*1*), hemes 7 and 8 are in branch (*2*). Both branches converge at heme 6, which passes electrons to catalytic heme 4. Numbers designate distances between iron ions of neighboring hemes.

the peroxidase reaction occurring on the oxidized (Fe(III)) form of the catalytic heme. The bond of phosphate with the Fe(II) form of the catalytic heme is less tight [40]; that is why nitrite TvNiR reductase activity was not inhibited by phosphate buffer.

In order to confirm the fact that the nitrite reductase and peroxidase reactions occur on the same catalytic heme 4, we checked the inhibition of the peroxidase reaction by nitrite ion (Fig. 4a). Linearization of the obtained data in the Lineweaver–Burk coordinates showed that nitrite is a competitive inhibitor of the peroxidase reaction. The effect of nitrite on peroxidase activity was more pronounced in HEPES buffer as compared to PPB at the same pH values.

It is known that the activity of some cytochrome *c* peroxidases is stimulated in the presence of Ca^{2+} ions. It has been supposed that purified preparations of these peroxidases are obtained in the Ca^{2+}–depleted form [38]. Ca^{2+} binding by peroxidases causes conformational changes that are accompanied by the removal of the distant ligand of the catalytic heme, which transfers to the five-coordinated state and is ready to bind the substrate—peroxide [36]. This Ca^{2+} ion presents in every TvNiR subunit [22]. It localizes at a distance of about 10 Å from the catalytic heme and participates in the coordination of catalytically important residues in the TvNiR active site, including His361 that situates above the catalytic heme. In contrast to peroxidases, this Ca^{2+} ion is tightly bound with the molecule and is not lost during the enzyme purification. Another binding site for Ca^{2+} ions in the TvNiR structure has been found near hemes 6 and 7 (Fig. 3). The low population of the second Ca^{2+} binding site in all structures of octaheme nitrite reductases suggests that the ion can be easily lost during purification. The role of the second Ca^{2+} ion in catalysis and structure is not clear. Its binding can affect the potentials of hemes 7 and 6 and, therefore, the rate of electron transport to the catalytic heme. However, in the case of TvNiR nitrite reductase activity, the introduc-

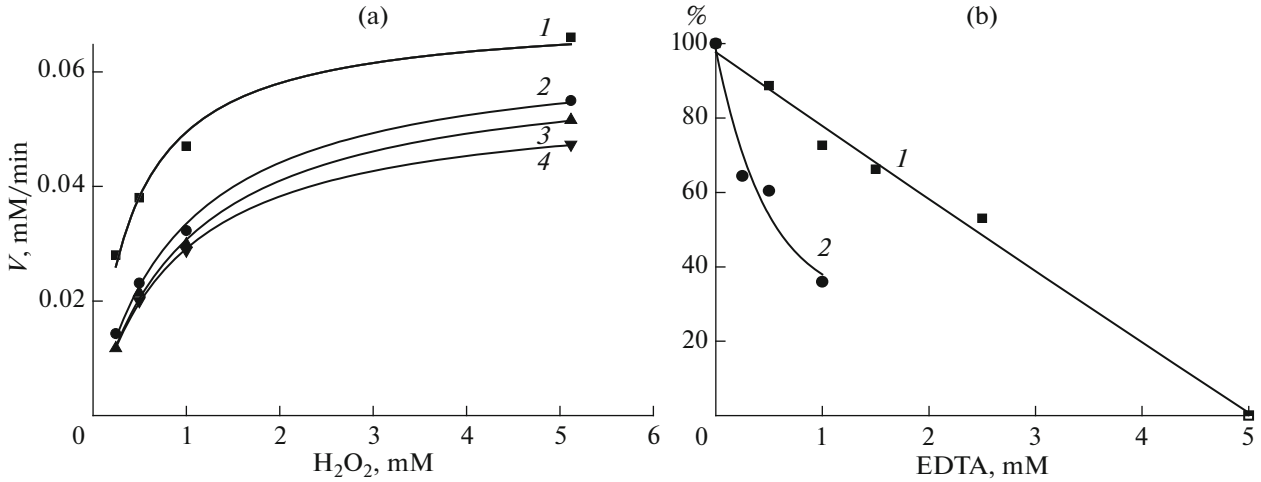

Fig. 4. Inhibition of TvNiR peroxidase activity (residual activity, %): a—by nitrite (*1*—0, *2*—0.25, *3*—0.5, *4*—1 mM of nitrite in 0.1 M HEPES buffer, pH 7.0); b—by EDTA in different buffers. (*1*) 0.1 M PPB, pH 7.0; (*2*) 0.1 M HEPES buffer, pH 7.0.

tion of calcium ions at a concentration of 0–5.0 mM did not affect the reaction rate. The treatment of the enzyme with EDTA at a concentration of 0–5.0 mM and the addition of EDTA to the reaction at a pH of 7.0 also did not affect the rate of the nitrite reductase reaction; this evidently confirms the tightness of the binding of the first Ca^{2+} ion and the inessentiality of the second for nitrite reduction in solution in the presence of reduced methyl viologen.

In the case of the peroxidase reaction, the introduction of Ca^{2+} ions to the reaction at a concentration of 0–5.0 mM caused inhibition of the peroxidase activity, which was 30% at a $CaCl_2$ concentration of 1.0 mM and about 45% at 5.0 mM. Different rate-limiting steps of two reactions is one possible explanation: in the case of the nitrite reductase reaction, nitrite reduction at the active site can be a rate-limiting step, as has been supposed for NrfA [40]. In the case of the peroxidase reaction, the rate can be limited by electron transfer along the intramolecular chain. Ca^{2+} ion binding with carboxyls of hemes 6 and 7 should cause an increase in the redox potential of these hemes, therefore affecting the rate of electron transport.

Treatment of the enzyme with EDTA and the addition of EDTA to the reaction also caused a decrease in TvNiR peroxidase activity; moreover, the decrease was more pronounced upon the use of HEPES buffer as compared to PPB (Fig. 4b). It is possible that the Ca^{2+} ion situated near the active site or the iron ion of the catalytic heme becomes available for EDTA in the presence of hydrogen peroxide.

The profile of the pH dependence of the rate of the peroxidase reaction catalyzed by TvNiR is described by a bell-shaped curve with a maximum at pH 4.7–5.0 and an additional shoulder in a pH region of 6.0–7.0. This is in accordance with the pH dependences obtained for other peroxidases with ABTS as a substrate [28, 41–43].

In order to estimate the effect of TvNiR oligomeric structure on the activity, we measured the peroxidase activity of trimers under analogous conditions. The trimer's activity turned out to be 6.60 ± 0.25 μmol of ABTS/min per mg of protein; this is higher than the hexamer activity by more than an order of magnitude. The catalytic constant for the trimer calculated per monomer concentration is 6.5 s^{-1} (table). The nitrite reductase activities of the trimer and hexamer were equal as was noted in Methods.

The pH dependences for the rate of peroxidase reactions for the hexamer and trimer have similar shapes (Fig. 5). This indicates that both oligomers have the same rate-limiting steps of the peroxidase reaction. We verified by gel filtration chromatography that the oligomerization degree of the protein did not change upon the protein concentrations used in a whole pH range.

Comparison of the activity of TvNiR hexamers and trimers with the activity of natural peroxidases and cytochrome c (table) showed that, in terms of catalytic efficiency (k_{cat}/K_M), TvNiR surpasses cytochrome *c* and its carboxymethylated derivative with a five-coordinated heme C (CM-cyt *c*), in which catalysis mainly occurs via peroxide activation upon its coordination with Fe(III) ion of the catalytic heme [27, 28], by several orders of magnitude. On the other hand, the TvNiR peroxidase activity is remarkably lower than the activity of natural peroxidases. This can be explained by a number of reasons. One of them is the effect of a ligand that coordinates the heme in the proximal position on the stability of ferryl cation radical. The substitution of histidine for a lysine residue, which is positively charged under the reaction conditions, should cause destabilization of ferryl cation rad-

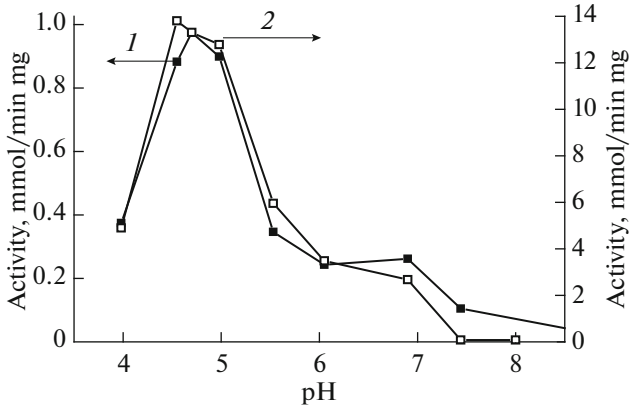

Fig. 5. Dependence of the rate of the peroxidase reaction catalyzed by hexameric (*1*) and trimeric (*2*) TvNiR forms on pH.

ical and a decrease in the reaction rate. Another cause involves the differences in the relative spatial position of catalytically important histidine and arginine residues in active sites of both enzymes (Fig. 1); this confirms the assertion on the importance of a fine adjustment of the active site in peroxidase catalysis [38]. However, the explanation based on properties of the electron transport chain in TvNiR seems to be more probable. Comparison of the size of the ABTS molecule (10.1 × 17.3 Å [44]) and the diameter of the TvNiR substrate channel (about 5 Å) showed that ABTS does not enter the substrate channel, and the possibility of the peroxidase reaction is defined by the presence of the electron transport chain from ABTS at the enzyme surface to the ferryl cation at the active site. This chain in TvNiR is possible due to heme contacts inside the monomer (Fig. 3) and the presence of hemes exposed to solvent on the surface of the protein globule. If the limiting stage of the peroxidase reaction is electron transport from ABTS to the enzyme, then the number of hemes are available for interaction with ABTS increases with the reaction rate. In the TvNiR hexamer, ABTS can interact only with one heme (heme 8; the available contact area for a sphere with a diameter of 10 Å is 127 Å^2 [22]) of every subunit. According to calculations, in the TvNiR trimer, heme 3 also becomes available for interaction with ABTS [22]; this can result in an increase in the reaction rate. Furthermore, upon the transfer of electrons from ABTS to heme 3, further electron transport to catalytic heme 4 occurs via the electron transport chain including hemes (1, 2) 3, 5, and 6; in the case of the electron transfer to heme 8, it occurs via the electron transport chain including hemes 8, 7, and 6 (Fig. 3). This can, in turn, affect the rate of electron transfer and, therefore, the rate of enzymatic reaction.

As was previously noted, the nitrite reductase activities of the trimer and hexamer are equal. It seems that the rate-limiting stage for this reaction upon the use of reduced methyl viologen as an electron donor is connected not with electron transport but with catalytic processes occurring at the active site, which is the same for the hexamer and trimer.

CONCLUSIONS

In order to estimate the possible participation of octaheme nitrite reductases from haloalkaliphilic bacteria of the *Thioalkalivibrio* genus in protection of the cell from oxidative stress, we characterized the peroxidase activity of TvNiR octaheme nitrite reductase from *T. nitratireducens*. Despite the similarity in the structure of active sites of TvNiR and cytochrome c peroxidases, TvNiR peroxidase activity measured under optimal conditions with ABTS as a substrate did not exceed 17 s^{-1} (k_{cat}/K_M = 855 mM^{-1} s^{-1}); this is 100–300 times lower than the activity of natural peroxidases and only slightly higher than the activity of modified cytochrome *c* with a five-coordinated heme, which is able to bond peroxide at the vacant sixth coordination place. Different values of peroxidase activities of TvNiR trimers and hexamers allowed the supposition that the rate-limiting stage of the process is not the catalytic reaction occurring at the active site but the electron transport from a high-potential donor (ABTS) to the active site via the electron transport chain consisting of hemes c.

ACKNOWLEDGMENTS

This work was supported by the Russian Scientific Foundation, project no. 14-24-00172.

REFERENCES

1. Simon, J., *FEMS Microbiol. Rev.*, 2002, vol. 26, pp. 285–309.
2. Einsle, O., *Methods. Enzymol.*, 2011, vol. 496, pp. 399–422.
3. Einsle, O., Messerschmidt, A., Stach, P., Bourenkov, G.P., Bartunik, H.D., Huber, R., and Kroneck, P.M.H., *Nature*, 1999, vol. 400, no. 6743, pp. 476–480.
4. Clarke, T.A., Mills, P.C., Poock, S.R., Butt, J.N., Cheesman, M.R., Cole, J.A., Hinton, J.C., Hemmings, A.M., Kemp, G., Soderberg, C.A., Spiro, S., Van Wonderen, J., and Richardson, D.J., *Methods Enzymol.*, 2008, vol. 437, pp. 63–77.
5. Tikhonova, T., Tikhonov, A., Trofimov, A., Polyakov, K., Boyko, K., Cherkashin, E., Rakitina, T., Sorokin, D., and Popov, V., *FEBS J.*, 2012, vol. 279, no. 21, pp. 4052–4061.
6. Tikhonova, T.V., Trofimov, A.A., and Popov, V.O., *Biochemistry* (Moscow), 2012, vol. 77, no. 10, pp. 1129–1138.
7. Einsle, O., Stach, P., Messerschmidt, A., Simon, J., Kroger, A., Huber, R., and Kroneck, P.M.H., *J. Biol. Chem.*, 2000, vol. 275, pp. 39608–39616.

8. Bamford, V.A., Angove, H.C., Seward, H.E., Thomson, A.J., Cole, J.A., Butt, J.N., Hemmings, A.M., and Richardson, D.J., *Biochemistry*, 2002, vol. 41, pp. 2921–2931.
9. Lockwood, C.W., Burlat, B., Cheesman, M.R., Kern, M., Simon, J., Clarke, T.A., Richardson, D.J., and Butt, J.N., *J. Am. Chem. Soc.*, 2015, vol. 137, no. 8, pp. 3059–3068.
10. Cunha, C.A., Macieira, S., Dias, J.M., Almeida, G., Goncalves, L.L., Costa, C., Lampreia, J., Huber, R., Moura, J.J.G., Moura, I., and Romao, M.J., *J. Biol. Chem.*, 2003, vol. 278, pp. 17455–17465.
11. Rodrigues, M.L., Oliveira, T.F., Pereira, I.A.C., and Archer, M., *EMBO J.*, 2006, vol. 25, pp. 5951–5960.
12. Youngblut, M., Judd, E.T., Srajer, V., Sayyed, B., Goelzer, T., Elliott, S.J., Schmidt, M., and Pacheco, A.A., *J. Biol. Inorg. Chem.*, 2012, vol. 17, pp. 647–662.
13. Kemp, G.L., Clarke, T.A., Marritt, S.J., Lockwood, C., Poock, S.R., Hemmings, A.M., Richardson, D.J., Cheesman, M.R., and Butt, J.N., *Biochem. J.*, 2010, vol. 431, no. 1, pp. 73–80.
14. van Wonderen, J.H., Burlat, B., Richardson, D.J., Cheesman, M.R., and Butt, J.N., *J. Biol. Chem.*, 2008, vol. 283, no. 15, pp. 9587–9594.
15. Angove, H.C., Cole, J.A., Richardson, D.J., and Butt, J.N., *J. Biol. Chem.*, 2002, vol. 277, pp. 23374–23381.
16. Lukat, P., Rudolf, M., Stach, P., Messerschmidt, A., Kroneck, P.M., Simon, J., and Einsle, O., *Biochemistry*, 2008, vol. 47, no. 7, pp. 2080–2086.
17. Rudolf, M., Einsle, O., Neese, F., and Kroneck, P.M., *Biochem. Soc. Trans.*, 2002, vol. 30, no. 4, pp. 649–653.
18. Kern, M., Volz, J., and Simon, J., *Environ. Microbiol.*, 2011, vol. 13, no. 9, pp. 2478–2494.
19. Muhlig, A., Kabisch, J., Pichner, R., Scherer, S., and Muller-Herbst, S., *Food Microbiol.*, 2014, vol. 42, pp. 26–33.
20. Pittman, M.S., Elvers, K.T., Lee, L., Jones, M.A., Poole, R.K., Park, S.F., and Kelly, D.J., *Mol. Microbiol.*, 2007, vol. 63, pp. 575–590.
21. Tikhonova, T.V., Slutsky, A., Antipov, A.N., Boyko, K.M., Polyakov, K.M., Sorokin, D.Y., Zvyagilskaya, R.A., and Popov, V.O., *Biochim. Biophys. Acta*, 2006, vol. 1764, pp. 715–723.
22. Polyakov, K.M., Boyko, KM., Tikhonova, T.V., Slutsky, A., Antipov, A.N., Zvyagilskaya, R.A., Popov, A.N., Bourenkov, G.P., Lamzin, V.S., and Popov, V.O., *J. Mol. Biol.*, 2009, vol. 389, pp. 846–862.
23. Sorokin, D.Y., Tourova, T.P., Sjollema, K.A., and Kuenen, J.G., *Int. J. Syst. Evol. Microbiol.*, 2003, vol. 53, pp. 1779–1783.
24. Sorokin, D.Y., Antipov, A.N., and Kuenen, J.G., *Arch. Microbiol.*, 2003, vol. 180, pp. 127–133.
25. Sorokin, D.Y., Tourova, T.P., Lysenko, A.M., Mityushina, L.L., and Kuenen, J.G., *Int. J. Syst. Evol. Microbiol.*, 2002, vol. 52, pp. 657–664.
26. Tikhonova, T.V., Slutskaya, E.S., Filimonenkov, A.A., Boiko, K.M., Kleimenov, S.Yu., Konarev, P.V., Polyakov, K.M., Svergun, D.I., Trofimov, A.A., Khomenkov, V.G., Zvyagil'skaya, R.A., and Popov, V.O., *Biochemistry* (Moscow), 2008, vol. 73, no. 2, pp. 164–170.
27. Yamada, S., Suruga, K., Ogawa, M., Hama, T., Satoh, T., Kawachi, R., Nishio, T., and Oku, T., *Biosci. Biotechnol. Biochem.*, 2002, vol. 66, no. 10, pp. 2044–2051.
28. Prasad, S., Maiti, N.C., Mazumdar, S., and Mitra, S., *Biochim. Biophys. Acta*, 2002, vol. 1596, pp. 63–75.
29. Suruga, K., Murakami, K., Taniyama, Y., Hama, T., Chida, H., Satoh, T., Yamada, S., Hakamata, W., Kawachi, R., Isogai, Y., Nishio, T., and Oku, T., *Biochem. Biophys. Res. Commun.*, 2004, vol. 315, no. 4, pp. 815–822.
30. Poulos, T.L., Freer, S.T., Alden, R.A., Edwards, S.L., Skogland, U., Takio, K., Eriksson, B., Xuong, N., Yonetani, T., and Kraut, J., *J. Biol. Chem.*, 1980, vol. 255, no. 2, pp. 575–580.
31. Poulos, T.L., *Curr. Opin. Biotechnol*, 1993, vol. 4, no. 4, pp. 484–489.
32. Choudhury, K., Sundaramoorthy, M., Hickman, A., Yonetani, T., Woehl, E., Dunn, M.F., and Poulos, T.L., *J. Biol. Chem.*, 1994, vol. 269, no. 32, pp. 20239–20249.
33. Kunishima, N., Fukuyama, K., Matsubara, H., Hatanaka, H., Shibano, Y., and Amachi, T., *J. Mol. Biol.*, 1994, vol. 235, no. 1, pp. 331–344.
34. Schuller, D.J., Ban, N., Huystee, R.B., McPherson, A., and Poulos, T.L., *Structure*, 1996, vol. 4, pp. 311–321.
35. Gajhede, M., Schuller, D.J., Henriksen, A., Smith, A.T., and Poulos, T.L., *Nat. Struct. Biol.*, 1997, vol. 4, pp. 1032–1038.
36. Erman, J.E. and Vitello, L.B., *Biochim. Biophys. Acta*, 2002, vol. 1597, pp. 193– 220.
37. Finzel, B.C., Poulos, T.L., and Kraut, J., *J. Biol. Chem.*, 1984, vol. 259, pp. 13027–13036.
38. Gazaryan, I.G., Khushpul'yan, D.M., and Tishkov, V.I., *Usp. Biol. Khim.*, 2006, vol. 46, pp. 303–322.
39. Trofimov, A.A., Polyakov, K.M., Boiko, K.M., Filimonenkov, A.A., Dorovatovskii, P.V., Tikhonova, T.V., Popov, V.O., and Koval'chuk, M.V., *Kristallografiya*, 2010, vol. 55, no. 10, pp. 1176–1182.
40. Einsle, O., Messerschmidt, A., Huber, R., Kroneck, P.M., and Neese, F., *J. Am. Chem. Soc.*, 2002, vol. 124, no. 39, pp. 11737–11745.
41. Kamal, J.K. and Behere, D.V., *J. Inorg. Biochem.*, 2003, vol. 94, no. 3, pp. 236–242.
42. Radi, R., Thomson, L., Rubbo, H., and Prodanov, E., *Arch. Biochem. Biophys.*, 1991, vol. 288, pp. 112–117.
43. Sakharov, I.Y., *Biochemistry* (Moscow), 2001, vol. 66, no. 1, pp. 515–519.
44. Chen, Y., Lykourinou, V., Hoang, T., Ming, L.-J., and Ma, S., *Inorg. Chem.*, 2012, vol. 51, pp. 12600–12602.

Translated by O. Maloletkina