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# Isolation and Oligomeric Composition of Cytochrome *c* Nitrite Reductase from the Haloalkaliphilic Bacterium *Thioalkalivibrio nitratireducens*

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**Abstract**—A new procedure for isolation of cytochrome *c* nitrite reductase from the haloalkaliphilic bacterium *Thioalkalivibrio nitratireducens* increasing significantly the yield of the purified enzyme is presented. The enzyme is isolated from the soluble fraction of the cell extract as a hexamer, as shown by gel filtration chromatography and small angle X-ray scattering analysis. Thermostability of the hexameric form of the nitrite reductase is characterized in terms of thermoinactivation and thermodenaturation.

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*Key words*: cytochrome *c* nitrite reductase, isolation, oligomeric composition, thermostability, thermodenaturation

Cytochrome *c*-containing nitrite reductases (NrfAs) are found in the cells of many bacteria [1]. The enzymes from the γ-proteobacterium *Escherichia coli* [2-7], δ-proteobacteria *Desulfovibrio desulfuricans* [8-12] and *Desulfovibrio vulgaris* Hildenborough [13-15], and from the ε-proteobacteria *Wolinella succinogenes* [16, 17] and *Sulforospirillum deleyianum* [18-21] have been studied in detail. All well studied NrfAs catalyze the six-electron reduction of nitrite to ammonia without producing intermediate products. The monomers of NrfAs are single-domain 50-kD proteins containing five *c*-hemes. The heme coordinated in the proximal position by the amino group of a Lys residue is involved in the catalysis, and the others participate in the electron transfer. It is suggested that NrfAs participate in respiratory processes in the cells of the bacteria using nitrite as the terminal electron acceptor [1]. The exception is the bacterium *D. vulgaris* Hildenborough [13] that is not capable of using nitrate and nitrite as the electron acceptors, where the function of NrfA is likely connected with the processes of cell detoxication.

Depending on the species of proteobacteria, NrfA was found only in the soluble fraction of the cell extract (*E. coli* [1, 6]), only in the membrane fraction (*D. desulfuricans* [12] and *D. vulgaris* Hildenborough [13]), or in both fractions (*W. succinogenes* and *S. deleyianum* [19, 20]). When isolating NrfA from the membrane fraction, the enzyme was obtained as a high-molecular-weight complex with the electron donor NrfH. From the soluble cell extract, the enzyme was isolated as a stable dimer $(NrfA)_2$ ($K_d$ is approximately 4 μM and 1-3 nM for the enzymes from *E. coli* and *W. succinogenes*, respectively [22]). Structural studies demonstrated that the dimer is a functional unit of nitrite reductase. Its formation yields the

*Abbreviations*: NrfA) bacterial cytochrome *c*-containing nitrite reductases coded by *nrfA* gene; SAXS) small angle X-ray scattering; TvNiR) cytochrome *c* nitrite reductase from *Thioalkalivibrio nitratireducens*.

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joint electron transfer chain consisting of 10 *c*-hemes and allows electron transfer from one monomer to the other, thus increasing the efficiency of catalysis [6, 11, 18].

Previously, we isolated and characterized a new cytochrome *c* nitrite reductase (TvNiR) from the soluble fraction of the cell extract of the haloalkaliphilic sulfur-oxidizing bacterium *Thioalkalivibrio nitratireducens* [23] found in sediments of the hypersaline lake Fazda (Egypt). The TvNiR possessed high nitrite reductase activity ($V_{max} = 2000 \pm 110$ μmol/min per mg of protein), and the only product of the reaction was ammonia. Investigation of the TvNiR by spectral, kinetic, chromatographic, and structural methods showed that the enzyme differs significantly from the previously described cytochrome *c* nitrite reductases [23, 24]: 1) the monomer of TvNiR is a double-domain protein containing eight *c*-hemes, five of which including the catalytic heme are homologous to five hemes of the known NrfA, and the additional three hemes are located in a separate domain; 2) in solution and in crystals, the TvNiR molecule exists as a highly symmetrical hexamer of 380 kD; 3) the formation of the hexamer containing 48 *c*-hemes does not result in a common electron transfer chain connecting several active sites (unpublished data). Considering these data, a question arises concerning the role of such a complex and stable structure in the catalysis, as well as the oligomeric composition of the protein in the cell.

While isolating TvNiR from cell extract by preparative electrophoresis [23], besides the hexameric form we observed active trimer and monomer (unpublished data). The formation of these forms was presumably connected with the procedure of the enzyme isolation: in an electric field in the presence of 2 M urea, the hexamer dissociates yielding trimers and monomers. However, another situation is also possible: the trimer (monomer) might be a physiologically significant form of the enzyme stabilized by some other protein, which is removed during electrophoresis. The goal of the present work was to elaborate an alternative method for isolation of TvNiR based on column chromatography and to characterize the oligomers of TvNiR and the contaminating proteins.

## MATERIALS AND METHODS

In the present work we used acrylamide, glycine, tetramethylethylenediamine, triphenyltetrazolium chloride, methyl viologen, and N-(1-naphthyl)ethylenediamine from Sigma (USA); sodium dithionite from Merck (Germany); bisacrylamide and 2-mercaptoethanol from Aldrich (USA). Other chemicals were of domestic production.

**Microorganisms and their cultivation.** The culture of *Tv. nitratireducens* strain ALEN 2 was maintained as described earlier [23]. The cells were grown at 28°C under microaerophilic conditions in mineral medium (pH 10) containing in 1 liter: 23 g of $Na_2CO_3$, 7 g of $NaHCO_3$, 5 g of NaCl, 1 g of $K_2HPO_4$, 1 g of $KNO_3$, 0.2 g of $MgCl_2 \cdot 6H_2O$, and the standard set of microelements [23]. A solution of thiosulfate (40 mM) was used as the source of energy. The inoculate was grown in the same medium supplemented with 5 mM $NH_4Cl$ in 750-ml shaken flasks (working volume 250 ml) using a rotary shaker (220 rpm). The maximal nitrite reductase activity was observed in the cells within the exponentional growth stage. In this stage, the cells were collected and used to prepare the cell extract as described earlier [23]. The soluble and periplasmic membrane fractions were obtained by high-speed centrifugation of the cell extract (100,000*g*, 2 h). The fraction of periplasmic membranes was washed three times with 25 mM potassium phosphate buffer, pH 7.0. Then both fractions were assayed for the nitrite reductase activity. The activity was detected only in the soluble fraction that was used for isolation of TvNiR.

**Isolation of nitrite reductase.** Nitrite reductase was purified by anion-exchange chromatography on a DEAE-Sepharose (Fast Flow) column (35 ml) at 4°C using a BioLogic LP chromatograph (BioRad, USA). The column was equilibrated with 25 mM potassium phosphate buffer, pH 7.0. After application of the extract onto the column and washing with the same buffer, the protein was eluted with a linear (0-1 M) NaCl gradient.

The subsequent step was gel-filtration chromatography on a Superdex$^{TM}$200 10/300 column using an AKTA FPLC chromatograph (Amersham Biosciences, USA). The column was equilibrated with 50 mM potassium phosphate buffer, pH 7.0, containing 0.15 M NaCl.

**Spectral studies** were performed using a Helios α Thermo Spectronic spectrophotometer (USA) equipped with a thermostatted cuvette holder.

**Protein assay.** Protein was determined by the Bradford's method [25].

**Measuring of the nitrite reductase activity.** To determine the nitrite reductase activity, methyl viologen reduced with dithionite was used as the electron donor. The reaction mixture contained 0.1 M potassium phosphate buffer, pH 7.0, 1 mM $NaNO_2$, 1.6 mM methyl viologen, and the investigated enzyme (2-20 μl of a solution of 0.1-0.2 mg/ml). The reaction was performed under anaerobic conditions in glass vials for gas chromatography. The reaction mixture and the dithionite solution were preliminarily purged with high purity argon for 15 min. The reaction was started by the addition of 15 μl of 1 M sodium dithionite to the reaction mixture preincubated at 30°C for 5 min. After definite time intervals, aliquots were taken from the reaction mixture to determine nitrite concentration using the method described in [26]. The unit of the nitrite reductase activity was determined as the amount of the enzyme catalyzing conversion of 1 μmol of $NO_2^-$ per min.

To investigate thermoinactivation and thermodissociation of TvNiR, a protein solution (0.02-0.2 mg/ml in

0.1 M potassium phosphate buffer, pH 7.0) was incubated at 30-80°C. After definite time intervals, aliquots were taken from the solution to measure the nitrite reductase activity under standard conditions and to determine the oligomeric composition of TvNiR by gel filtration.

**SDS-PAGE** was performed according to Laemmli [27]. To evaluate the molecular weight of the subunits, a kit of protein standards from Amersham Biosciences was used (galactosidase (116 kD), bovine serum albumin (66.2 kD), ovalbumin (45 kD), lactate dehydrogenase (35 kD), endonuclease (25 kD), and lysozyme (14 kD)).

**Determination of the subunit composition of nitrite reductase by HPLC.** The oligomeric composition of nitrite reductase was determined by gel filtration on a Superdex$^{TM}$200 10/300 GL column (separation range of 10-300 kD). The proteins were eluted with 50 mM potassium phosphate buffer, pH 7.0, containing 0.15 M NaCl. Protein was monitored at 280 nm.

To determine the molecular weights of the eluted proteins, the column was preliminarily calibrated using protein standards (Amersham Biosciences): thyroglobulin (669 kD), ferritin (440 kD), bovine serum albumin (67 kD), and ribonuclease (13.5 kD). Blue dextran (2000 kD) was used to determine the exclusion volume of the column.

**Mass spectra** were obtained on a Bruker Ultraflex II MALDI TOF mass-spectrometer (Germany) equipped with a UV laser. Protein samples in the polyacrylamide gel were preliminarily subjected to trypsinolysis. The mass spectra of the peptides were obtained in the positive-ion mode using a reflectron. The accuracy of the measured masses was 0.01% after additional calibration using the peaks of trypsin autolysis.

**Protein identification.** The proteins were identified using the Mascot program (www.matrixscience.com) and the NCBI database (sub-base bacteria) with the indicated accuracy assuming the possible oxidation of the methionines with oxygen and modification of the cysteines with acrylamide.

**Differential scanning calorimetry (DSC).** Calorimetric studies were performed using a DASM-4M instrument (Institute for Biological Instrumentation, Russian Academy of Sciences, Pushchino) with capillary platinum 0.47-ml cells as described previously [28]. The measurements were made at the heating rate of 1.0°/min. To check the reversibility of thermodenaturation, after the first scanning and subsequent cooling, samples were heated again. Thermodenaturation of TvNiR was completely irreversible. The DSC curves obtained during the repeated heating were subtracted from the original curves obtained during the first scanning. The resulting differential heat capacity curves reflected the process of protein thermodenaturation. The measurements were made at different protein concentration (0.5-3 mg/ml).

**Small angle X-ray scattering (SAXS) analysis.** The experiments were done at the Hamburg branch of the European Molecular Biology Laboratory (EMBL) at beamline X33 of the DORIS Storage Ring of the DESY Synchrotron Center [29]. Homogeneous nitrite reductase in Tris-borate buffer, pH 8.5, was investigated at three different concentrations: 1.5, 7.5, and 15 mg/ml. The data were recorded using a MAR345 image plate detector. The sample–detector distance was 2.7 m, this covering the scattering vector range $0.012 < s < 0.45$ Å$^{-1}$, where $s = 4\pi\sin(\theta)/\lambda$, $2\theta$ is the scattering angle, and $\lambda$ is the X-ray wavelength of 1.5 Å. To test for possible radiation damage of the sample, two sequential measurements during 2 min were made. No differences were revealed between the two measurements, and, consequently, no damage of the sample was detected. The data were averaged radially and normalized to the intensity of the incident beam. The scattering of the buffer was subtracted from the scattering of the samples. To make the corrections for interparticle interactions, the differential data were extrapolated to zero concentration according to standard procedures. All data processing procedures were made using the PRIMUS program [30]. The gyration radius and the maximal particle size were calculated using the GNOM program [31]. Theoretical scattering curves were created using the CRYSOL program based on the crystallographic structures of the monomer, dimer, trimer, and hexamer of TvNiR [32]. To evaluate quantitatively the oligomeric composition of the TvNiR samples, the scattering intensity was presented as the superposition of the signals from different oligomers of TvNiR, where the contribution of the each state must be proportional to its volume ratio in the solution. The values of the volume ratios of different oligomeric components providing the best approximation of the experimental data by the theoretical curves were determined using the OLIGOMER program [33].

## RESULTS AND DISCUSSION

**Isolation and characterization of TvNiR and concomitant proteins.** The first step in the procedure of TvNiR purification was anion-exchange chromatography on a DEAE-Sepharose column. The proteins were eluted with a 0-1 M NaCl gradient (Fig. 1a). The protein fractions exhibiting the nitrite reductase activity and possessing the typical for cytochrome *c* red-brown coloration were eluted at NaCl concentration of 0.5-0.58 M (peak II in Fig. 1a). The fractions with maximal specific nitrite reductase activity were pooled and analyzed by SDS-PAGE (Fig. 2, lane *3*), concentrated and applied to a Superdex$^{TM}$200 10/300 gel-filtration column equilibrated with 50 mM potassium phosphate buffer, pH 7.0, containing 0.15 M NaCl (Fig. 1b). The gel filtration procedure resulted in two peaks with the absorption bands that are characteristic for cytochromes *c* (410 and 532 nm for the oxidized form and 421, 525, and 554 nm after the reduction with

sodium dithionite). Peak *1* (retention volume 10.6 ml) contained proteins of 380-400 kD and exhibited the nitrite reductase activity. Peak *2* (retention volume 14.2 ml) contained proteins of 50-70 kD exhibiting no nitrite reductase activity.

The fractions of peak *1* with the maximal nitrite reductase activity were pooled, concentrated, and characterized. An aliquot of the preparation was again applied to the gel filtration column to analyze the homogeneity of the protein. The procedure resulted in a symmetric peak (Fig. 1c) with the retention volume of 10.6 ml, this corresponding to the molecular weight of the TvNiR hexamer (380-390 kD). No trimeric forms were revealed in the process of TvNiR isolation from the fresh cell homogenate. The results of SDS-PAGE confirmed the homogeneity of the TvNiR (Fig. 2, lane *6*). MALDI-TOF analysis confirmed the identity of the TvNiR preparation to that obtained previously by the preparative electrophoresis. The TvNiR obtained by gel filtration exhibited the same specific activity (150 μmol $NO_2^-$/min per mg protein) as the enzyme obtained by preparative electrophoresis. The two-step procedure of TvNiR isolation is presented in the table. It should be noted that the yield of the homogeneous protein using the new procedure was significantly higher than that using preparative electrophoresis (23 and 5%, respectively).

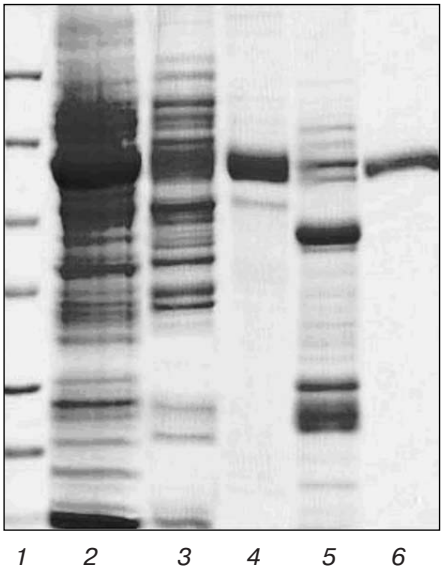


**Fig. 2.** SDS-PAGE analysis of TvNiR fractions at different steps of purification. Lanes: *1*) protein standards; *2*) cell extract; *3*) DEAE-chromatography, peak II; *4*) gel filtration, peak *1*; *5*) gel filtration, peak *2*; *6*) homogeneous TvNiR preparation.

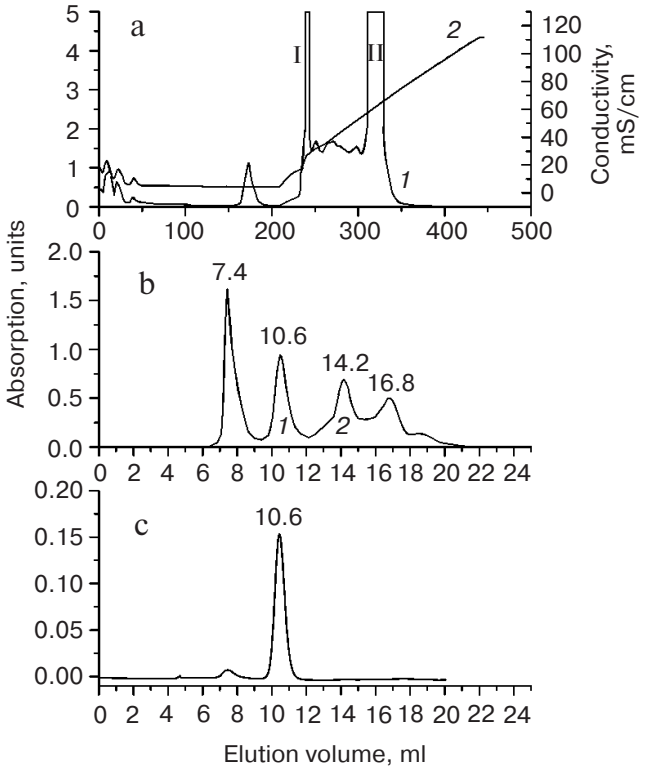


**Fig. 1.** a) Anion-exchange chromatography of the extract of *Tv. nitratireducens*: *1*) absorbance at 280 nm; *2*) conductivity (NaCl gradient). b) Gel chromatography of peak II obtained after the anion-exchange chromatography: peak *1*, proteins of 380-390 kD (TvNiR hexamer); peak *2*, proteins of 50-70 kD. c) Gel chromatography of the homogeneous TvNiR preparation.

It is known that the role of the electron donor for NrfAs isolated from different species of proteobacteria is played by cytochrome *c*-containing proteins: the transmembrane cytochrome *c* NrfH containing four hemes (δ- and ε-proteobacteria) [1, 12, 13, 15, 19, 34] or the soluble NrfB containing five hemes (γ-proteobacteria) [1, 20, 35, 36]. The nitrite reductase complex of γ-proteobacteria also contains periplasmic [4Fe-4S]-ferredoxin NrfC [1]. To find the possible electron donor of TvNiR, the protein fraction copurifying with TvNiR in the stage of anion-exchange chromatography (Fig. 1b, peak *2*) exhibiting no nitrite reductase activity but possessing the typical for cytochrome *c* spectrum was analyzed by SDS-PAGE (Fig. 2, lane *5*) and MALDI TOF spectrometry. According to the results of SDS-PAGE, this fraction contains four main protein bands with molecular mass about 60, 45, 25 and diffuse band at 20 kD. Only the proteins of ~20 kD exhibited the typical for cytochrome *c* red-brown coloration (this can be seen in the 3-fold overloaded gel compared to that presented in Fig. 2). The results of MALDI TOF spectrometry for these proteins did not reveal significant homology with the bacterial cytochromes from the NCBI database (subbase bacteria). A plausible reason is inhomogeneity of the protein in the band. According to the data of MALDI TOF spectrometry, of the other proteins on lane *5* (Fig. 2), the 60-kD protein exhibited the maximal homology with [4Fe-4S]-ferredoxin (61 kD) from the propionate-oxidizing sulfate-reducing bacterium *Syntrophobacter*

Two-step procedure for isolation of TvNiR

| Purification step | Protein, mg | Specific activity, U/mg | Purification degree | Activity, U | Yield, % |
|---|---|---|---|---|---|
| Cell extract | 93 | 2.6 | 1.0 | 241.8 | 100 |
| DEAE-Sepharose | 40 | 4.8 | 1.85 | 192.8 | 80 |
| Superdex 200 | 0.38 | 150 | 57.7 | 57 | 23.5 |

Note: 1 U corresponds to 1 μmol $NO_2^-$/min.

*fumaroxidans* MPOB, where this protein is involved in electron transport. These two proteins with molecular masses of 20 and 60 kD are of special interest for our further work.

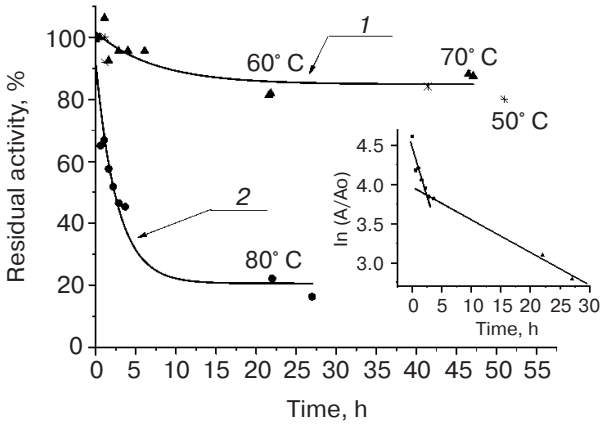


**Fig. 3.** Thermoinactivation of TvNiR: 1 μM protein solution in 0.1 M potassium phosphate buffer, pH 7.0, was incubated at 50, 60, and 70°C (curve *1*) and at 80°C (curve *2*). The insert shows the semi-logarithmic plot of curve *2*.

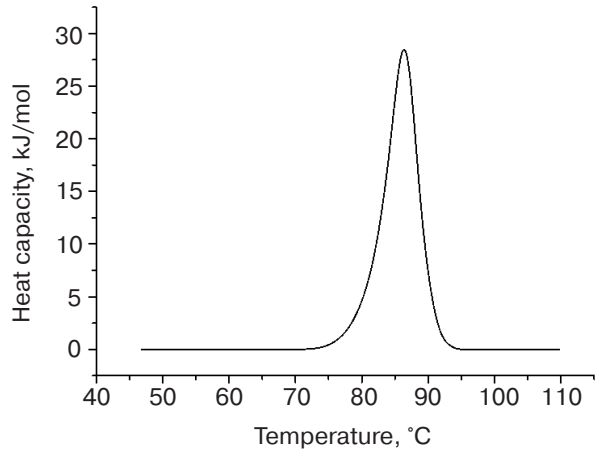


**Fig. 4.** Thermodenaturation curve of TvNiR obtained by DSC. The analyzed solution contained 1 mg/ml (16 μM) of the enzyme in 0.1 M potassium phosphate buffer, pH 7.0.

**Thermostability of TvNiR.** As seen from the data, the procedure of chromatographic purification of TvNiR yielded only the hexameric form of the enzyme.

Attempts to dissociate the hexamer under moderate conditions (dilution, changing in pH and temperature) showed that the dilution of a TvNiR solution to concentrations of 0.7-1.5 μM did not result in a noticeable dissociation of the hexamer. A prolonged (20-80 h) incubation of the diluted TvNiR solutions under enhanced temperatures (50-70°C) resulted in dissociation of 10-15% of the hexameric molecules yielding trimers, which was detected by gel filtration. No other products (dimers or monomers) were detected. The disadvantage of this method is low accuracy under low enzyme concentrations, as well as the additional dilution of the protein during the chromatography. The change in the nitrite reductase activity during the enzyme dissociation could allow more accurate evaluation of the dissociation extent of TvNiR. However, as we demonstrated earlier, the hexamer and the trimer possess the same nitrite reductase activity [23]. The activity of the enzyme was stable during incubation at 50-70°C for at least 50 h; at 80°C, the half-inactivation period was 2.2 h (Fig. 3). The enzyme inactivation at 80°C is described by a two-step scheme with the rate constants of $(6.7 \pm 1.4)\cdot10^{-5}$ $sec^{-1}$ and $(1.2 \pm 0.05)\cdot10^{-5}$ $sec^{-1}$ for the fast and slow steps, respectively (inset to Fig. 3). The enzyme retained residual activity of ~20% even after 25 h of incubation at 80°C. The exclusively high stability of the hexamer was also confirmed by DSC studies: melting of the TvNiR molecule is an irreversible highly cooperative process with the transition temperature of 86°C and the transition enthalpy of 5600 ± 200 kJ/mol of hexamer (Fig. 4). The transition temperature did not depend on the protein concentration in the range of 0.5-3.0 mg/ml, indicating that there was no intermediate reversible dissociation of the hexamer into the trimers or monomers. The denatured protein retained about 20% of the activity in the reaction of nitrite reduction. The residual activity is likely connected with the fact that in the denatured protein five-coordinated heme *c* exposed to the aqueous medium. Similar nitrite reductase activity was detected in cytochrome *c* subjected to denaturation or limited proteolysis [37].

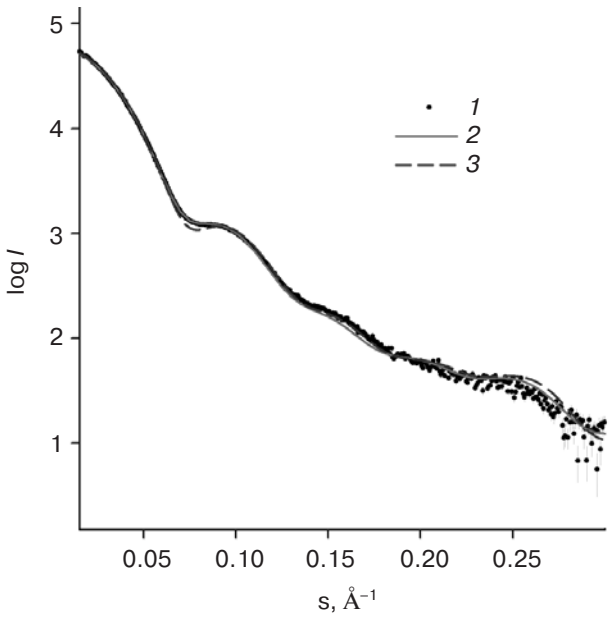


**Fig. 5.** SAXS curves of TvNiR. The experimental data are shown by points; the values of experimental errors are shown by bars (*1*). The curve obtained by the OLIGOMER program is shown by the solid line (*2*). The theoretical curve for TvNiR hexamer is shown by the dotted line (*3*). The plot is the dependence of the common logarithm of the scattering intensity (relative units) on the scattering vector $s = 4\pi\sin(\theta)/\lambda$, where $2\theta$ is the scattering angle, and $\lambda$ is the wavelength of X-ray radiation (1.5 Å).

**Characterization of the oligomeric composition of TvNiR by small-angle X-ray scattering (SAXS).** For additional examination of the oligomeric composition of TvNiR in solution, we used SAXS analysis. Besides the oligomeric structure, the method allows evaluation of some geometrical and weight characteristics of biological samples, and sometimes determination of their structure with the resolution of 10 Å.

The experimental SAXS curve for the TvNiR sample is presented in Fig. 5 (curve *1*). The gyration radius and the maximal protein size determined from the experimental data constituted 46 ± 1 and 130 ± 10 Å. These values are close to the theoretical parameters of the crystal structure of the TvNiR hexamer (47 and 142 Å, respectively).

Using the CRYSOL program [32], the experimental scattering curves were compared with the theoretical curves created for different oligomeric states of TvNiR. The analysis of the curves demonstrated that the experimental curve is described reasonably well by the theoretical curve obtained for the hexameric form (Fig. 5, curve *3*). However, there is some mismatch in the region of the first maximum. Corrections for the possible presence in the solution of dissociated forms (dimers and monomers) of the hexamer (~10% of volume ratios) made with the use of the OLIGOMER program [33] provided a better approximation of the experimental data by the theoretical curve including the region of the first maximum. Therefore, SAXS analysis confirmed that in solution TvNiR exists predominantly as the hexamer. Nevertheless, it should be noted that the results of the experiment do not exclude the presence in the solution of small amount of monomeric and dimeric forms of TvNiR.

Thus all the data confirm that TvNiR is a soluble protein, and the hexamer is stable and predominant if not the unique form of the enzyme in solution and presumably in the cell.

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