Biochemistry (Moscow), Vol. 70, No. 7, 2005, pp. 809-814. Translated from Biokhimiya, Vol. 70, No. 7, 2005, pp. 980-986.
Original Russian Text Copyright © 2005 by Morozkina, Nosikov, Zvyagilskaya, L'vov.
Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM04-140, December 5, 2004.

Isolation, Purification, and Characterization of Nitrate Reductase from a Salt-Tolerant *Rhodotorula glutinis* Yeast Strain Grown in the Presence of Tungsten

E. V. Morozkina*, A. N. Nosikov, R. A. Zvyagilskaya, and N. P. L'vov

Bach Institute of Biochemistry, Russian Academy of Sciences, Leninsky pr. 33, 119071 Moscow, Russia; fax: (7-095) 954-2732; E-mail: Chicelena@pisem.net

Received June 9, 2004

Abstract—The salt-tolerant *Rhodotorula glutinis* yeast strain grew in medium containing nitrate, 1 mM tungsten, and trace amounts of molybdenum (as impurities from the reagents used). Isolation of electrophoretically homogenous preparation of nitrate reductase from the *Rh. glutinis* cells grown under these growth conditions is described. The isolated nitrate reductase is a molybdenum-containing homodimer with molecular mass of 130 kD, containing 0.177 mol of Mo per mol of the enzyme. The activity of the enzyme is maximal at pH 7.0 and 35-45°C and is inhibited by low concentrations of azide and cyanide. The enzyme is almost insensitive to 1 mM tungsten.

Key words: nitrate reductase, tungsten, molybdenum

Nitrate reductase, a key enzyme in nitrate assimilation in eukaryotes [1] as well as in both nitrate assimilation and dissimilation in prokaryotes [2], contains in the active site molybdenum (Mo) as molybdenum cofactor (Mo-co).

A large body of research on plants and microorganisms has shown that tungsten (W) is an antagonist of molybdenum (Mo), and the replacement of Mo in the growth medium by W results in the synthesis of inactive nitrate reductase and some other Mo-containing enzymes [3-5]. Therefore, W, sharing physical and chemical properties with Mo and replacing Mo in Mo-containing enzymes to form catalytically inactive enzyme analogs, is a useful tool in elucidating the properties and functions of Mo in Mo-enzymes [6].

Recently, W-containing enzymes (formate dehydrogenase, aldehyde:ferredoxin-oxidoreductase, formaldehyde:ferredoxin-oxidoreductase, etc.), in which W could not be replaced by Mo, were described [7, 8]. Pterin-containing W-cofactor, an analog of Mo-cofactor of Mo-containing enzymes, was found as the active site of these enzymes [7, 9, 10].

However, there are some enzymes that exhibit catalytic activity with both Mo and W, albeit to a lesser extent with W (formyl-methanofurane dehydrogenase, trimethylamine-N-oxidoreductase, etc.) [11-13]. Molybdopterin, coordinating (liganding) both Mo and W, is the active site of these enzymes.

Finally, there is now ample evidence that W positively affects nitrate reductase activity. Low (1.36-5.44 nM) W concentrations, being added to the soil, stimulated nitrate reductase of plants [14]. The stimulatory effect of W was postulated to be due to its stabilizing effect on the nitrate reductase, thus preventing its inactivation and allowing the replacement of W by trace amounts of ambient Mo with concomitant activation of the W-containing analog of the enzyme. It is also conceivable that the enhanced activity of the nitrate reductase in plants grown in the presence of equimolar concentrations of Mo and W relies on hypersynthesis (immunochemically detected) of the nitrate reductase apoprotein [15].

Previously [16], we have shown that a salt-tolerant *Rhodotorula glutinis* yeast strain, unlike many other microorganisms, including the salt-sensitive *Rh. glutinis* strain, is able to grow in the presence of high (1 mM) equimolar concentrations of W and Mo. Moreover, these conditions promoted enhanced cell growth and elevated nitrate reductase activity. This prompted us to examine the effect of W in the absence of added Mo on cell growth, activity, and intracellular distribution of nitrate reductase(s), as well as to purify and characterize nitrate reductase(s).

The nitrate reductase isolated from the salt-sensitive *Rh. glutinis* strain belongs to the class of NAD(P)H: nitrate-oxidoreductases [17].

* To whom correspondence should be addressed.

0006-2979/05/7007-0809 ©2005 Pleiades Publishing, Inc.

MATERIALS AND METHODS

Glycerol, calcium pantothenate, inositol, nicotinic acid, *p*-aminobenzoic acid, riboflavin, thiamine hydrochloride, biotin, folic acid, and EDTA were purchased from Sigma (USA); methyl viologen, N-(1-naphthyl)ethylenediamine, and sodium dithionite from Serva (Germany); Coomassie Brilliant Blue G-250 from Ferak (Germany); Toyopearl HW-55F from Toyo Soda (Japan). All other chemicals of analytical grade (of highest quality available) were obtained from domestic suppliers.

Organism and growth conditions. The salt-tolerant *Rhodotorula glutinis* yeast strain was obtained as a pure isolate from leaf surfaces of salt-excreting *Atriplex halimus*, a plant found in the Negev desert (Israel) growing under high soil salinity and sharp daily fluctuating temperature and humidity. The strain was purified using selective media and identified as *Rhodotorula glutinis* (Fresen) on the basis of its morphological, physiological, biochemical, and chemotaxonomic properties. The validity of the identification was confirmed by the International Mycological Institute (UK).

Cells were routinely grown at 28-30°C with orbital agitation (220 rpm) in 750-ml flasks in 100-150 ml of liquid modified Rider's medium, pH 4.5-4.6, containing (g/liter): $MgSO_4 \cdot 7H_2O$, 0.5; NaCl, 0.1; $CaCl_2 \cdot 2H_2O$, 0.05; KH_2PO_4, 8.6; KNO_3, 0.3; glycerol, 7.5; $Na_2WO_4 \cdot 2H_2O$, 0.33, supplemented with microelements and vitamins as described earlier [16]. In the control experiments, $Na_2WO_4 \cdot 2H_2O$ was omitted and the medium contained only trace amounts of Mo as impurities from the reagents used. Cell growth was monitored turbidimetrically by measuring the absorbance at 540 nm (OD_{540}). Cells were harvested during exponential growth at specified time points corresponding to maximal nitrate reductase activity.

Cell extract was obtained by cell disruption in a French press at 100-150 atm as described previously [16]. The homogenate was centrifuged at 19,000*g* for 20 min at 4°C, and the supernatant (the cell-free extract) was used for nitrate reductase purification.

Nitrate reductase purification. The enzyme was purified to homogeneity by a combination of several procedures, including ammonium sulfate precipitation (0-40%) and ion-exchange chromatography on a DEAE-Toyopearl 650M (2.1 × 2.6 cm) column. The precipitated material was desalted, resuspended in 10 mM sodium phosphate buffer, pH 7.0, and loaded onto the column. The column was washed with 40 mM sodium phosphate buffer, and the nitrate reductase was eluted with a 40-100 mM sodium phosphate gradient. The buffers were supplemented with 10% glycerol as a stabilizer. The partially purified nitrate reductase was further purified using gel chromatography on a Sephacryl S-200 (2.5 × 93 cm) column, which had been equilibrated with 50 mM sodium phosphate buffer, pH 7.0, and then by ion-exchange chromatography on a DEAE-cellulose DE-52 (1.1 × 2.5 cm) column equilibrated with 20 mM sodium phosphate buffer, pH 7.0. The enzyme was eluted with a 0-0.2 M KCl gradient. The buffers were supplemented with 10% glycerol and 2 mM 2-mercaptoethanol as stabilizers.

All purification steps were performed at 4°C. The enzyme was purified 280- and 43-fold on protein and specific activity basis, respectively. The specific activity of the purified enzyme was rather high (for eukaryotic nitrate reductases), attaining 1.3 μmol NO_2^- formed for 1 min per mg of protein.

Assay of nitrate reductase activity. Nitrate reductase activity was assayed with dithionite-reduced methyl viologen or bromophenol blue as electron donors. The incubation medium contained 80 mM sodium phosphate buffer, pH 7.0, 0.8 mM methyl viologen (or 0.5 mM bromophenol blue), 20 mM KNO_3, 2 mM sodium dithionite, and 20-100-μl aliquots of enzyme preparations. The reaction was initiated by the addition of dithionite and terminated by the addition of a mixture containing 500 μl of 0.6% sulfanilic acid in 20% HCl and 500 μl of 2 mM N-(1-naphthyl)ethylenediamine. The absorbance at 548 nm was measured after 15 min required for color development. One unit of enzyme activity was defined as the amount of enzyme forming 1 nmol of NO_2^- per min at 40°C.

Protein was determined by the method of Bradford [18] with bovine serum albumin as standard.

Gel chromatography for separation of nitrate reductase isoforms, determination of their molecular masses, and molybdenum cofactor content and W distribution was performed at 4°C using a Toyopearl HW-55 (fine) (2.6 × 89 cm) column equilibrated with 50 mM sodium phosphate buffer, pH 7.5, supplemented with 100 mM KCl and 0.5 mM Na-EDTA.

Molybdenum cofactor was determined as described earlier [19]. Aliquots of the homogenous nitrate reductase were heated at 90°C for 90 sec to ensure molybdenum cofactor dissociation. The reaction medium contained 2 mM reduced glutathione and 2 mM ascorbic acid to protect the molybdenum cofactor. Then the treated preparations were incubated at 5°C for 20 h in the presence of 2 mM sodium molybdate and 100 μl of the cell-free extract from *N. crassa* nit-1 mutant bearing the nitrate reductase with defective molybdenum cofactor. After complementation, activity of the resulting nitrate reductase was determined with NADPH as the electron donor.

Non-denaturing polyacrylamide gel electrophoresis (native PAGE) was carried out using a linear (5-15%) gel gradient in the plate (11 × 11 cm, 1-mm thick) by the method of Davis [20]. Tris-HCl, pH 8.8, was used as the electrophoresis buffer. To determine the molecular mass of the homogenous nitrate reductase we used the following Electrophoresis HMW Calibration standards (with their M_r): thyroglobulin (669 kD), ferritin (440 kD),

catalase (232 kD), lactate dehydrogenase (140 kD), and bovine serum albumin (67 kD) (Pharmacia, Sweden).

SDS-PAGE with a 5-20% linear gel gradient was performed according to the Laemmli method [21]. To determine the molecular masses of the enzyme subunits we used the following Electrophoresis HMW Calibration standards (with their molecular masses): thyroglobulin (330 kD), ferritin (220 kD), bovine serum albumin (67 kD), catalase (60 kD), lactate dehydrogenase (36 kD), and ferritin (18.5 kD) (Pharmacia).

Proteins resolved by SDS-PAGE were visualized with silver by the method of Nesterenko [22].

Tungsten in the protein fractions was determined by the kinetic method designed in our laboratory [23].

Metal determination. Metals in the homogenous nitrate reductase were analyzed by flame atomic emission spectrometry and flame mass-spectrometry with an Optima 2000 DV and ELAN 9000 (Perkin Elmer, USA), respectively.

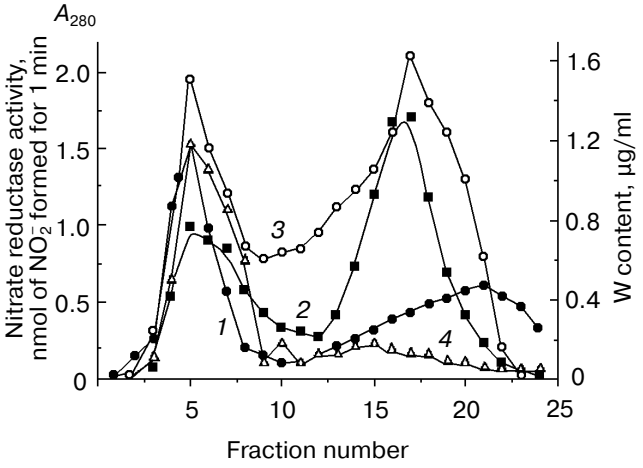

Fig. 2. Gel chromatography of the *Rh. glutinis* cell-free extract on the Toyopearl HW-55 (fine) column: *1*) absorbance at 280 nm; *2*) activity of nitrate reductase, nmol of NO_2^- formed for 1 min; *3*) activity of molybdenum cofactor, nmol of NO_2^- formed for 1 min; *4*) tungsten (W) content, μg/ml.

RESULTS AND DISCUSSION

Previously we have shown [16] that 1 mM W, being added to the medium containing nitrate and Mo, enhanced growth of the salt-tolerant *Rh. glutinis* yeast strain and stimulated its nitrate reductase activity. Figure 1 depicts that addition of 1 mM W to the medium, containing nitrate and only trace amounts of Mo (as impurities from the reagents used), inhibited growth of the same *Rh. glutinis* strain as compared to the control (Mo-containing) medium and retarded the growth onset inducing a 40-60-h lag-phase. Following optimization of growth conditions of yeast cells, maximal nitrate reductase activity (13.9-15.4 nmol of NO_2^- formed for 1 min per mg of protein) was found in exponentially (100-110 h) growing cells. Cells harvested at this growth phase were used for obtaining the cell-free extract and then for isolation and purification of nitrate reductase.

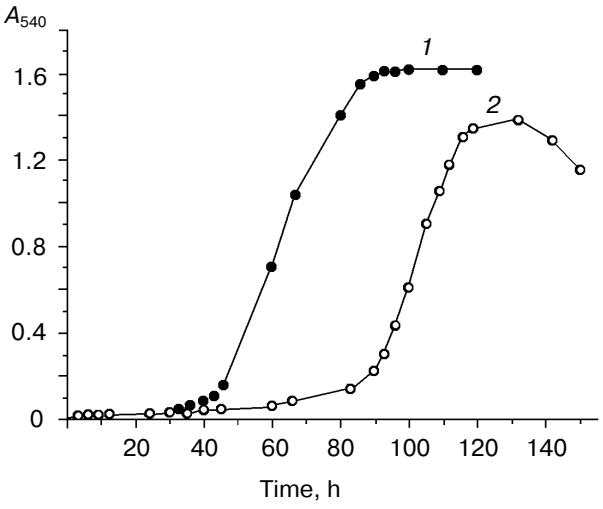

Fig. 1. Growth curves of *Rh. glutinis* in the control medium (*1*) and in the presence of 1 mM Na_2WO_4 (*2*).

Based on gel-filtration chromatography on a Toyopearl HW-55 (fine) column, the *Rh. glutinis* cell-free extract contained two peaks of nitrate reductase activity, suggesting the existence of two enzyme isoforms with different molecular masses (Fig. 2).

The fractions pooled from the Toyopearl HW-55 (fine) column were examined for the presence of molybdenum cofactor. After complementation with the apo-nitrate reductase of the *Neurospora crassa* nit-1 mutant, the elution profile of nitrate reductase activities matched that of molybdenum cofactor activities, suggesting the presence of two nitrate reductase isoforms incorporating molybdenum cofactor in their active sites. Tungsten was found only in the higher molecular mass nitrate reductase fraction. This means that the low molecular mass fraction held only Mo, while the high molecular mass fraction contained Mo and possibly W. As expected, in the control experiments (not shown), when yeast cells were grown in medium supplemented with 1 mM W and 5 mM ammonium (as the sole source of nitrogen), the high molecular mass fractions separated by gel chromatography did not display nitrate reductase activity (ammonium is known to repress synthesis of nitrate reductase). Nevertheless, they contained W, probably in the form of the W-binding protein. Thus, the W-containing fraction revealed by gel chromatography most probably was composed of the W-binding protein and the high molecular mass isoform of nitrate reductase containing Mo or both Mo and W. Inasmuch as the latter lost most of its W during enzyme

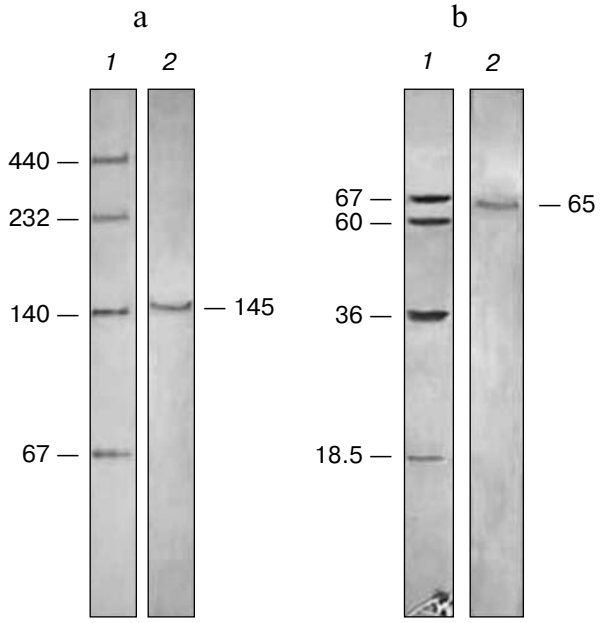

Fig. 3. Native (a) and denaturing SDS-PAGE (b) of the homogenous nitrate reductase (*2*). Lanes *1*: standard protein markers (kD).

purification and because its contribution to the total cellular nitrate reductase activity was insignificant (from 14.4 to 35.0% depending on the cell grinding method used), we have focused our efforts on studying the low molecular mass Mo-containing isoform of nitrate reductase.

Using a combination of ammonium sulfate (0-40%) precipitation, ion-exchange chromatography on a DEAE-Toyopearl 650M column, gel chromatography on a Sephacryl S-200 column, and ion-exchange chromatography on a DEAE-cellulose DE-52 column (for detail, see "Materials and Methods"), nitrate reductase was purified to homogeneity (Fig. 3a).

Gel chromatography on the Toyopearl HW-55 (fine) column showed an apparent molecular mass of the purified nitrate reductase of 127-143 kD. SDS-PAGE revealed a single protein band with molecular mass of 65 kD (Fig. 3b), thus indicating that the enzyme is a homodimer with molecular mass of 130 kD.

Tungsten is known to be a biological antagonist of Mo, and addition of W to medium generally results in formation of inactive analogs of Mo-containing enzymes. The ability of the *Rh. glutinis* yeast to grow in Mo-deprived W-containing medium suggests either synthesis of alternative enzymes or tolerance of the existing enzymes to W. The detection of molybdenum cofactor in the active site of the isolated nitrate reductase, and insensitivity of nitrate reductase to usually inhibitory action of 1 mM W are arguments in support of the second suggestion.

The purified nitrate reductase was W-free and contained 0.177 mol Mo per mol of the enzyme. The relative low content of Mo in the enzyme may be due to a well-known ability of W to induce overexpression of the structural gene of nitrate reductase in organisms grown in W-containing medium, giving an increase in the level of apo-nitrate reductase [15, 16].

The purified nitrate reductase from *Rh. glutinis* has an elevated temperature optimum (35-45°C), which is not typical for mesophilic organisms. Activity of the nitrate reductase was the highest at pH 6.5-7.5 (maximal at 7.0), which coincides with the pH optimum of most intracellular enzymes.

Among the most important kinetic characteristics of an enzyme is its affinity for the substrate. Dixon (a) and Lineweaver–Burk (b) plots describing the rate of reaction as a function of nitrate concentration (Fig. 4) indicated rather low affinity of the *Rh. glutinis* nitrate reductase for substrate (an apparent K_m value for nitrate of 0.74 ±

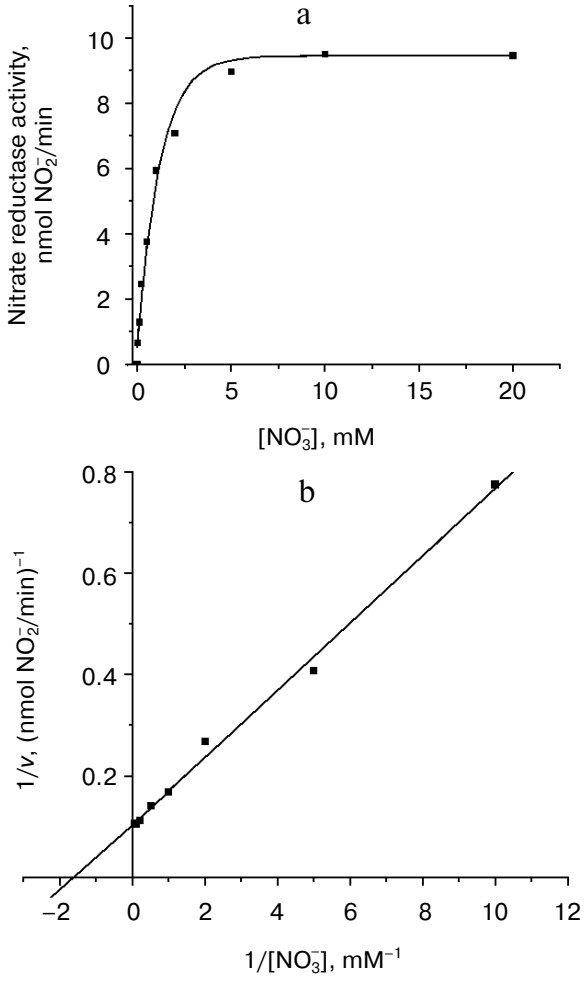

Fig. 4. Dependence of activity of the homogenous nitrate reductase on nitrate concentration expressed as Dixon (a) and Lineweaver–Burk (b) plots.

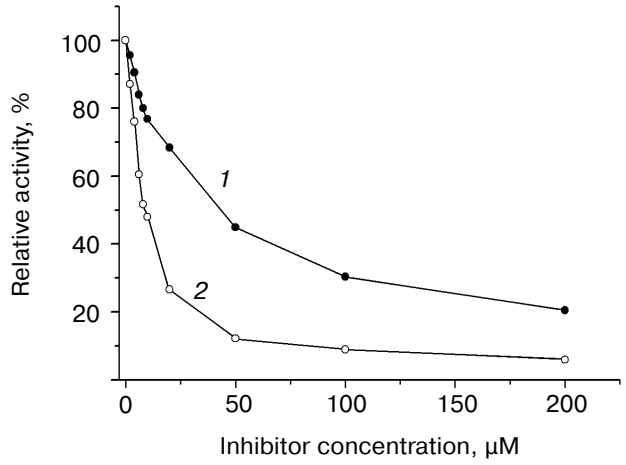

Fig. 5. Effect of azide (*1*) and cyanide (*2*) on activity of nitrate reductase.

0.08 mM) [24]. V_{max} attained 9.35 ± 0.31 nmol of NO_2^- formed per min.

Cyanide and azide inhibit enzyme activity, liganding metals in the enzyme active site. Activity of the nitrate reductase from *Rh. glutinis* was inhibited by low concentrations of cyanide (K_i = 9.4 μM) and azide (K_i = 44.0 μM) (Fig. 5), which is in a good accordance with the data available for the nitrate reductase from the salt-sensitive *Rh. glutinis* strain [17].

Thus, the used salt-tolerant *Rh. glutinis* strain, unlike the salt-sensitive *Rh. glutinis* strain, grew in medium containing 1 mM W and synthesized nitrate reductase, differing from the salt-sensitive counterpart by an elevated temperature optimum and surprisingly high tolerance to W.

What are the reasons for the high tolerance of the nitrate reductase to W? Previously it has been shown that microorganisms [25] and higher plants [26] grown in the presence of high concentrations of toxic metals can synthesize metal-binding proteins and peptides. It is possible that the yeasts used possess the same defense mechanisms.

The high molecular mass fraction of the *Rh. glutinis* nitrate reductase was found to contain W (see Fig. 2), its maximal level attaining 0.8-1.7 μg/ml, thus giving good indirect evidence for the potential biosynthesis of W-binding protein(s). Biosynthesis of the W-binding protein did not depend on the nitrogen source (it occurred in the presence of ammonium, known to repress biosynthesis of nitrate reductases), and was induced (promoted) by high W concentrations in the medium. All our attempts to isolate the W-binding protein using routine approaches such as applying detergents, e.g., Triton-X-100, Tween-80, Tween-85, hexadecyltrimethyl ammonium bromide, sodium cholate, and urea (up to 5 M), or purification by ion-exchange and affinity gel chromatography have not been successful because of rapid loss of W. On exposure to lipolytic enzymes, the W was released, and on exposure to solvents (chloroform–isopropanol) the protein was found in the chloroform fraction, suggesting a high level of lipids in this protein.

We have found that the W-containing protein binds both W and Mo, which may stem from similar physicochemical properties of these metals. Tungsten and Mo have equal atomic (1.40 Å) and ionic (0.68 Å) radii, similar electronegativity (1.4 and 1.3 for W and Mo, respectively), and some other coordination chemistry characteristics are also very similar [27].

It is also conceivable that the observed resistance of the isolated nitrate reductase to W may be a reflection of general resistance of enzymes to various stress factors in the salt-tolerant *Rh. glutinis* strain. Salt-tolerant yeasts are known to possess an elevated level of Hsp26p and Hsp12p (heat-shock proteins) and/or of a protein factor with molecular mass of 140 kD fraction liganding the CCCCT-element in the promoter region of genes encoding different enzymes [28].

An insight into the mechanisms underlying adaptation of living organisms to adverse environmental factors (high concentrations of heavy metals) is of considerable theoretical and applied interest. Further studies on the rather unusual (nontrivial) resistance of the *Rh. glutinis* yeast to 1 mM tungsten will make possible clearer understanding and explanation of this phenomenon.

This study was supported by the Russian Academy of Sciences (grant on cellular and molecular biology).

REFERENCES

1. Mendel, R. R., and Hansch, R. (2002) *J. Exp. Botany*, **53**, 1689-1698.
2. Zumft, W. G. (1997) *Microbiol. Mol. Biol. Rev.*, **61**, 553-616.
3. Haimer, Y. M., Wray, J. L., and Filner, P. (1969) *J. Plant. Physiol.*, **44**, 1197-1199.
4. Notton, B. A., and Hewitt, E. J. (1971) *Biochem. Biophys. Res. Commun.*, **44**, 702-710.
5. Ljungdahe, L. G. (1976) *Trends Biochem. Sci.*, **1**, 63-65.
6. L'vov, N. P. (1989) *Molybdenum in Nitrogen Assimilation in Plants and Microorganisms. 43rd Bach Meeting* [in Russian], Nauka, Moscow.
7. Johnson, M. K., Rees, D. C., and Adams, M. W. W. (1996) *Chem. Rev.*, **96**, 2817-2839.
8. L'vov, N. P., Nosikov, A, N., and Antipov, A. N. (2002) *Biochemistry (Moscow)*, **67**, 196-200.
9. Johnson, J. L., Rajagopalan, K. V., Mukund, S., and Adams, M. W. W. (1993) *J. Biol. Chem.*, **268**, 4848-4852.
10. Chan, M. K., Mukund, S., Kletzin, A., Adams, M. W. W., and Rees, D. C. (1995) *Science*, **267**, 1463-1469.
11. Bertram, P. A., Schmitz, R. A., Linder, D., and Thauer, R. K. (1994) *Arch. Microbiol.*, **161**, 220-228.
12. Schmitz, R. A., Richter, M., Linder, D., and Thauer, R. K. (1992) *FEBS Lett.*, **309**, 78-81.

13. Buc, J., Santini, C. L., Giordani, R., Czjzek, M., Wu, L. F., and Giordano, G. (1999) *Mol. Microbiol.*, **32**, 159-168.
14. Notton, B. A. (1983) in *Metals and Micronutrients. Uptake and Utilization by Plants* (Rob, D. A., and Pierpoint, W. S., eds.) Academic Press, New York, pp. 219-239.
15. Deng, M., Moureax, T., and Caboshe, M. (1989) *Plant Physiol.*, **91**, 304-309.
16. Nosikov, A. N., Chichikalo, E. V., Golubeva, L. I., Zvyagilskaya, R. A., and L'vov, N. P. (2000) *Biochemistry (Moscow)*, **65**, 204-207.
17. Guerrero, M. G., and Gutierrez, M. (1977) *Biochim. Biophys. Acta*, **482**, 271-285.
18. Bradford, M. A. (1976) *Analyt. Biochem.*, **72**, 248-254.
19. Hawkes, T. R., and Bray, R. C. (1984) *Biochem. J.*, **219**, 481-493.
20. Davis, B. J. (1965) *Ann. N. Y. Acad. Sci.*, **121**, 404-407.
21. Laemmli, U. K. (1970) *Nature*, **227**, 680-683.
22. Nesterenko, M. V. (1994) *Biochim. Biophys. Meth.*, **28**, 231-242.
23. Nosikov, A. N., Chichikalo, E. V., and L'vov, N. P. (2001) *Prikl. Biokhim. Mikrobiol.*, **37**, 739-741.
24. Sengupta, S., Shaila, M. S., and Rao, G. R. (1996) *Biochem. J.*, **317**, 147-155.
25. Antipov, A. N., Lyalikova, N. N., and L'vov, N. P. (2000) *IUBMB Life*, **49**, 137-141.
26. Kalakutsky, K. L., L'vov, N. P., and Zabolotny, A. I. (1991) *Biokhimiya*, **56**, 1220-1227.
27. Kletzin, A., and Adams, M. W. (1996) *FEMS Microbiol. Rev.*, **18**, 5-63.
28. Andreishcheva, E. N., and Zvyagilskaya, R. A. (1999) *Prikl. Biokhim. Mikrobiol.*, **35**, 243-256.