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The Effect of ATP Sulphurylase on the Prooxidant Properties of Selenate in Yeast *Schizosaccharomyces pombe*

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Summary

Selenium is an essential microelement in human and animal nutrition, whose intake can be in inorganic (*e.g.* selenite, selenate) or organic form (*e.g.* selenomethionine). The prooxidant effect of inorganic selenium sources in the animal nutrition has been found as a great disadvantage. Therefore, in this study the effect of the ATP sulphurylase on the prooxidant properties and toxicity of selenate in the fission yeast model organism *Schizosaccharomyces pombe* has been studied. Two strains of yeast *Schizosaccharomyces pombe* were used, selenate resistant (Se^R) ZIM 1889 and selenate sensitive (Se^S) ZIM 1878 strains, with inactive and active ATP sulphurylase, respectively. During the yeasts' exposure to selenate growth, intracellular oxidation, cell viability and antioxidative defence systems were determined. Also, activities of antioxidative enzymes (catalase, superoxide dismutases, glutathione peroxidase, glutathione reductase and glucose-6-phosphate dehydrogenase) and intracellular content of reduced glutathione were measured. The results show altered enzymatic activities and decreased intracellular content of reduced form of glutathione in the selenate sensitive strain as a consequence of active ATP sulphurylase, which enables selenate reduction leading to oxidative stress. During the selenate reduction, reactive oxygen species (ROS) are generated and therefore antioxidative defence systems are induced. In contrast, in the selenate resistant strain with inactive ATP sulphurylase, where selenate reduction does not occur, no induction of antioxidative defence systems was found. Consequently, the active ATP sulphurylase is the key enzyme for the prooxidant properties of selenate and it seems to be the main reason for selenate toxicity and ROS formation during the selenate reduction.

Key words: ATP sulphurylase, *Schizosaccharomyces pombe*, selenate, prooxidant properties

Introduction

Selenium is a powerful micronutrient constituting the active centre of about 20 eukaryotic proteins highly relevant in biochemistry, mostly for redox state regulation. In the last 50 years it has been extensively studied, particularly its metabolic functions and the consequences of its deficiency in human and animal diets (*1*,*2*).

Dietary selenium, primarily as selenomethionine and selenocysteine for humans, fulfills the dietary requirement for selenoenzymes and proteins. In humans and animals excessive dietary selenium may be toxic, especially inorganic selenium in animal nutrition where the prooxidant properties of selenite were found as a great disadvantage (*3*). According to the free radical theory of selenium toxicity based on the reaction of selenium compounds such as selenite, selenium dioxide and

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diselenides, with thiols such as glutathione, selenotrisulphides are formed, which react with other thiols to generate superoxide and other reactive oxygen species. This catalytic reaction of selenium compounds with thiols likely accounts for selenium toxicity. Selenium compounds such as selenates and selenoethers (R-Se-R), which do not react with thiols, become toxic only after reduction to selenite or selenol (*4*).

Selenate toxicity is believed to be connected with the formation of reactive oxygen species (ROS), namely superoxide anions ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2), during selenate reduction. Superoxide anions might be processed during nonenzymatic selenate reduction with reduced glutathione (*5,6*). It is known that H_2O_2 can react with Fe^{2+} or Cu^+ (Fenton's reaction), so more ROS can be formed, namely hydroxyl radical ($OH^{\cdot}$) (*7*). It was demonstrated that enzyme ATP sulphurylase plays an important role in the reduction of selenate to selenite (*8*). Since selenite is more toxic than selenate (*5*), selenate tolerance of yeasts depends on the amount of selenite produced in the presence of functionally active ATP sulphurylase during the selenate reduction process (*8*).

Selenate has similar chemical properties as sulphate, so it is assimilated by sulphur assimilation pathway (*9*), where it is co-transported into the cell with $3H^+$ by sulphate permease, which is the first step in sulphur assimilation pathway. Selenate needs to be activated by enzyme ATP sulphurylase before the reduction process can begin. It converts selenate to adenosine 5'-phosphoselenate (APSe), which is reduced by APS kinase to 3'-phospho-5'-adenosine phosphoselenate (PAPSe). Selenite is reduced from PAPSe by PAPSe reductase (*10*). Selenide (H_2Se) can be formed from selenite by sulphite reductase (*11*) and can also be reduced nonenzymatically with reduced glutathione (GSH) to elemental selenium (*9,11*).

If the levels of ROS produced during enzymatic as well as nonenzymatic selenate reduction exceed the antioxidant capacity of the cells, the cells face an oxidative stress (*12*). ROS can react with cellular macromolecules (DNA, lipids and proteins), which are protected by primary (neutralising ROS) and secondary antioxidative defence systems (repairing damages, degrading oxidised molecules). Cells possess enzymatic and nonenzymatic primary defence systems to protect cellular components, GSH being perhaps the best-known example of nonenzymatic antioxidative defence system (*12*). $O_2^{\cdot -}$ can be enzymatically removed by manganese containing superoxide dismutase (MnSOD) in mitochondria, and copper and zinc superoxide dismutase (Cu/ZnSOD) in cytoplasm (*13*). The enzyme catalase (CAT) catalyses the dismutation of H_2O_2. Glutathione peroxidase (GPX) reduces H_2O_2 using GSH as a reductant. Subsequently, oxidised glutathione (GSSG) can be reduced by NADPH-dependent glutathione reductase (GR), which is responsible for the maintenance of the GSH/GSSG ratio in the cell (*12*). NADPH, the reducing power, is mainly generated by glucose-6-phosphate dehydrogenase (G6PDH) *via* the pentose phosphate pathway (*14*).

In this study the effect of ATP sulphurylase on the prooxidant properties of selenate in selenate resistant (Se^R) and selenate sensitive (Se^S) yeast strains *Schizosaccharomyces pombe* has been investigated. To elucidate selenate toxicity in the Se^R and Se^S strains during the selenate reduction and ROS generation, estimation of intracellular oxidation, cell viability, enzymatic activities of the enzymes mentioned above and intracellular content of GSH were determined.

Materials and Methods

Yeast strains

Two *Schizosaccharomyces pombe* strains were used. *Schizosaccharomyces pombe* ZIM 1889 (B-579 Se^R-2) is selenate resistant/sulphate non-utilizing strain (Se^R) with the inactive sulphate reduction pathway. This mutant has defective ATP sulphurylase and therefore it is unable to convert sulphate to adenosine 5'-phosphosulphate. *Schizosaccharomyces pombe* ZIM 1878 (B-579 h^- Se^S) is selenate sensitive/sulphate utilizing strain (Se^S) with an active sulphate reduction pathway by which sulphate ions can cross the cell membrane and enter the pathway (*10*). Yeast strains are deposited at the Collection for Industrial Microorganisms (ZIM), Ljubljana, Slovenia.

Growth medium and culture conditions

Yeasts were cultured at 28 °C on a shaker (200 rpm) in the liquid YEPD medium (yeast extract 1 % (Biolife, Italy), peptone 1 % (Biolife, Italy) and glucose 2 % (Sigma-Aldrich, USA)) in 500-mL Erlenmeyer flasks. Initial pH of the medium was adjusted to pH=4. 6 with 1 M HCl or NaOH (Sigma-Aldrich, USA). Selenate concentrations of 0. 04, 0. 4 and 4 mM in the medium were obtained with the addition of appropriate amounts of 0. 2 M stock solutions as Na_2SeO_4 (Merck, Germany) at the beginning of cultivation.

For viability assays, YEPD agar (yeast extract 1 %, peptone 1 %, glucose 2 %, and agar 2 % (Biolife, Italy)) was used.

Cell viability determination

For viability assays in the mid-exponential phase, the cells were diluted in physiological solution (0. 9 % NaCl (Merck, Germany)), plated on YEPD agar and counted after 3 days of incubation at 28 °C. The cell viability results are expressed as colony forming units per mL (CFU/mL), where one CFU is defined as a cell capable of replication (*15*). The total cell number was determined by measuring absorbance (A_{650}) and calculated from standard curve equation.

Estimation of intracellular oxidation

Intracellular oxidation was estimated in the mid-exponential phase using a method described by Jakubowski and Bartosz (*16*), where compound 2',7'-dichlorofluorescin (H_2DCF) (Sigma-Aldrich, USA) is able to react with oxidants. It is given as 2',7'-dichlorofluorescin diacetate (H_2DCFDA), which easily penetrates the plasma membrane and is hydrolyzed inside the cells by non-specific esterases. Therefore, the non-fluorescent H_2DCF is oxidized to fluorescent 2',7'-dichlorofluorescin (DCF), which is determined fluorimetrically (Tecan Safire2, Switzerland). Cell suspensions were prepared as described previously by Jamnik and Raspor (*17*), and incubated with H_2DCFDA in the dark (200 rpm, 120 min, 28 °C).

Results are expressed in relative values with the values of control samples presenting 100 %.

Preparation of yeast cell-free extracts

The yeast cell-free extracts were prepared from the culture in the mid-exponential growth phase using liquid nitrogen. The biomass was harvested (4000 rpm, 5 min) and washed twice with 15 mM phosphate buffer (pH=4. 0) (Merck, Germany), covered with liquid nitrogen and smashed three times. Phosphate buffer of 50 mM (pH=7. 0) with protease inhibitor cocktail (Roche, Switzerland) was added to the biomass at a ratio 5 to 1. Centrifugation at 20 000×*g* and 4 °C for 20 min followed. The supernatant was collected and protein levels were determined by the method of Bradford (*18*) with BSA as standard. The supernatant was stored at –80 °C for further analysis for a maximum of three days.

Measurement of the efficiency of antioxidative defence systems

Enzymatic activities of superoxide dismutases, catalase, glutathione peroxidase, glutathione reductase and glucose-6-phosphate dehydrogenase were determined in yeast cell-free extracts. The total SOD activity was assayed using the method of Ravindranath and Fridovich (*13*), where one unit of SOD activity is defined as the quantity of enzyme that reduces the superoxide-dependent colour change by 50 %. The MnSOD activity was measured after adding 0. 6 M KCN to the same reagents as for measuring total SOD activity (*19*). CAT activity was determined in terms of decomposition of hydrogen peroxide, which was followed directly by the decrease in absorbance at 240 nm (*20*). The activities of GPX and G6PDH were determined by monitoring NADPH oxidation and $NAPD^+$ reduction at 340 nm, respectively (*21,22*). For determining GR activity, method of Pinto *et al*. (*23*) was used. The reduced glutathione (GSH) content was measured as described by Jamnik and Raspor (*17*).

Experiments were done in duplicates. The data obtained were analyzed using Student's *t*-test for significant differences between the two sample mean values ($N \geq 6$).

Results and Discussion

The enzyme ATP sulphurylase of sulphur assimilation pathway has an important role not only in sulphate, but also in selenate reduction processes (*8,22,24*). ATP sulphurylase activates selenate to APSe, which can be further reduced (*24*). Because selenate-resistant (Se^R) strain of *S. pombe* used in our study has inactive ATP sulphurylase, selenate cannot be activated to form APSe and further reduced to toxic selenite. Therefore, selenate has no inhibitory effect on the growth (*8*) and cell viability (Fig. 1) of the Se^R strain. Raspor *et al*. (*8*) demonstrated that in the presence of selenate, the Se^R cells accumulated (3163±30) μg of Se per g of dry mass, which is 10-fold more bioaccumulated selenium than by selenate-sensitive (Se^S) strain ((307±37) μg of Se per g of dry mass) of yeast *S. pombe* if 4 mM selenate were added to the growth medium, while its growth did not decrease. It was also demonstrated that selenite added to the growth medium of Se^R and Se^S strains caused similar inhibitory effects on both strains. This effect was similar to that when selenate was added to the growth medium of the Se^S strain (*8*).

Inhibitory effect of Se(VI) on yeast viability is seen in Fig. 1. Total cell number and colony forming units (CFU) did not change in selenate resistant (Se^R) strain due to Se(VI) supplementation. Cell viability decreased significantly in the selenate sensitive (Se^S) strain, where 0. 04 mM Se(VI) reduced total cell number and CFU by 63 and 79 %, respectively. Both 0.4 and 4 mM Se(VI) concentrations caused total inhibition of cell growth; therefore, CFU was reduced by 98 % compared to the control CFU.

Selenate sensitive (Se^S) strain has an active ATP sulphurylase, and that is why selenate can be activated to APSe and reduced to selenite, which can reduce yeast growth (*8*). Therefore, it was expected that cell viability in the mid-exponential phase would decrease. Results showed that total cell number and cell viability decreased significantly when higher selenate concentrations were added to the medium (Fig. 1).

Due to selenate toxicity, microorganisms have developed different detoxification mechanisms, which involve enzymatic reduction of selenite to elemental selenium, as described for moulds and yeast (*24–26*). Nonenzymatic

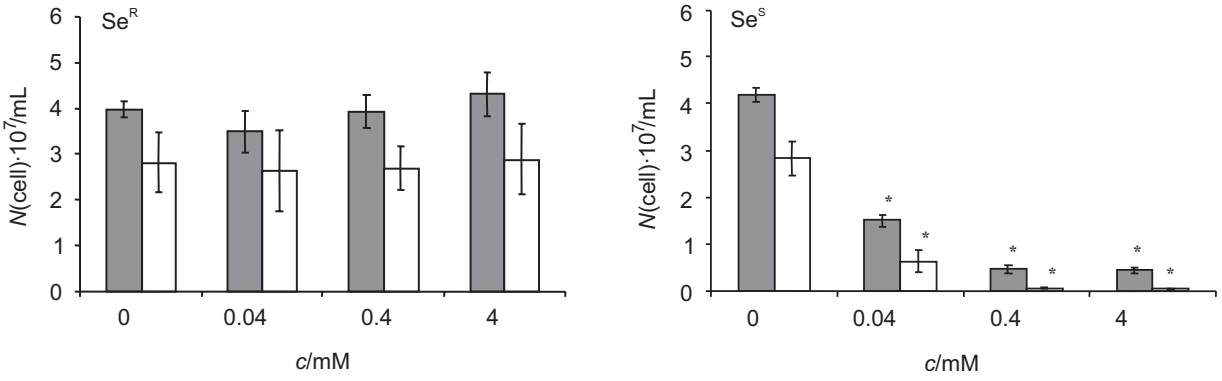

Fig. 1. Effect of Se(VI) on cell viability of yeast *S. pombe* ZIM 1889 (Se^R) and ZIM 1878 (Se^S) in the mid-exponential phase of the aerobic cultivation. The results are presented as the mean of at least two independent cultivations±SD. (Symbols: ■ total cell number, □ CFU, *p<0. 001)

detoxification mechanism includes reduced form of glutathione for conversion of selenate to elemental selenium (*27*). Antioxidative defence systems also have important role in affecting selenite resistance, since it has been shown that overexpression of enzyme glutathione reductase increased the resistance to selenite in the yeast *Saccharomyces cerevisiae* (*5*). To get a better understanding of the ATP sulphurylase in connection with oxidative stress during the selenate reduction, we investigated the efficiency of antioxidative defence systems in the fission yeast, *S. pombe*, with active and inactive ATP sulphurylase.

Toxicity of selenate when reduced to selenite is believed to be due to ROS formation during selenate reduction process (*5,6,9*), which can lead to oxidative stress (*12*). If ROS are not removed by antioxidative defence systems, they can damage cellular macromolecules, cell replication is stopped and cell death can result. According to this fact we estimated intracellular oxidation, which was not altered in the Se^R strain, while it was significantly increased in the Se^S strain during the selenate supplementation (Table 1). From these results, it can be concluded that active ATP sulphurylase is necessary for selenate reduction when ROS are generated and oxidative stress occurs.

Therefore, Se(VI) added at the beginning of the batch cultivations caused no significant differences in enzyme activities of antioxidative defence system nor in reduced glutathione content of Se^R strain (Table 1). On the contrary, in Se^S strain (Table 1) only total activity and Cu/ZnSOD activity were not altered. MnSOD activity increased approx. 3-fold when 0. 4 and 4 mM concentrations of Se(VI) were added, but not in the samples with 0. 04 mM Se(VI). All three (0. 04, 0. 4 and 4 mM) Se(VI) concentrations led to significantly higher activities of CAT, GPX and GR. Activities of CAT and GPX increased in proportion to higher Se(VI) concentrations. The concentrations of Se(VI) of 0.04, 0.4 and 4 mM caused increased CAT activity by 3-, 4.7- and 5-fold, respectively. Lower increases were found in GPX activity, in the range from 1. 3 to 1. 9 in comparison with control sample without selenium. GR activity was 10-fold higher when samples were treated with 0. 04 mM Se(VI), but only 4. 4- and 3. 7-fold when 0. 4 and 4 mM Se(VI) were added, respectively. G6PDH activity was also measured. However, only 0. 04 mM Se(VI) led to significant, 18-fold increase (Table 1). Reduced glutathione (GSH) content did not change in selenate resistant strain, but 0. 04, 0. 4 and 4 mM Se(VI) altered the reduction of GSH by 6-, 11- and 14-fold in the Se^S strain, respectively.

Superoxide dismutases are the main antioxidative defence systems against superoxide anions (*28*). Total SOD and Cu/ZnSOD activities were not changed significantly during the selenate exposure. Only MnSOD activity increased in the selenate-treated samples of the Se^S strain (Table 1), which indicates the increase of superoxide anion generation as a by-product of oxidative phosphorylation, probably due to higher ATP consumption during Se(VI) exposure needed for selenate activation.

Se(VI) ions added to the Se^S strain increased catalase and glutathione peroxidase activity (Table 1), which are responsible for removing hydrogen peroxide (H_2O_2) from the cells. It is believed that hydrogen selenide (H_2Se), produced from selenite by sulphite reductase (*11*), can react with GSH from which H_2O_2 is formed (*6*), which causes oxidative stress. Since CAT has lower affinity for H_2O_2 than GPX, it plays an important role when higher intracellular concentrations of H_2O_2 are induced (*29*). It can be postulated that Se(VI) contribute to the increase of H_2O_2 to such extent that CAT activity has to be induced. Removing H_2O_2 is critical for the cell, since it can react with metal ions (*e.g.* Fe or Cu) resulting in the formation of hydroxyl radical ($OH^{\cdot}$), which is known as Fenton reaction (*7*).

Table 1. Effect of Se(VI) on intracellular oxidation and antioxidative defence systems (including the activity of total superoxide dismutase (SOD), manganese-dependent superoxide dismutase (MnSOD), copper/zinc-superoxide dismutase (Cu/ZnSOD), catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (GR), glucose-6-phosphate dehydrogenase (G6PDH) and intracellular content of glutathione (GSH)) of yeast *S. pombe* ZIM 1889 (Se^R) and ZIM 1878 (Se^S) in the mid-exponential phase of the aerobic cultivation

	Control		0.04 mM		0.4 mM		4 mM	
	Se^R	Se^S	Se^R	Se^S	Se^R	Se^S	Se^R	Se^S
[a]Intracellular oxidation	100	100	88±16	**(152±23)***	86±14	**(200±44)***	83±23	**(153±27)***
[b]Total SOD	13.2±0.4	13.0±1.1	13.0±0.8	13.9±3.6	13.8±1.1	13.3±1.7	13.3±0.6	13.6±1.2
[c]MnSOD	0.9±0.4	1.0±0.6	0.7±0.4	1.3±0.7	1.0±0.5	**(3.1±0.6)***	1.0±0.6	**(3.3±0.6)***
[b]Cu/ZnSOD	12.4±0.3	12.0±1.4	12.3±0.3	12.5±4.1	12.8±1.1	10.2±1.6	12.3±0.3	10.3±1.4
[b]CAT	8.7±1.9	22.4±4.7	10.4±4.4	**(66.9±3.0)***	9.2±1.5	**(106.3±8.3)***	8.3±2.5	**113.0±4.3***
[c]GPX	28±1	28±4	28±1	**(38±5)****	28±2	**(51±3)***	28±2	**52±6***
[c]GR	17±11	10±1	18±10	**(101±4)***	19±10	**(44±3)***	15±5	**(37±2)***
[b]G6PDH	0.41±0.01	0.32±0.02	0.34±0.02	**(0.57±0.05)***	0.36±0.02	0.30±0.08	0.36±0.02	0.33±0.01
[d]GSH	2.56±0.09	4.30±0.16	2.64±0.40	**(0.73±0.03)***	2.95±0.46	**(0.40±0.16)***	2.89±0.40	**(0.29±0.04)***

Symbols: [a]relative oxidation compared to control (%); [b]specific activity in U/mg of proteins; [c]specific activity in mU/mg of proteins; [d]μmol of reduced glutathione per g of wet biomass; $^{**}p<0.01$, $^*p<0.001$ ($N\geq6$). The results are presented as the mean of at least two independent cultivations±SD

Oxidized glutathione, produced from GPX catalyzed reaction, has to be reduced by the NADPH-dependent glutathione reductase (*12*), when overexpression of GR increases resistance to selenite (*5*). In contrast to CAT and GPX activity, higher concentration of Se(VI) decreased GR activity (Table 1) of Se^S strain. As already mentioned, since the addition of 4 mM Se(VI) caused higher selenium bioaccumulation in Se^S strain (*8*), intracellular consumption of ATP and NADPH was increased due to higher selenate transport and its reduction. Consequently, possible lower content of ATP in NADPH required for GR activity caused lower GR activity in 4 mM than in the case of 0. 04 mM Se(VI) and therefore, the level of GR induction was lower. Additionally, activity of glucose-6-phosphate dehydrogenase (G6PDH), which catalyses the rate-limiting NADPH-producing step in the pentose phosphate pathway (*14*), was significantly higher only when 0. 04 mM Se(VI) were added to Se^S strain (Table 1).

Besides enzymatic defence system, the role of reduced form of glutathione in the antioxidative defence systems was also investigated. According to the measurement of intracellular content of GSH (Table 1), it can be concluded that the addition of Se(VI) does not cause any significant differences between the Se(VI) treated samples and the control Se^R strain. On the contrary, it was found that in Se^S strain selenate supplementation decreased intracellular content of GSH (Table 1). It is known that GSH has a dual role in selenite-induced stress. It can act as an antioxidant, protecting the cells against oxidative stress, or as a prooxidant, facilitating oxidative stress (*27*). Selenite can react with GSH in nonenzymatic reduction, when ROS are believed to be formed, especially hydrogen peroxide and superoxide anion (*11,30*). GSH content in Se^S strain can decrease due to probable selenite transport in the vacuole of the cell as a glutathione conjugate resulting in depletion of GSH in the cytoplasm. Therefore, GSH in the vacuole could not be efficiently regenerated by GR (*5*). Due to higher selenate bioaccumulation when higher concentrations of selenate were added to the growth media in Se^S strain (*8*), more GSH could react in nonenzymatic reaction, leading to lower concentrations of GSH in the case of 4 mM selenate in comparison with 0. 04 mM selenate concentration.

Conclusion

In this study the influence of selenium prooxidative properties on the antioxidative defence system level in the fission yeast *Schizosaccharomyces pombe* was presented. It can be seen from Table 1 that Se(VI) did not alter enzymatic activities of Se^R strain due to inactive ATP sulphurylase. Therefore, the results are consistent with the work of Raspor *et al*. (*8*), who found that active ATP sulphurylase is necessary for the selenate reduction when ROS are formed (*5,6*). The model study of analysis of selenate sensitive and resistant strains showed that active ATP sulphurylase has a key role in selenate prooxidative properties. Comparison of antioxidative enzyme activities and intracellular oxidation of dichlorofluorescin (DCFH) of Se^S and Se^R strains showed that selenate toxicity is caused by induction of ROS formation when active ATP sulphurylase is present, which can be clearly observed as decreased cell viability.

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