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Effects of hexavalent chromium on the plasma membranes of sensitive and tolerant mutants of *Schizosaccharomyces pombe*. An EPR study

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Abstract

The interactions of chromium(VI) with the plasma membranes of chromium-sensitive (*chr-51S*) and chromium-tolerant (*chr1–66T*) mutants and their parental strain (*6chr*$^+$) of a *Schizosaccharomyces pombe* strain were studied by electron paramagnetic resonance (EPR) spectroscopy. 5-doxylstearic acid (5-SASL) and 3-doxylbutyric acid (HO-185) spin probes were used to label the membranes. The order parameter *S* from the EPR spectra was calculated at different temperatures (0–25 °C) in order to characterize the internal dynamics of the membranes. In control experiments, both mutants exhibited differences in structural transitions in the both 5-SASL- and the HO-185-labeled membranes in comparison with their parental strain, suggesting differences in the membrane composition and/or rotational dynamics of these mutants. Addition of $K_2Cr_2O_7$ (225 μM) induced small decreases in the phase transition temperatures of the 5-SASL-labeled membranes of the parental and chromium-sensitive strains. More pronounced effects of the chromium compound on the HO-185-labeled membranes were detected as evidence that the membrane perturbations are mostly localized in the environment of the lipid–water interface.
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Keywords: Chromium(VI); Spin label; Plasma membrane; EPR; *Schizosaccharomyces pombe*; Fission yeast

1. Introduction

Besides the very significant role of chromium in industry, its compounds are indispensable for the metabolism of living creatures. Nevertheless, its toxic effects on biological objects are a serious problem worldwide. The concentration of chromium compounds in the environment was earlier tolerable, but as a consequence of human activities, the level has now increased. The international data indicate that the current high levels of chromates, which contain Cr(VI), exceed the reducing capacity of the environment. Unfortunately, chromate compound, as major environmental pollutants, are among the best-documented mutagens and carcinogens [1–3]. In addition to the relevance of metal toxicity as concerns the environmental/industrial applications of yeast and other fungi, these organisms also serve as useful models for the characterization of certain aspects of metal toxicity, and yeast strains are excellent eukaryotic models for delineation of the mechanism of metal toxicity. Chromium exists in many oxidation states, but only Cr(VI) and Cr(III) form stable compounds under environmental conditions. Cr(VI), the most toxic chromium species, penetrates into cells as the tetrahedral chromate anion (CrO_4^{2-}) [4] by facilitated diffusion, using a nonspecific anion carrier, the permease system; this anion is then rapidly reduced intracellularly to Cr(III) [5]. In contrast, Cr(III), which can cross the membranes only very slowly in the form of cations, is much less mobile and exists mostly bound to organic matter in the soil and the aquatic environment. In vitro experiments have revealed that Cr(III) forms many complexes with biologically relevant ligand molecules: it may be involved in DNA cross-linking, DNA–protein cross-linking, DNA condensation and decreasing DNA replication fidelity [6–9]. It is believed that the reduction of Cr(VI) to its lower oxidation states is an important step in chromate toxicity [2]. The one-electron reduction of Cr(VI) by cellular reductants produces different reactive oxygen species (ROS). The mutagenic and carcinogenic effects of Cr(VI) compounds may be attributed to the fact that they are able to induce hydroxyl, superoxide anion, thiyl and glutathionyl free radicals [10]. Cellular molecules such as lipids, proteins and DNA are highly susceptible to oxidative attack and degradation. This multiplicity of inter-

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action between chromate compounds and cellular processes has been discussed in earlier publications [11–13]. However, few studies have been reported on the interactions of Cr(VI) with plasma membranes. In order to examine such interactions, electron paramagnetic resonance (EPR) spectroscopy experiments were performed in the present study on chromium-sensitive and chromium-tolerant mutants and their parental strain of *Schizosaccharomyces pombe*. EPR spectroscopy in combination with the use of probe molecules seems to be a useful technique with which to study the dynamic and structural changes in the lipid region of membranes. Cr(VI) readily interacts with the lipid components of the membranes and the perturbations are located very close to the lipid–water interface.

2. Materials and methods

2.1. Chemicals

$K_2Cr_2O_7$ and other chemicals were of analytical grade and purchased from Sigma (Germany) and Reanal (Hungary). $K_2Cr_2O_7$ solutions were always freshly prepared before the experiments. Stock solutions (5 mg/ml) were prepared from the probe molecules 5-(4′4-dimethyloxazolidine-*N*-oxyl)stearic acid, also known as 5-doxylstearic acid (5-SASL) and 4-(2-*n*-undecyl-3-oxyl-4′,4-dimethyloxazolidine-*N*-oxyl)butyric acid (HO-185) in ethanol, and the solutions were kept at − 18 °C until use.

2.2. Strains and culture conditions

The chromium-tolerant (*chr1–66T*) and chromium-sensitive (*chr-51S*) mutants of *S. pombe* strains, earlier designated *CT-6.66* and *CS-6.51*, were used [14]. The mutants were obtained by induced mutagenesis from a parental ($6chr^+$) auxotrophic strain (*lys1–131*, h^+), earlier designated *CW-6*. The media used throughout the experiments contained 0.5% yeast extract, 3% glucose, 150 mg/ml lysine and 100 mg/ml leucine (YEL). Media were solidified with 2% agar (YEA). Five-day-old stock cultures were used to prepare overnight mid-log-phase cultures. The mutants were obtained and tested with the methods described by Moreno et al. [15].

2.3. Preparation of samples for spin labeling

Mid-log-phase cells were collected by centrifugation and washed twice with 0.6 M KCl solution. For spheroplast formation, the suspension was incubated with 2% lyophilized snail enzyme prepared from *Helix pomatia* in 0.6 M KCl as osmotic stabilizer. After incubation for 2 h at 37 °C, the spheroplasts were washed twice in stabilizer solution and diluted five-fold in 0.6 M KCl prior to the addition of spin probes. For control experiments, 10 μl of 5-SASL or 14 μl of HO-185 (5 mg/ml in ethanol) was added to 500 μl of cell suspension, and the mixture was gently shaken for 3 min at room temperature to facilitate spin probe incorporation. Thereafter, the spheroplast suspension was sedimented for 3 min at 3000 × *g* and resuspended in 300 μl of 0.6 M KCl. The suspension was transferred to a 100 μl capillary tube and centrifuged again at 4 °C, and the supernatant was carefully removed. The capillary tubes contained 10^8 spheroplasts in each experiment. Under these conditions, no isotropic triplet arising from unincorporated spin probes was detected. In order to study the effects of Cr(VI) on the plasma membranes, $K_2Cr_2O_7$ solution (225 μM as final concentration) was added before spin labeling. After treatment with $K_2Cr_2O_7$ for 120 min, the cell suspension was washed again with 0.6 M KCl.

2.4. EPR measurements

EPR spectra were recorded with an ESP 300E spectrometer (Bruker BioSpin, Germany) equipped with a 412 VT temperature regulator. The EPR spectra of the fatty acid spin probes (5-SASL and HO-185) incorporated into the membranes were taken in the temperature range from 0 to 25 °C for both control and chromate-treated samples. The conventional EPR spectra were obtained at a microwave power of 5 mW and at a field modulation of 100 kHz with an amplitude of 0.2 mT. The spectra were scaled to the same peak-to-peak amplitude or were normalized to an identical double integral. In biological membranes, molecular ordering exists. The order parameter (*S*), which characterizes the mean orientation of the spin labels in the membrane, is usually calculated in order to describe the effects of different substances on membranes [16,17]. The spectra at lower temperatures did not give resolved turning points from which the inner splitting could be estimated. The calculated order parameter *S* proposed by Israelachvili et al. [18] was therefore used, which is particularly useful in cases where the inner splitting is not measurable from the experimental spectra:

$$S = 1/2(3(A'_{zz} - A_{\perp})/(A_{zz} - A_{\perp}) - 1)$$

where $A'_{zz} \approx A'_{||}$ is the outer hyperfine splitting measured in the experimental spectrum, $A_{zz} \approx A_{||}$ and $A_{\perp} = 1/2(A_{xx} + A_{yy})$, where A_{xx}, A_{yy} and A_{zz} are the principal values of the *A* tensor. The order parameters were calculated by using the values $A_{||} = 3.36$ mT and $A_{\perp} = 0.605$ mT [19]. For the chromate-treated 5-SASL membranes, the Cr(V) signal overlapped with the high-field extreme. A simple parameter was therefore introduced instead of $2A_{||}$ so as characterize the changes in mobility of the probe molecules as a function of temperature. This parameter (spectral parameter *P*) was defined as the distance between the low-field maximum and the crossover point of the Cr(V) signal. In order to compare the differences between the strains as concerns the effects of Cr(VI), the plot of the parameters against temperature was approximated by two straight lines. The straight lines were obtained by using a computer program that calculated the breakpoint by searching for the minimum of the residual sum

of squares of the two regression lines. In accordance with Jones and Molitoris [20], an approximate F value was calculated to verify that a broken line gave a significantly better fit than a single straight line. Data analysis revealed that the two-line model resulted in a significantly better fit in all cases.

3. Results and discussion

3.1. Characterization of strains

In order to examine the interaction between Cr(VI) and the plasma membrane, we used chromium-tolerant and chromium-sensitive mutants and their parental *S. pombe* strain. The mutants were derived from the heterothallic parental strain by using *N*-nitroso-*N'*-nitro-*N*-methylguanidine (NTG) treatment at a dose producing about 10% survival. The mutation frequency for NTG was 5×10^{-4}. These mutants could be applied well in our experiments because they proved to be stable for each marker [14].

The mutants exhibited different minimal inhibitory concentrations (MICs) for Cd^{2+}, Cu^{2+}, Ni^{2+} and Zn^{2+} (see Table 1 for Cr(VI), Cd^{2+} and Cu^{2+}). The MICs of $K_2Cr_2O_7$ on YEA medium were 225 μM for the parental strain, 125 μM for its chromium-sensitive mutant and 275 μM for its chromium-tolerant one. The chromate uptake and viabilities of the parental strain and the chromium-tolerant mutant were the same, while the chromium-sensitive mutant exhibited a significantly higher bioaccumulation and a lower viability on YEL liquid medium [14].

These well-characterized yeast strains seemed to be suitable for study of the dynamic properties of the membranes and their significance in the interactions with Cr(VI). The application of 225 μM $K_2Cr_2O_7$, i.e. the MIC of the parental strain on complete medium, seemed to be justified to carry out measurements on the interaction of the plasma membranes with Cr(VI) by EPR.

The EPR measurements were carried out in yeast extract-free solutions. The integrity of the plasma membranes did not change during treatment with 225 μM $K_2Cr_2O_7$; neither lysis nor shrinking of the spheroplasts was observed. In order to characterize the differences between the strains, the change in the order parameter S was examined at different temperatures. The data points could be approximated by two straight lines as a function of temperature. The breaks in the temperature profile were at 11 °C for the parental strain and at 12.5 °C for the sensitive mutant, while no break point was observed for the resistant mutant when the 5-SASL spin label was applied. Significantly larger differences were calculated in the case of the HO-185 probe molecule (Table 2). The changes in the spectra were independent of whether the spectral parameter ($2A'_{zz}$) versus temperature was measured with increasing temperature up to 25 °C or with decreasing temperature from 25 to 0 °C; no hysteresis in the hyperfine splitting and order parameter was detected.

Table 1
Minimal inhibitory concentrations of the different metals of the *S. pombe* strains

Code of strains	Minimal inhibitory concentration (μM)		
	$K_2Cr_2O_7$	$CdCl_2 \times 2H_2O$	$CuSO_4 \times 5H_2O$
6chr$^+$ parental strain	225	1250	3500
chr-51S chromium-sensitive mutant	125	1500	4000
chr1–66T chromium-tolerant mutant	275	1250	3500

The medium (YEA) used in this experiment varied only as concerns the concentrations of the different metals.

Table 2
The change in phase transition temperature produced by Cr(VI) treatment of the *S. pombe* strains

Code of strains	Phase transition temperature (°C)			
	Control		Chromium-treated	
	Labeled with 5-SASL	Labeled with HO-185	Labeled with 5-SASL	Labeled with HO-185
6chr$^+$	11	12	10.5	8.5
chr-51S	12.5	14	11.5	10
chr1–66T	No break point	8	No break point	8.5

The phase transition temperature can be obtained from the break in the plot of the parameters against temperature. The plots are approximated by two straight lines.

3.2. Interaction of Cr(VI) with plasma membranes

Before spin probe incorporation, the cells were treated in buffer solution containing 225 μM $K_2Cr_2O_7$ to achieve the maximum concentration of Cr(VI) in the cells. An EPR signal at $g = 1.985$, arising from Cr(V), evidenced the rapid reduction of Cr(VI) to Cr(V) (Fig. 1). The concentration of the Cr(V) species generated did not remain constant: the double integral of the newly formed Cr(V) species decreased in time due to chemical reactions that resulted in the further reduction of Cr(V) to Cr(III). It was demonstrated earlier that a very broad line width could be expected from Cr(IV) and Cr(III). Moreover, neither the line width nor the g-factor allowed a clear distinction between Cr(IV) and Cr(III) complexes [21]. The appearance of the Cr(V) signal and its change demonstrated the fast incorporation of Cr(VI) into the membrane, and its interaction with reducing agents in the membrane interior. EPR technique cannot distinguish the Cr(V) ions located in the membrane and in the interior of the cell. Very likely, the decrease of the Cr(V) signal can be accounted for the reducing of yeast cells. Approximately 120 min was needed to reduce Cr(VI) to Cr(III) in the absence of extracellular Cr(VI) (data not presented). The treatment of the cells with 225 μM $K_2Cr_2O_7$ did not significantly impair the membrane function, at least during the time of the incubation and the spectroscopic measurements.

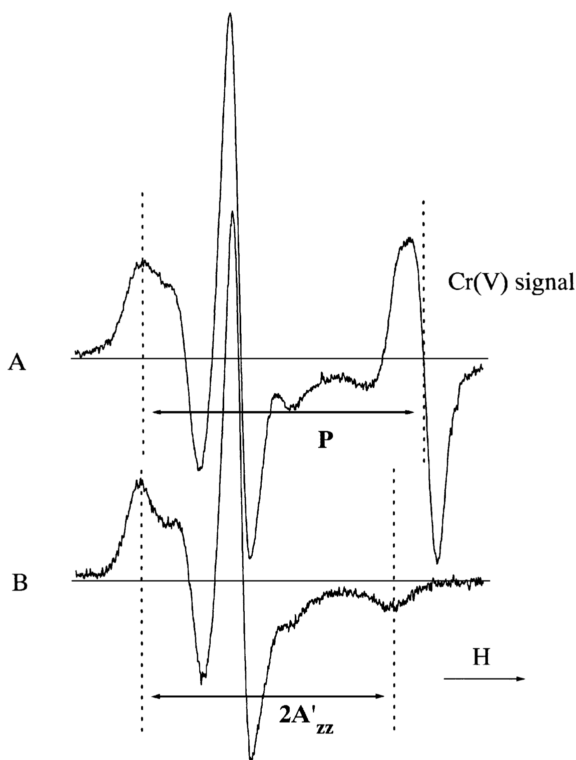

Fig. 1. EPR spectra of samples of the parental strain (*6chr*$^+$) of *S. pombe* at 14 °C before (B) and after (A) Cr(VI) treatment. The spectral parameter *P* was defined as the distance between the low-field maximum and the crossover point of the Cr(V) signal (g = 1.985). The field scan was 10 mT.

A slow, temperature-dependent decrease in the intensity of the 5-SASL and HO-185 EPR signals with time was also observed, but no effect on the spectral line shape during the temperature scans was detected. Since the stearic acid spin labels are able to react with other substances, this was very probably a consequence of the metabolic activity in the cell interior, or was due to the effects of various biological reductants in the membranes.

3.3. Effects of Cr(VI) treatment on membranes

When incorporated into the membranes of the spheroplast, both spin labels (5-SASL and HO-185) reflected different mobilities depending on the strain, and the mobility was affected to different extents on the addition of Cr(VI). The nitroxide moieties of the two labels are located in different regions of the membrane, and it is therefore to be expected that they exhibit different mobility changes, depending on the lipid environment and the temperature. The 5-SASL label indicated only a small difference between the membranes of the parental and sensitive strains. For the tolerant mutant labeled with 5-SASL, no break point was found (Table 2). In contrast, when the membrane was labeled with HO-185, marked changes in the order parameter were observed (see Table 2 and Figs. 2–4). The sensitive mutant labeled with HO-185 exhibited a smaller fluidity in comparison with its parental cells. The spectral parameter (P) and the hyperfine splitting constant that characterize the rotational mobility of the 5-SASL and HO-185 probe molecules were a little larger for the sensitive mutant below the phase transition temperature studied in these experiments (Table 3).

The break points in the temperature plots were significantly different after treatment with Cr(VI). The addition of Cr(VI) to the samples led to decreases in the phase transition temperatures of the 5-SASL-labeled sensitive and parental strains, but not in that of the tolerant mutant. The probe molecule HO-185 revealed an increased mobility of the membrane interior of the parental and the sensitive strains, whereas only a small change of rigidity could be detected in the lipid phase of the tolerant mutant. The difference between the results obtained with the different labels might be due to the nonuniform distribution of the probe molecules, and possibly to the different distributions of the different chromium species along the normal to the membrane. In the case of the HO-185 spin probe, where the nitroxide moiety is situated near the surface of the membrane, and its fatty acid chain is shorter than that of the 5-SASL label, the spectra reflected changes in the lipid region close to the lipid–water interface. Cr(III) displays a preferred localization in the negatively charged regions of the membrane [22,23], and

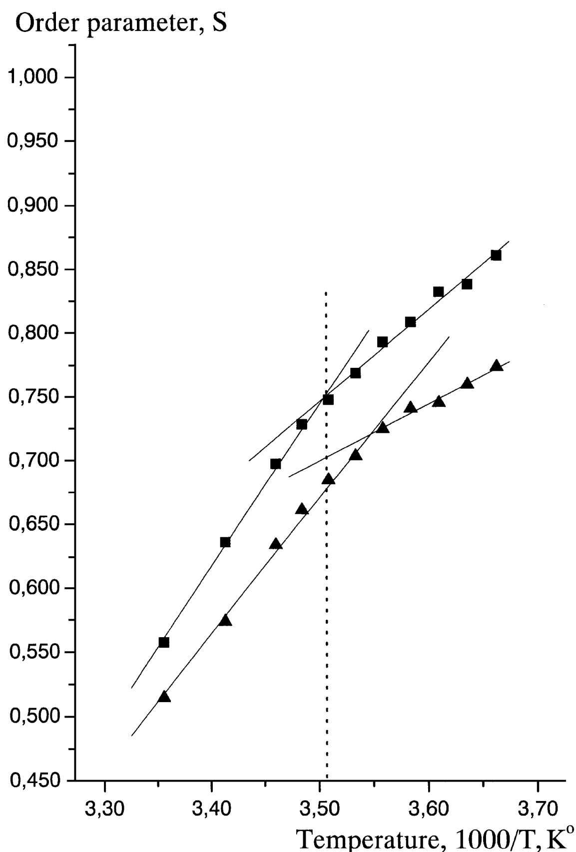

Fig. 2. Plot of order parameter *S* for the parental strain (*6chr*$^+$) of *S. pombe* (labeled with HO-185) as a function of temperature (symbols: ■, control; ▲, chromate-treated).

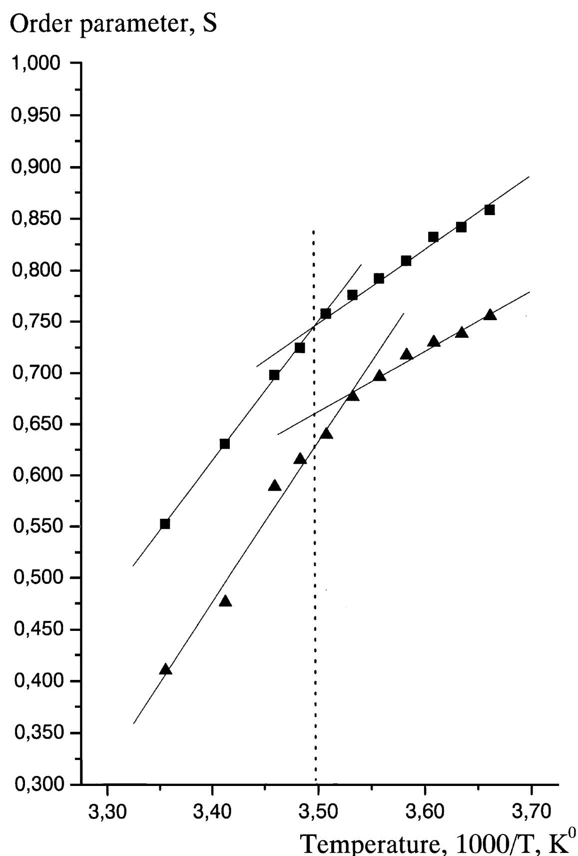

Fig. 3. Plot of order parameter *S* for the chromium-sensitive mutant (*chr-51S*) of *S. pombe* (labeled with HO-185) as a function of temperature (symbols: ■, control; ▲, chromate-treated).

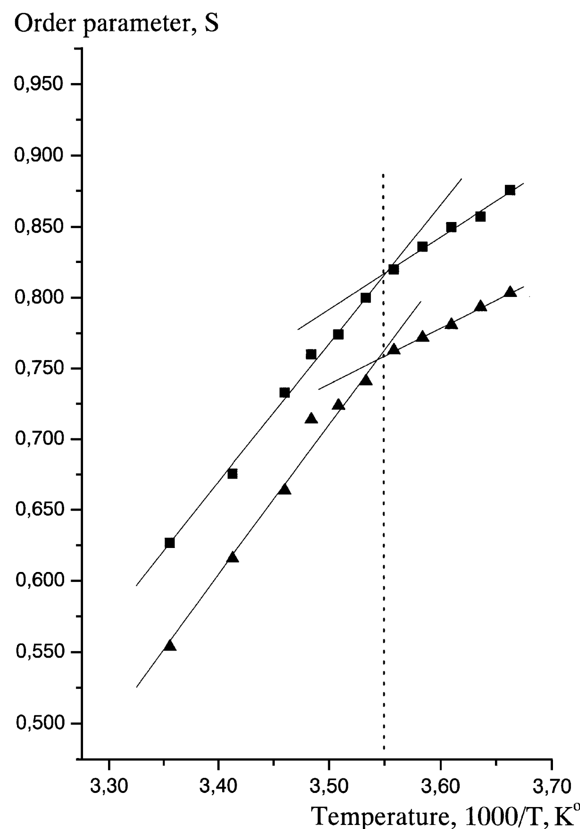

Fig. 4. Plot of order parameter *S* for the chromium-tolerant mutant (*chr1–66T*) of *S. pombe* (labeled with HO-185) as a function of temperature (symbols: ■, control; ▲, chromate-treated).

this might tend to create bridges between phospholipid molecules and the side chains of the negatively charged amino acid residues. These interactions can reduce the stability of the head group regions of the membrane, and can affect the boundary layer of phospholipids around the membrane proteins. In such a way, the receptor and transport activities that are usually associated with the protein components of the membrane can be modulated by the altered properties of the lipid matrix [23]. On the other hand, one of the most widely observed effects of metal toxicity is a sudden increase in membrane permeability, which is generally manifested as a rapid loss of intracellular ion pools, and an enhanced accumulation of extracellular ions. Similarly to Cr(III), Cr(VI) readily interacts with components of the membrane, and may also perturb the hydrophobic region of the membrane [24]. A comparison of the EPR results obtained on the 5-SASL- and HO-185-labeled membranes suggests that the interactions and lipid perturbation of the Cr(VI) species with the membrane components are mostly localized close to the lipid–water interface.

The Cr(VI) tolerance of budding yeast *Saccharomyces* sp., *Candida* sp. and *Rhodosporidium* sp. was found to be due to a reduced chromate uptake. The chromium-sensitive mutant of the fission yeast *S. pombe* exhibited a significantly increased rate of Cr(VI) uptake and bioaccumulation, accompanied by a decreased viability in comparison with its parental strain and the chromium-tolerant mutant. The viability of an ergosterol-less *erg-2* membrane mutant of *Candida albicans* was lower at various Cr(VI) concentrations (0.6–2.5–5.0 mM) than that of its ergosterol-producing *33 erg*$^+$ parental strain, suggesting the existence of Cr(VI)–plasma membrane interactions [25].

In contrast with chromate ion, the plasma membrane is hardly permeable for the hexaquo-Cr(III) ion. Cr(III) causes a strong fluidizing effect on the membranes of yeasts, the effect exhibiting time and concentration dependences. The interactions result in a loss of metabolites adsorbing at 260 nm: this loss was 40% for the *33 erg*$^+$ strain and 60% for its

Table 3
Hyperfine splitting constants of the EPR spectra of different *S. pombe* strains

Code of strains	Hyperfine splitting (G)	
	Labeled with 5-SASL	Labeled with HO-185
6chr$^+$	56.55	57.85
chr-51S	56.49	58.2
chr1–66T	55.67	58.83

EPR spectra were recorded at 12 °C. The values are the means of four determinations. The calculated error does not exceed 0.03 mT.

erg-2 mutant of *C. albicans*. A similar, but significantly slower process was caused by Cu^{2+} in *Saccharomyces cerevisiae*. Cd^{2+} also exhibited a protracted strong fluidization of the plasma membrane of energized cells of *S. pombe*, but Zn^{2+} caused only a marginal membrane fluidization, suppressing the fluidizing action of Cd^{2+}. Moreover, the uptake, bioaccumulation, mode of action and processes of detoxification are different for each of the above-mentioned ions, whereas most metal ions promote lipid peroxidation in yeasts. It was hypothesized that chromium-mediated ROS generation causes persistent oxidative stress and that this leads to lipid peroxidation. The oxidative stress may contribute to Cr(VI)-induced carcinogenesis [2,26].

In summary, it was shown that Cr(VI) uptake and reduction was fast, but the consecutive steps of Cr(V) reduction were relatively slow, not only for *Candida* strains, but also for *S. pombe* fission yeast cells [25]. We found by means of EPR that there is a difference between the membrane structures and dynamics of the mutants in comparison with their parental strain. The labels used for the study are located in different regions of the membranes, and reported changes in membrane fluidity are induced by incorporated Cr(VI) species. The effects of chromate depend on the internal structure and dynamics of the membranes. Cr(VI) exerted dissimilar effects on the different strains; it is very likely that there is connection between the membrane components, their dynamic and structural properties and chromium tolerance and sensitivity.

The changes in local structure produced by Cr(VI) and its unstable intermediates, the presence of Cr(III) in the outer and inner headgroup regions of the membrane, and the changes in the electric charge of the cell surface could all affect the physiological function of the cells. The altered permeability for different substances, the toxicity of the various substances and the ion binding potentials might influence the signaling processes, the cell–cell interactions, and hence the viability of the cells.

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