# Biological Activity of Copper(II) *N*-Salicylideneaminoacidato Complexes. Reduction of Chlorophyll Content in Freshwater Alga *Chlorella vulgaris* and Inhibition of Photosynthetic Electron Transport in Spinach Chloroplasts

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The effects of $CuCl_2$ and 12 Cu(II) complexes on reduction of chlorophyll content in statically cultivated green alga *Chlorella vulgaris* and inhibition of photosynthetic electron transport in spinach chloroplasts were studied. The studied complexes were six chelate cuprates of the composition $M^+[Cu(TSB)(X)]^-$ containing tridentate Schiff base dianion ligands ($TSB^{2-}$) of *N*-salicylideneaminoacidato type (derived from $\alpha$-alanine or $\beta$-alanine, valine, phenylalanine), additional pseudohalogeno ligands ($NCS^-$ or $NCO^-$), and M (K, $NH_4$ or Na) as well as six molecular (*N*-salicylidene-$\beta$-alaninato)copper(II) complexes of the composition [Cu(sal-$\beta$-ala)(L)] with additional organic molecular ligands (L = imidazole, pyrazole, pyridine, quinoline, urea or thiourea). The toxic effects of the investigated Cu(II) complexes were compared with those of $CuCl_2$ and copper(II) acetate and the influence of the coordination mode of ligands in the tested Cu(II) complexes on the biological activity was discussed. It was found that in the set of Cu(II) cuprates, the inhibitory activity of the compound concerning reduction of chlorophyll content in *Ch. vulgaris* strongly depended on the applied amino acid and decreased in the following order: $\beta$-alanine, $\alpha$-alanine, phenylalanine, valine. The differences between inhibitory effectiveness of six molecular (*N*-salicylidene-$\beta$-alaninato)copper(II) complexes with additional molecular ligands against *Ch. vulgaris* were not too high, indicating that the effect of additional organic ligands on the inhibitory activity is not significant. The lower inhibitory effect of both types of Cu(II) chelates in comparison to that of $CuCl_2$ and copper(II) acetate probably results from their higher stability in aqueous solutions. The Cu(II) compounds also decreased fluorescence intensity of chlorophyll *a* that is present in pigment-protein complexes of photosynthetic centres (mainly in photosystem 2) of spinach chloroplasts. It could be assumed that the toxic effects of the studied copper(II) complexes are probably due to the substitution of their additional ligands with *N*-, *S*- or *O*-donor ligands present in proteins of algal and higher plant cells.

Copper belongs to the essential metals, which are indispensable for plants. These metals, in general called essential bioelements, in optimal concentration provide important functions in plant metabolism: they are components of the enzymes, structural proteins, assimilation pigments, they maintain osmotic potential in the cells. However, after application of the higher concentration, they become toxic. At elevated concentrations, copper can act strongly on chromatin, the photosynthetic apparatus, growth, and senescence processes [1]. Copper is a very potent inhibitor of photosynthetic activity with several sites of action in the photosynthetic electron transport chain [2, 3]. It is known to damage cell membranes by binding to the —SH groups of membrane proteins and by inducing lipid peroxidation [4, 5]. The results of the study of *Kupper et al.* [6], focused on $Cu^{2+}$ attack on the photosynthetic apparatus of the green alga *Scenedesmus quadricauda,* showed that the reaction occurring under low irradiance (shade reaction) was characterized by heavy metal substitution of $Mg^{2+}$ in chlorophyll molecules bound predominantly in the light harvesting complex II (LHC II). On the other hand, under high irradiance (sun reaction), the LHC II chlorophylls

were inaccessible to substitution and the damage occurred in the photosystem 2 reaction centre instead.

In the copper(II) complexes with tridentate Schiff bases (TSB) derived from salicylaldehyde and various amino acids, the TSB dianionic ligand is coordinated *via* the donor atoms of phenolic (O), azomethine (N), and carboxylic (O) group forming two fused chelate rings [7]. Some copper(II) chelates with tridentate Schiff base dianions of the *N*-salicylideneaminoacidato type can be considered as simple models of the Cu, Zn-superoxide dismutase active site and some of them showed remarkable SOD-like activity [8].

In our previous papers we investigated the inhibitory effects of aqua(aryloxyacetato)copper(II) complexes on the photosynthetic electron transport (PET) in spinach chloroplasts [9—11] and green alga *Chlorella vulgaris* [12, 13]. These compounds interact with the intermediate $Z^+/D^+$, *i.e.* with tyrosine radicals $Tyr_Z$ and $Tyr_D$, which are situated in the 161st position in $D_1$ and $D_2$ proteins, located on the donor side of photosystem 2 [9, 11, 13]. The study of PET-inhibiting activity of copper(II) complexes markedly depended on the coordination mode of the studied compounds [10, 14].

The aim of this paper was to investigate the toxic effects of some cuprates with *N*-salicylideneaminoacidato ligands (derived from $\alpha$-alanine or $\beta$-alanine, valine, phenylalanine), with additional anionic ligands ($NCS^-$ or $NCO^-$) as well as the (*N*-salicylidene-$\beta$-alaninato)copper(II) complexes with additional molecular ligands (L = imidazole, pyrazole, pyridine, quinoline, urea or thiourea) on PET in spinach chloroplasts and chlorophyll content in green alga *Ch. vulgaris*. The effect of these compounds on fluorescence intensity of the emission band at $\lambda$ = 686 nm, corresponding to chlorophyll *a* in pigment-protein complexes of photosynthetic centres was investigated in the suspensions of spinach chloroplasts as well.

## EXPERIMENTAL

$CuCl_2 \cdot 2H_2O$ (*I*), anal. grade, was purchased from Lachema. The studied copper(II) complexes were $Cu(CH_3COO)_2 \cdot H_2O$ (*II*), K[Cu(sal-DL-val)(NCS)] (*III*), K[Cu(sal-DL-phal)(NCS)] (*IV*), K[Cu(sal-DL-$\alpha$-ala)(NCO)] (*V*), K[Cu(sal-DL-$\alpha$-ala)(NCS)] (*VI*), $NH_4$[Cu(sal-$\beta$-ala)(NCS)] (*VII*), $Na_4[Cu_2(sal\text{-}\beta\text{-}ala)_2(NCS)_2](SCN)_2 \cdot 4H_2O$ (*VIII*), Cu(sal-$\beta$-ala)(Py) (*IX*), Cu(sal-$\beta$-ala)(Im) (*X*), $Cu(sal\text{-}\beta\text{-}ala)(Pz)_2 \cdot 2H_2O$ (*XI*), Cu(sal-$\beta$-ala)(Quin)$\cdot H_2O$ (*XII*), Cu(sal-$\beta$-ala)(Ur) (*XIII*), Cu(sal-$\beta$-ala)(Tu) (*XIV*) where val = valine, phal = phenylalanine, ala = alanine, sal = *N*-salicylidene, Py = pyridine, Im = imidazole, Pz = pyrazole, Quin = quinoline, Ur = urea, and Tu = thiourea. The Cu(II) complexes were synthesized according to *Krätsmár-Šmogrovič et al.* [15, 16] and *Švajlenová et al.* [17]. The brackets were used only for Cu(II) complexes with solved X-ray structure. Anal. grade chemicals were employed for the preparation of all solutions. Freshly distilled water was used in all experiments.

Chloroplasts were prepared according to the procedure described by *Walker* [18]. The effect of tested compounds on the photochemical activity of spinach chloroplasts was investigated spectrophotometrically in the presence of the electron acceptor 2,6-dichlorophenol-indophenol (DCPIP) according to *Kráľová et al.* [19]. The chlorophyll (Chl) content in these experiments was 30 mg $dm^{-3}$.

The alga *Ch. vulgaris* was statically cultivated (7 d, photoperiod 16 h light/8 h dark; irradiance 100 $\mu$mol $m^{-2}$ $s^{-1}$ PAR; pH = 5.8) at mean air temperature 25 °C according to the method described previously [13]. The Chl content in the alga suspension was determined spectrophotometrically after extraction into methanol according to *Wellburn* [20]. All tested concentrations were triplicated and $IC_{50}$ values with 95 % confidence limits ($C.L._{0.05}$) were calculated. Chl content in the suspensions at the beginning of cultivation was 0.1 mg $dm^{-3}$.

The fluorescence emission spectra of spinach chloroplasts were recorded on fluorescence spectrophotometer F-2000 (Hitachi, Tokyo, Japan) using excitation wavelength $\lambda_{ex}$ = 436 nm for monitoring fluorescence of Chl *a*, excitation slit of 20 nm, and emission slit of 10 nm. The required concentration of the studied compounds was achieved using the appropriate amounts of their ethanol solutions and subsequent evaporation of the solvent. Then chloroplast suspension was added and the samples were kept in the dark for 10 min before measurements. Chl content in these experiments was 10 mg $dm^{-3}$.

## RESULTS AND DISCUSSION

The effects of six cuprates containing *N*-salicylideneaminoacidato ligands (derived from $\alpha$-alanine (*V, VI*) or $\beta$-alanine (*VII, VIII*), valine (*III*), phenylalanine (*IV*)) and additional anionic ligands ($NCS^-$ or $NCO^-$) as well as six molecular (*N*-salicylidene-$\beta$-alaninato)copper(II) complexes with additional molecular ligands (imidazole (*X*), pyrazole (*XI*), pyridine (*IX*), quinoline (*XII*), urea (*XIII*) or thiourea (*XIV*)) on reduction of Chl content in statically cultivated green alga *Ch. vulgaris* and PET inhibition in spinach chloroplasts were studied. The corresponding $IC_{50}$ values (*i.e.* concentrations of the compound causing a 50 % decrease of the activity of the control sample) reflecting the toxic effects of Cu(II) compounds on the studied photosynthesizing organisms are summarized in Table 1. The inhibitory activities of the investigated Cu(II) complexes were compared with those of *I* and *II* and the influence of the coordination mode of ligands in the tested Cu(II) complexes on the biological activity was evaluated.

**Table 1.** $IC_{50}$ Values of the Studied Cu(II) Compounds Concerning Inhibition of Photosynthetic Electron Transport in Spinach Chloroplasts and Reduction of Chlorophyll Content in *Chlorella vulgaris* Suspensions

| Compound | Spinach chloroplasts $IC_{50}$ / mmol dm$^{-3}$ | *Chlorella vulgaris* $IC_{50} \pm C.L._{0.05}$ / $\mu$mol dm$^{-3}$ | Compound | Spinach chloroplasts $IC_{50}$ / mmol dm$^{-3}$ | *Chlorella vulgaris* $IC_{50} \pm C.L._{0.05}$ / $\mu$mol dm$^{-3}$ |
|---|---|---|---|---|---|
| *I* | 0.012 | 14.0 (12.2—16.1) | *VIII* | 1.289 | 37.4 (32.3—42.9) |
| *II* | 0.011 | 20.1 (18.5—22.4) | *IX* | 2.606 | 37.5 (34.7—40.6) |
| *III* | 2.631 | 571.7 (502.1—657.8) | *X* | 0.719 | 30.5 (27.7—34.2) |
| *IV* | 2.040 | 196.8 (176.9—217.2) | *XI* | 2.334 | 34.6 (30.8—39.0) |
| *V* | 2.835 | 198.3 (172.3—231.0) | *XII* | 1.993 | 21.0 (16.9—24.5) |
| *VI* | 1.401 | 82.5 (72.5—98.8) | *XIII* | 3.640 | 38.3 (32.5—45.5) |
| *VII* | 1.363 | 40.8 (36.4—45.7) | *XIV* | 0.637 | 58.7 (49.2—65.6) |

The structures of copper(II) complexes contained the essential structural motive: square-pyramidal coordination of the Cu(II) central atom. The copper(II) central atom, coordinated with three Schiff base donor atoms (nitrogen atom of the azomethine group, phenolic oxygen of the salicylaldehyde moiety, and the oxygen atom of the amino acid carboxyl group), results in two metallochelate rings (one six-membered and one five-membered, or two six-membered chelate rings, depending on the type of amino acid used). The fourth site in the basal plane is occupied by the donor atom of the corresponding additional ligand. Further positions in the coordination polyhedron are, in crystalline state, occupied by coordinated water molecules or by other donor atoms from the neighbouring coordination unit [7, 21, 22]. If the additional ligand is bridging ligand ($\mu$-NCS), dimer anionic complexes are formed [21]. In the dissolved state the above-discussed coordination position will be occupied by solvent molecules (in our experiments water).

The effects of six molecular Cu(II) complexes with additional organic ligands and *N*-salicylidene-$\beta$-alaninato(2-) ligand (*IX*—*XIV*) on reduction of chlorophyll content in green alga *Ch. vulgaris* were comparable to each other ($IC_{50}$ values varied in the range from 21.0 $\mu$mol dm$^{-3}$ (*XII*) to 58.7 $\mu$mol dm$^{-3}$ (*XIV*)). The contribution of the additional molecular ligand to toxicity of the compounds was not too high and decreased in the following order:

Quin (*XII*), Im (*X*), Pz (*XI*), Py (*IX*), Ur (*XIII*), Tu (*XIV*).

In the set of Cu(II) cuprates, the inhibitory activity against *Ch. vulgaris* strongly depended on the applied amino acid and it decreased in the following order: $\beta$-alanine, $\alpha$-alanine, phenylalanine, valine. The $IC_{50}$ values determined for compounds *VII* and *VIII* (40.8 $\mu$mol dm$^{-3}$ and 37.4 $\mu$mol dm$^{-3}$, respectively) were approximately two times lower than the $IC_{50}$ value determined for *VI* and five times lower than the corresponding $IC_{50}$ value determined for *V* (82.5 $\mu$mol dm$^{-3}$ and 198.3 $\mu$mol dm$^{-3}$, respectively). Planar arrangement of ligands around the Cu(II) ion, as in the Schiff bases derived from salicylaldehyde and $\alpha$-amino acids, stabilizes its bivalence [23]. In case of the 6-membered ring (in the complex derived from $\beta$-alanine), the planarity of the ring is impaired [22], and in this case the Cu(II) ion should more easily interact with potential "biological" targets (ligands). This is reflected also in the stability constant of the complex of copper with $\beta$-alanine ($\log K = 7.13$), which is by one order lower than that with $\alpha$-alanine ($\log K = 8.12$) [24].

On the other hand, from the comparison of the $IC_{50}$ values of *IV* and *V* (196.8 $\mu$mol dm$^{-3}$ and 198.3 $\mu$mol dm$^{-3}$, respectively), it is evident that the substitution of DL-$\alpha$-ala by more lipophilic DL-phal in the *N*-salicylideneaminoacidato ligand did not affect the biological activity. Relatively low inhibitory activity of *III* (571.7 $\mu$mol dm$^{-3}$) could be assigned to the sterical restriction of branched isopropyl substituent of valine [21] which can complicate a satisfactory approach of the central metal ion to the potential "biological ligands" in the cell. The decrease of Chl content in alga can be caused by inhibition of photosynthetic electron transport and/or by inhibition of certain biochemical pathways interfering with the formation of this important photosynthetic pigment [1], it may be also connected with changes in the biosynthesis of Chl by replacement of $Mg^{2+}$ ions by $Cu^{2+}$ [6, 25].

The differences in immediate toxic effects of all studied Cu(II) complexes on the inhibition of photosynthetic electron transport in spinach chloroplasts

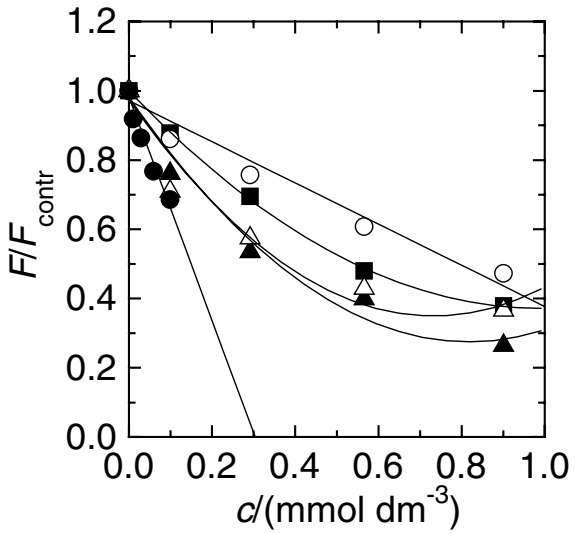


**Fig. 1.** Dependence of the fluorescence quenching on the concentration of *I* (full circles), *III* (open circles), *IV* (full squares), *XI* (open triangles), and *XII* (full triangles). $F_{\mathrm{contr}}$ – fluorescence intensity of the untreated suspension of spinach chloroplasts at $\lambda = 686$ nm, $F$ – fluorescence intensity of the suspension of spinach chloroplasts treated with Cu(II) compounds at $\lambda = 686$ nm ($\lambda_{\mathrm{ex}} = 436$ nm).

were relatively small. More significant effect of individual ligands on the biological activity was not observed. The inhibitory effectiveness of the majority of the tested compounds (with the exception of compounds *X* and *XIV*) was approximately by two orders lower than that of *I* and *II*. The lower inhibitory effect of both types of Cu(II)-chelate complexes probably resulted from their higher stability in aqueous solutions. In the long-term tests with *Chlorella vulgaris* (7 days), an eventual dissociation of Cu(II) compounds cannot be excluded. On the other hand, it can be assumed that at short-term PET measurements the complexes remain stable during the experiment.

For the study of the effects of Cu(II) compounds upon photosynthetic centres, the emission fluorescence spectra of spinach chloroplasts in aqueous suspensions were recorded. When chloroplasts were irradiated with the light of $\lambda_{\mathrm{ex}} = 436$ nm, an emission band with the maximum at $\lambda = 686$ nm was observed. This band belongs to the pigment-protein complexes present mainly in photosystem 2 [26, 27]. It was found that chloroplasts treated with Cu(II) compounds exhibited quenching of the emission of Chl *a* molecules. Fig. 1 presents the dependence of $F/F_{\mathrm{contr}}$ in the suspension of spinach chloroplasts ($F_{\mathrm{contr}}$ – fluorescence intensity at $\lambda = 686$ nm in the control, $F$ – fluorescence intensity at $\lambda = 686$ nm in the presence of Cu(II) compound) on the concentration of compounds *I, III, IV, XI*, and *XII*. The greater is the fluorescence quenching, the more efficient is the interaction of the inhibitor with pigment-protein complexes in photosystem 2. The most intensive above-mentioned interaction was exhibited by *I*. The smallest effect on the fluorescence intensity of the chloroplast suspension was shown by Cu(II) cuprates with Schiff bases derived from valine (*III*) and phenylalanine (*IV*). The results are in agreement with the findings concerning the biological activity of investigated Cu(II) compounds presented in Table 1.

It could be assumed that the toxic effects of the studied *N*-salicylideneaminoacidatocopper(II) complexes could be assigned to the substitution of their additional ligands with *N*-, *S*- or *O*-donor ligands occurring in proteins of algal and higher plant cells. The interaction of diaqua-(*N*-pyruvidene-$\beta$-alaninato)copper(II) monohydrate with aromatic amino acids present in photosynthetic centres has been already confirmed [11]. Organic ligands could also affect the transport of metal ions through the lipophilic regions of cell membranes. The obtained results are in agreement with the previously published papers [9—14].

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