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Different possibilities for the formation of complexes of copper and zinc with chlorophyll inside photosynthetic organelles: chloroplasts and thylakoids

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Abstract: The possibility of the formation of copper and zinc complexes with chlorophyll in photosynthetic organelles (chloroplasts) and suborganelles (thylakoids) was studied. The visible and fluorescence spectra obtained from chloroplasts and thylakoids in the presence of the two metals confirmed complex formation in the case of copper, while such possibility appears to be very minor in the case of zinc. The reason for this distinction lies in the different type of complexes which chlorophyll forms with the two metals: only "central" or "substitution" copper–chlorophyll complexes may be formed inside the two isolated entities, while the formation of a possible zinc–chlorophyll "peripheral" type of complex is prevented for steric reasons. The latter fact is of high biological relevance, since both complexes may cause an irreversible impairment and damage of photosynthetic function.

Keywords: chlorophyll, copper, zinc, fluorescence, chloroplasts, thylakoids.

INTRODUCTION

Chlorophyll (Chl), the major photosynthesis pigment, in chemical terms is a chlorin, a dihydroporphyrin derivative containing an isocyclic cyclopentanone ring (fused to a pyrrole ring between C-13 and C-15 positions), where central metal Mg atom coordinates four symmetric pyrrole rings (Fig. 1). The major function of chlorophyll in photosynthesis is related to light collection and light conversion processes. Significant progress has been made in understanding of the *in vitro* properties of Chl and this contributed to a better understanding of the role of Chl in photosynthesis on the molecular level.[1,2]

Plants easily absorb many toxic heavy metals.[3,4] Once absorbed, they penetrate to plant tissues (including leaves) and at higher concentrations, they may inhibit photosynthesis.[5–7] Heavy metals can replace the relatively weakly bound central magnesium atom (Mg) of chlorophyll, to form heavy metal complexes (Chl––HMS).[8–10] The Chl–HMS complexes may cause an impairment of photosynthetic function, which can have fatal consequences.[11] The detailed consequences of this substitution for higher plants and green algae have been discussed by Küpper *et al*.[8,12,13]

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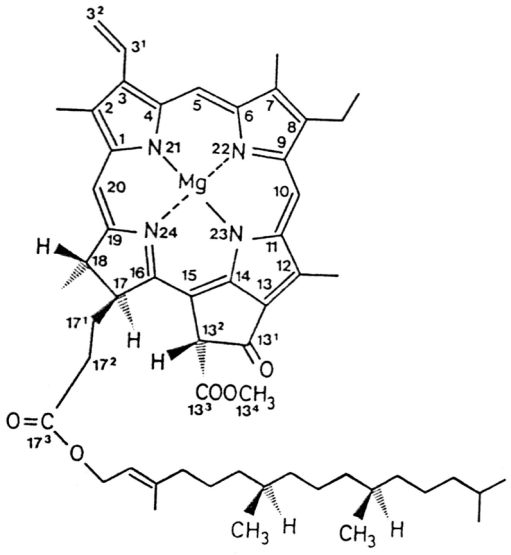


Fig. 1. Structure of chlorophyll *a*, with numerated C-positions.

The general reactions of Chl with heavy metals *in vitro*[11] and *in vivo*[14,15] have already been reported. Early *in vivo* studies of Chl–HMS formation in higher plants and green algae[8,12] showed that this reaction was strongly dependent on the light intensity: in the dark, the majority of antenna Chls are accessible for Chl–HMS formation[13] ("shade reaction"), while under high light conditions, only a small fraction of antenna Chls undergoes it ("sun reaction").[8,11,12] Chl–HMS complexes can be prepared by heating Chls with metal salts in acid or organic solutions.[16] Also, acidification of Chl solutions with hydrochloric acid followed by the addition of a heavy metals solution leads to the formation of Chl–HMS.[7,8,16–18] Küpper performed experiments *in vitro* by adding solutions of heavy metals to 96 % ethanolic grass extracts.[8]

Chl–HMS shows a spectroscopic behavior different to that of Chl itself,[8,17–21] qualitatively (shift of the characteristic band maxima), as well as quantitatively (different intensities of the corresponding bands). For example, Cu–Chl formation is followed by a hypsochromic ("blue") shift of the long wavelength ("red") absorption band,[8,12,17,18] with significantly lower coefficient of absorption value compared to Chl itself.[17] A not so expressed "blue" shift of the "red" Chl band was observed for Zn–Chl.[19,22]

In this work, the possible formation of Chl–HMS complexes in isolated photosynthetic organelles, chloroplasts and their sub-units, thylakoids, with two chosen heavy metals, zinc and copper, was studied. In lower concentrations, copper is an essential micronutrient for higher plants and algae and it is even a constituent of the primary electron-donor of photosystem I, the Cu–protein, plastocyanin.[23] However, high external Cu concentrations (and those of Zn as well) – like the ones in this work – may produce many damaging effects. Zinc may be inclu-

ded in the degradation of chloroplasts stromal proteins.[24] Copper may affect all kinds of photosynthetic activities, such as electron-transport and ATP production,[25–28] or oxygen evolution.[29]

The complexes Cu–Chl and Zn–Chl were examined in this study by visible and fluorescence spectroscopy.

EXPERIMENTAL

The Chl–HMS formation may occur under low light ("shade reaction") and under high light conditions ("sun reaction").[13] Since just a minority of antenna chlorophylls (*in vivo*) is accessible to Chl–HMS formation under high light conditions,[13] the experimental part of this work was performed under shade conditions as much as possible.

Isolation of intact chloroplasts

Intact chloroplasts from spinach were prepared using a described procedure.[30] Healthy, intact spinach leaves floating in a basin of water were exposed to bright light for 30 min, before removing the midribs. The plant material (50 g) was immersed in 200 cm^3 of grinding medium, which had previously been brought to a consistency of melting snow. The grinding was done in a couple of seconds. The suspension was then squeezed through two layers of muslin and filtered through 8 layers of muslin and a layer of cotton-wool. The chloroplasts were quickly separated from the supernatant by centrifugation in four tubes at 4000 rpm for 100 s. The supernatant was decanted and the pellet, resuspended in about 80 cm^3 of grinding medium, was recentrifuged in two tubes at 4000 rpm for 100 s.

A low cation medium was employed as the grinding and resuspending medium.[31,32] It contained 12.035 g of sorbitol, 2 cm^3 of 1 M KCl, 0.8 cm^3 of 0.25 M EDTA, 95 cm^3 of water and 2.383 g of Hepes (4-(2-hydroxyethyl)-1-piperazine ethanesulphonic acid) buffer, pH 7.8. The grinding medium was prepared daily by mixing about 6 cm^3 of the low cation medium with 18 g sorbitol and 276 cm^3 of water.

The clean, final pellet of chloroplasts was resuspended in 0.5–1.0 cm^3 of resuspending medium, containing 5 cm^3 of the low-cation medium, 0.02 cm^3 of 1 M $MgCl_2$, 0.02 cm^3 of 1 M $MnCl_2$, 0.1 g of serum albumin and 5 cm^3 of water.

The chlorophyll content (Chl*a* – Chl*b*) in the suspension of chloroplasts was calculated as reported (2.2×10^{-3} M).[33]

Content of intact chloroplasts in the suspension of chloroplasts

The content was calculated by comparing the reduction of $K_3Fe(CN)_6$ in a suspension of intact chloroplasts and broken chloroplasts (spectrophotometrically). The mixture contained 10×10^{-3} cm^3 of chloroplasts suspension, 1 cm^3 of the low-cation medium, 1 cm^3 of water, 20×10^{-3} cm^3 of 1 M $MgCl_2$, and 40×10^{-3} cm^3 of a solution containing 164 mg of $K_3Fe(CN)_6$ dissolved in 1 cm^3 of water. The absorbance of the mixture was measured at 420 nm (following a 1 min exposure to bright light) and then upon the subsequent addition of 10×10^{-3} cm^3 of 1 M NH_4Cl. The same procedure was also applied to broken chloroplasts (the chloroplasts were broken by hard mixing in an excess of water). Calculated content of intact chloroplasts was 65 %.

Incubation of chloroplasts and thylakoids with zinc and copper

The reaction mixture containing 1.5 cm^3 of the low-cation medium, 20×10^{-3} cm^3 of chloroplasts or thylakoids suspension, 1.25 cm^3 of water and 0.25 cm^3 of 0.05 M aqueous solution of $ZnSO_4$ or $CuSO_4$ was prepared. After the incubation period (t_{inc} = 3 h – 7 d), the copper or zinc complexes with chlorophyll from the incubated chloroplasts or thylakoids were extracted by adding acetone (4 cm^3), and then cyclohexane (5 cm^3) in 2 cm^3 of the reaction mixture. The acetone releases the chlorophyll from the incubated chloroplasts and thylakoids (making them "disorganized") and increases their solubility in the cyclohexane phase, in which the complex formation is stopped.[8]

Finally, the Vis spectra of the complexes were recorded in cyclohexane using the Vis spectrophotometric method for *in vitro* conditions.[22]

Vis spectroscopy

The Vis spectra of "disorganized" Cu–Chl and Zn–Chl complexes (obtained from the incubated chloroplasts and thylakoids) were recorded on a Varian Cary-100 spectrophotometer in the wavelength range of 350 to 750 nm with a constant concentration of ions (Cu^{2+} or Zn^{2+}), after different t_{inc} periods.

Fluorescence spectroscopy

The fluorescence spectra of the chloroplasts and thylakoids in the resuspension medium were recorded on a Fluorolog Jobin Yvon Horiba spectrofluorimeter. The excitation wavelength was 430 nm.

RESULTS

Before the addition of any metal ions to the chloroplasts and thylakoids, a control experiment was performed in order to assign all possible absorption changes in the absorption of Chl to factors other than the formation of the complexes. The control was performed over a period of one week. A hardly detectable change occurred just at the end of this period (not shown). This means that changes in the Vis spectra of "disorganized chlorophylls" and the fluorescent spectra of treated chloroplasts and thylakoids occurring in a period of less than one week, shown in Figs. 2–4 could be assigned only to the formation of the complexes.

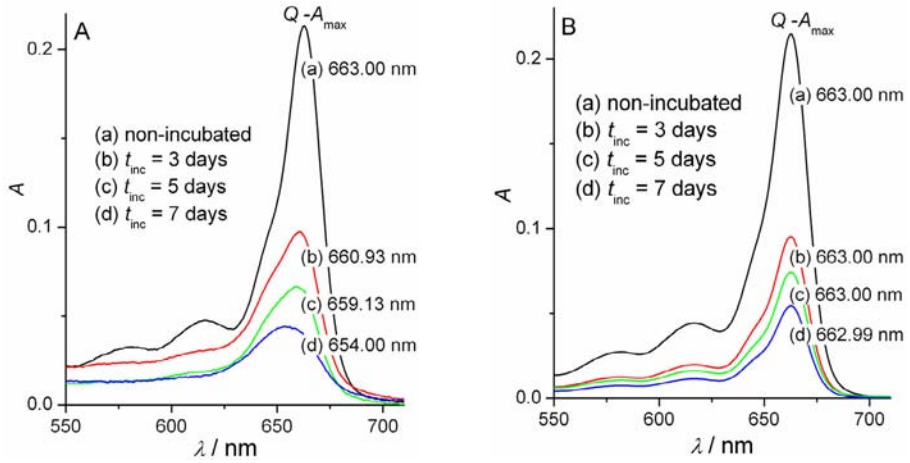


Fig. 2. Vis spectra of "disorganized chlorophylls" obtained from isolated intact chloroplasts (65 %) incubated for two time periods ($t_{inc.}$) with (A) copper and (B) zinc. The Chl concentration in the chloroplasts was Chl_{a+b} = 0.015 mmol dm^{-3}, while the two concentrations ratio was $n(Cu^{2+},Zn^{2+})/n(Chl_{a+b})$ = 3000:1.

The Vis spectra of "disorganized chlorophylls" obtained from chloroplasts incubated with zinc and copper ions for several different periods of time are shown in Figs. 2A and 2B, respectively. The spectra of "disorganized chlorophylls" obtained from non-incubated chloroplasts serve as blanks. The *Q* or "red band" is an exclusive ("diagnostic") characteristic of chlorophyll and its derivatives,[2,34,35]

thus eventual changes in its intensity or position should be attributed only to Chl and not to any other pigment (such as for Soret or the *B* ("blue")-band, where accessory pigments, carotenoids, can make a significant contribution).

Fluorescence spectra obtained from isolated photosynthetic organelles, chloroplasts, and sub-organelles, thylakoids, incubated with copper ions for two different periods of time, are shown in Figs. 3a and 3b, respectively. The fluorescence of non-incubated chloroplasts again plays the role of the "blank". While the position of the fluorescence maximum (F_{max}) barely shows any change during the incubation, the intensity of the F_{max} shows a huge drop (> 75 %).

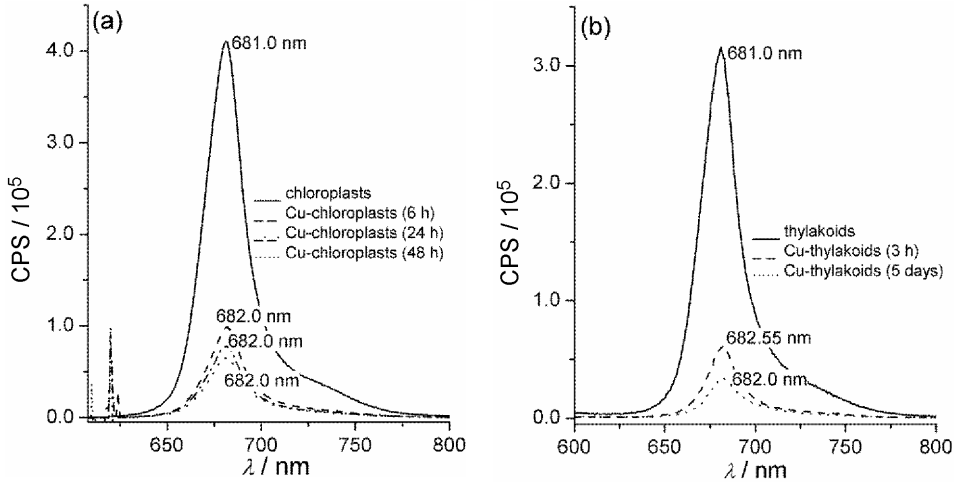


Fig. 3. The chlorophyll *Q*-band fluorescence spectra of (a) chloroplasts, and (b) thylakoids in the resuspended medium in the presence of copper for two different time periods following the beginning of the formation of the complexes (t_{inc}). Clearly, a very similar type of change can be seen. The fluorescence (cps – counts per second) from non-incubated chloroplasts served as the blank.

The completely opposite behavior was observed with the chloroplasts and thylakoids incubated with Zn^{2+} ions (Figs. 4a and 4b, respectively), *i.e.*, the intensity of F_{max} of the chloroplasts did not change significantly even after 2 days of incubation (Fig. 4a), or even a certain rise was evidenced after 5 days of incubation of the thylakoids (Fig. 4b).

DISCUSSION

Vis spectra

The Vis spectra of isolated chloroplasts incubated with copper, and then converted into disorganized chlorophylls, clearly proved the formation of the Cu complex. The hypsochromic ("blue") shift of 9 nm after 7 days of incubation (followed by a hypochromic effect, fall of the intensity) is clearly visible (Fig. 2A). The same effect was detected with the isolated thylakoids (shown in the Appendix). The results shown in Fig. 2A were obtained with an about 10 times higher con-

centration of Cu^{2+} ions, compared to that used for recording the fluorescent spectra; an additional experiment performed with a comparable Cu^{2+} concentration also expressed a "blue" shift (a concentration effect, in addition to the hypsochromic effect, shown in Appendix), although two times smaller than the one shown in Fig. 2A. On the other hand, isolated chloroplasts incubated with zinc, and then also converted in disorganized chlorophylls, did not prove the formation of a Zn complex (Fig. 2B), at least not one of the same type as the one formed with Cu, since a hypsochromic shift was hardly visible. This is in good agreement with the results found in a study of isolated Chl fractions,[22] where a clear and significant hypsochromic effect (also followed by a hypochromic effect, proving the instability of complexes) was found for the Chl fractions incubated with copper, confirming "central" complex formation (in which the central Mg atom of chlorophyll was replaced by copper); at the same time, the "peripheral" complex (6-membered chelate cycle fused at the periphery of the Chl structure) was not clearly confirmed in the case of Cu–Chl, but was in the case of Zn–Chl.[22]

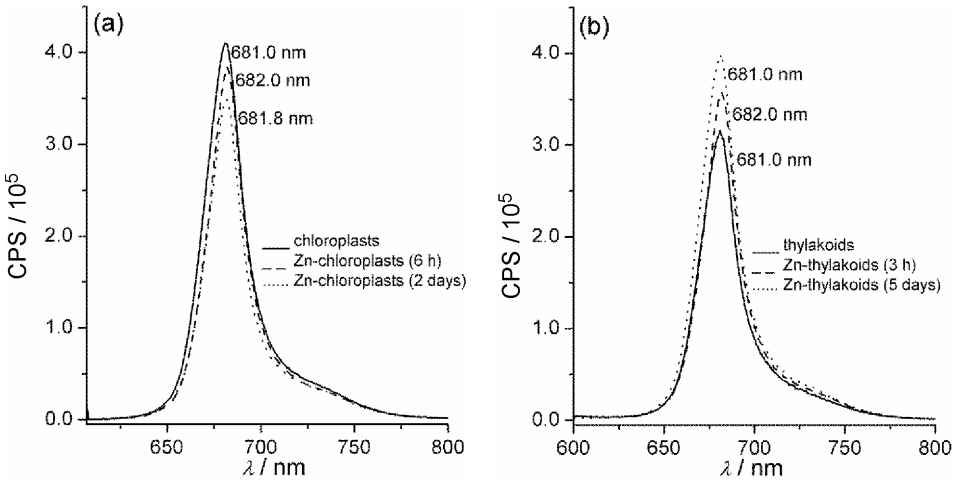


Fig. 4. The chlorophyll *Q*-band fluorescence spectra of (a) chloroplasts, and (b) thylakoids in the resuspended medium in the presence of zinc for two different time periods following the beginning of the formation of the complexes (t_{inc}). Clearly, while a very similar type of change can be seen (a–b), the behavior is quite the opposite compared to that shown in Figs. 3a and 3b (copper effect). The fluorescence (cps – counts per second) from non-incubated chloroplasts served as the blank.

Fluorescence spectra

Bearing in mind that Chl fluorescence represents an intrinsic probe of photosynthesis,[36] it was reasonable to expect another proof for Chl–HMS formation from fluorescence spectra (*in vitro*). Generally, it is well known that Chl–HMS formation leads to a decrease of the intensity of the fluorescence emission, indicating the formation of an unstable first excitation state, which relaxes to a greater extent thermally.[13] Considering that chlorophyll fluorescence emission and ki-

netics *in vivo* are influenced by toxic heavy metals, including copper and zinc,[12,37] and bearing in mind the high complexity of the involved isolated photosynthetic organelles and sub-organelles, it is to be expected that the fluorescent spectra of chloroplasts and thylakoids would also be affected by the action of toxic metals. This is clearly seen with copper through a rough observation of the magnitude of the change in ΔF_{max} obtained with chloroplasts and thylakoids, compared to non-incubated chloroplasts and thylakoids (Figs. 3a and 3b) (F_{max} of Chl fluorescence in chloroplasts and thylakoids was red-shifted, to 681 nm, compared to isolated Chls due to the aggregation of the Chl molecules in the antennas).[2] The fluorescence emission was greatly suppressed in Cu-treated chloroplasts and thylakoids: the organization of the antennas of the photosystems favors the formation of non-fluorescent "central complexes", even after 3 h (thylakoids – Fig. 3b) or 6 h (chloroplasts – Fig. 3a). Prolongation of the incubation period (t_{inc}) up to 2 days (chloroplasts – Fig. 3a) or to 5 days (thylakoids – Fig. 3b) did not change the situation very much, even the almost negligible "red shift" of F_{max} (681 nm for non-incubated chloroplasts and thylakoids *vs.* 682 nm for the complex) remains the same. This remarkable decrease of fluorescence intensity (>75 %) induced by the action of copper is in accordance with copper-induced suppression of fluorescence induction in spinach chloroplasts,[6] or the induction kinetics in isolated thylakoids.[38] It is also necessary to note that the great similarity of the fluorescence spectra obtained from the chloroplasts and thylakoids (Figs. 3a and 3b) proves a predominance of the Cu–Chl interaction inside the photosynthetic antennas compared to the potential Cu interactions with the stromal lipo-protein matrix of the chloroplasts.

On the other hand, the fluorescence spectra of the chloroplasts and thylakoids exposed to the action of Zn^{2+} exhibited the completely opposite behavior (Figs. 4a and 4b). The magnitude of F_{max} decreased just a little after 6 h, and another few percents after 48 h, compared to the non-incubated chloroplasts (Fig. 4a). On the other hand the magnitude of the change in F_{max} for the thylakoids was of a higher magnitude but in the opposite direction (more than 20 % after 5 days, Fig. 4b). There is no clear explanation for this distinction, although Zn–Chl complexes have been reported to fluoresce at least as much as Chl itself in benzene and diethyl ether.[39] However, Zn–Chl can not replace Chl in higher plants and green algae – comparable systems to chloroplasts and thylakoids – because its "blue-shifted" absorbance detected in solutions[39] could reduce the spectral overlap of fluorescence/absorbance bands required for excitation transfer to the reaction centers of the photosynthetic apparatus of plants.[13] Therefore, in both cases – chloroplasts and thylakoids – there is not an extremely large drop in the Chl fluorescence intensity, as is the case with copper (Figs. 3a and 3b). A possible explanation could come from a nature of aggregated antennas structures. Chl molecules inside photosynthetic antennas are aggregated most probably through (C-131) =

= O···Mg interaction and this could potentially be the key factor preventing the formation of Zn–Chl "peripheral" complexes,[22] rather than a possible interaction with the stromal lipo-protein matrix (otherwise copper would react in a similar manner); the formation of "central" complexes in the case of zinc is certainly excluded.

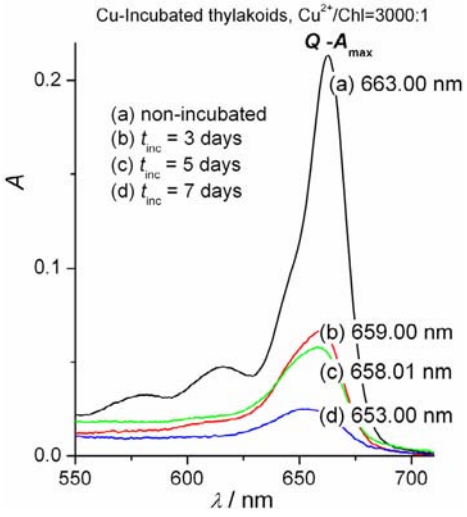
Finally, the question is how much this *in vitro* behavior of the Chl–HMS complexes can mimic the expected *in vivo* situation, where plants and algae absorb copper and zinc from the surrounding environment, permitting the penetration within photosynthetic apparatus and the formation of Zn– and Cu–Chl complexes within it. One of the main limiting factors is the sensitivity of chlorophyll: Chl can undergo "light" and "dark" reactions. The first ones are mostly photo-oxidation reactions involving the chlorin structures, resulting in different porphyrin modifications.[40] The other ones involve the periphery of the chlorin structure but do not change the chlorin nucleus.[16] While the first ones can be prevented by simply keeping chlorophyll in the dark, the second ones can not be avoided and potentially, they can influence chelate formation.[22] The peripheral chelate formation is especially sensitive to oxidation reaction at the C-13[2] position (the allomerization reaction – see Fig. 1) yielding several derivatives incapable of enolization at the isocyclic ring.[16] Structures of the allomer products may exclude any possibility for the chelate formation ("peripheral complexes"). Generally, allomerization products are less abundant in fresh spinach samples than in the older ones; in the latter case, prolonged senescence may lead to chlorophyll breakdown and the appearance of monopyrrolic compounds.[41]

However, from a biological point of view, the formation of either type of complex, "central" or "peripheral", has pathological consequences on the functioning of chloroplasts and thylakoids, ranging from irreversible impairment (in the "light phase") to complete breakdown of photosynthesis, a fatal end for the plants.[8,11] The two complexes are selectively connected to particular steps in this pathological process. Thus while "central" complexes are generally related to primary photosynthesis photochemistry,[13] "peripheral" complexes certainly affect the process of photon transfer inside the photosynthesis antennas, by widening the gap in the spectral overlapping between neighboring Chl molecules.[12]

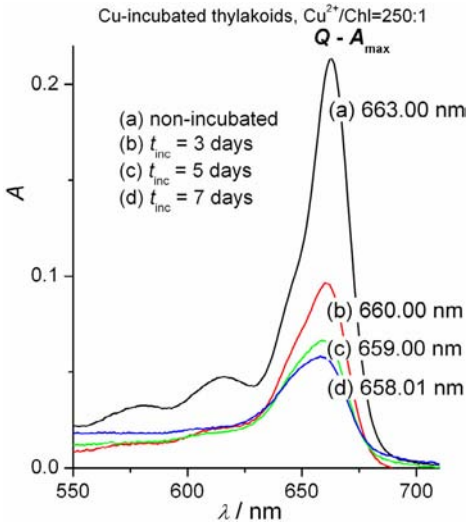
CONCLUSIONS

Of the studied metals, only copper forms complexes with Chl inside the photosynthetic antennas of isolated chloroplasts and thylakoids. The complexes are of "central" or "substitution" type. Zinc cannot form this type of complex and the other, "peripheral" type of Zn–Chl complex is not possible in chloroplasts and thylakoids for steric reasons.

APPENDIX



Incubation of isolated thylakoids by Cu^{2+} ions: the effect on A_{max} position of disorganized chlorophylls' *Q* ("red") band. The Cu^{2+}/Chl ratio the same as for Fig. 2A (3000:1).



Incubation of isolated thylakoids by Cu^{2+} ions: the effect on A_{max} position of disorganized chlorophylls' *Q* ("red") band. The Cu^{2+}/Chl ratio is an order of magnitude smaller (250:1) than the one for Fig. 2A.

И З В О Д

РАЗЛИЧИТЕ МОГУЋНОСТИ ЗА ФОРМИРАЊЕ КОМПЛЕКСА БАКРА И ЦИНКА СА ХЛОРОФИЛОМ У ФОТОСИНТЕТИЧНИМ ОРГАНЕЛАМА: ХЛОРОПЛАСТИМА И ТИЛАКОИДИМА

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У овом раду су испитиване могућности за формирање комплекса бакра и цинка са хлорофилом у фотосинтетичним органелама (хлоропластима) и суб-органелама (тилакоидима). Флуоресцентни спектри добијени из хлоропласта и тилакоида у присуству два метала потврдили су формирање комплекса у случају бакра, док је у случају цинка та могућност минорна. Разлог за овакву различитост је у различитим типовима комплекса које хлорофил формира са ова два метала: само "централни" или "супституциони" бакар–хлорофилни комплекси могу бити формирани унутар два изолована ентитета, док је формирање цинк–хлорофилног "периферног" типа комплекса спречено из стерних разлога. Последња чињеница је од велике биолошке важности пошто оба типа комплекса могу проузроковати неповратну дисфункцију и оштећење фотосинтетичког процеса.

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