The Role of Cu in Respiration of Pea Plants and Heterotrophically Growing *Scenedesmus* Cells

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In *Scenedesmus* about half of NADH oxidation proceeds *via* a cyanide-sensitive and the other half *via* a cyanide-insensitive respiratory pathway. In contrast, respiration is completely cyanide sensitive in pea indicating that the alternative respiratory pathway is absent. Cu deficiency in pea plants and in heterotrophically grown *Scenedesmus* cells interferes with respiratory activity of mitochondria. In both organisms, the cyanide-sensitive NADH oxidation was strongly decreased during cultivation in low Cu media. Cu sensitivity was also observed for the alternative respiratory pathway in *Scenedesmus*. These results suggest that a Cu-containing component is involved in the alternative respiratory pathway. This is the main reason why alternative respiration cannot be regarded as a compensation for low cytochrome-oxidase activities during Cu starvation.

The Cu dependency of the cyanide-sensitive respiration was located at the site of cytochrome oxidase. A strong coordination of the biosynthesis of the Cu-containing cytochrome-oxidase complex was evident. When the endogenous Cu pool was low, formation of cytochrome aa_3, another component of cytochrome oxidase, was also decreased.

Introduction

Many redox reactions are catalyzed by enzymes which contain redox-active metals as functional groups [1]. Besides of Fe, Cu plays an essential role in the electron-transfer reactions of chloroplasts and mitochondria. In the latters, the prominent Cu-containing protein is cytochrome oxidase, the terminal oxidase of the respiratory electron-transport chain [2]. It contains the cytochromes *a*, a_3 and two atoms of copper [3].

Studies with yeast [4], sycamore cells [5], clover [6, 7] and a green alga [8] have shown that Cu deficiency inhibits formation of cytochrome oxidase. This decreased synthesis in general results in low respiration rates with a subsequent negative impact on the energy metabolism. Similar Cu-deficiency effects on synthesis and function of cytochrome oxidase have also been observed with animal mitochondria [9].

In a recent publication, we have investigated the influence of Cu deficiency on photosynthesis and respiration in the green alga *Dunaliella* and shown that both processes are strongly affected [8]. In addition to direct effects of Cu deficiency on cytochrome oxidation, we found an indirect one due to inhibited photosynthesis which supplies carbohydrates, a substrate for the respiratory process. This dependency is characteristic for autotrophic organisms. Therefore, we extended our studies on heterotrophic cultures of *Scenedesmus* and report here on the influence of Cu deficiency on respiration of this green alga cultivated on exogenous sugar in the dark. Furthermore, *Scenedesmus* exhibits cyanide-insensitive respiration [10]. It has been discussed that a Cu-containing redox component participates in this alternative pathway [11, 12]. If this is the case, Cu deficiency should also decrease cyanide-insensitive respiration. The results on respiratory activity of *Scenedesmus* will be compared to data obtained with strongly Cu-depleted pea plants.

Materials and Methods

Pea seeds (*Pisum sativum*, cv. Progress) were germinated in moistened sand at 28 °C for ten days and then hydroponically grown in a Hewitt full nutrient solution supplemented with different Cu^{2+} concentrations; 0.03 μM for deficient plants and 1 μM for the control plants. The details have been described recently [13].

The plants denoted as strongly Cu depleted originated from seeds produced by Cu-deficient plants.

Scenedesmus acutus (strain 276-3a, Algal Culture Collection, University of Göttingen) was grown

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under heterotrophic conditions in the dark. Growth conditions and composition of the sterile cultivation medium were as described [14]. The composition of the nutrient solution was modified by the addition of glucose (5 g/l), vitamin B_{12} (30 nM), and riboflavin (42.5 μM). The copper content of the standard medium was 10^{-6} M, and in the Cu-deficient cultures as indicated. The algae were harvested after a 8-day growth period. Cell mass of cultures was determined as packed cell volume (pcv) in graduated microcentrifuge tubes of 80 μl capacity.

Electron-transfer activities

In vitro respiratory electron-transport activities were measured out with washed mitochondria from pea leaves and *Scenedesmus* cells.

Pea mitochondria were isolated as described by Douce *et al.* [15]. *Scenedesmus* mitochondria were obtained according to Sharpless and Butow [16]. The cells were broken with glass beads (0.5 mm diameter) for 30 sec at maximum speed with a Braun-Merckenschläger homogenizer in an isolation medium containing 0.3 M sorbitol, 0.5 mM EDTA, and 25 mM HEPES buffer, pH 7.0. From the resulting homogenate, mitochondria were isolated by differential centrifugation.

For determination of cytochrome aa_3, the mitochondria were further purified on a density gradient with three steps of 30%, 45%, and 55% (w/w) sucrose. Centrifugation was for 90 min at 100,000 × *g* in a swing-out rotor. The mitochondrial fractions were collected between the 45% and 55% sucrose steps.

Respiratory electron-transport activities from NADH to oxygen were determined in an assay medium containing 1 mM NADH, 0.1 mM ADP and aliquots of the organelle fraction in a reaction medium containing 0.3 M sucrose, 10 mM KCl, 5 mM $MgCl_2$, 10 mM KH_2PO_4, and 10 mM HEPES buffer, pH 7.2. Oxygen uptake was recorded with a Clark-type oxygen electrode. Cytochrome-oxidase activity was determined as KCN-sensitive oxidation of reduced horse-heart cytochrome *c*-550 in a double-beam spectrophotometer at 550 nm, as previously described [8]. The reaction mixture contained 40 mM Tris-HCl buffer, pH 7.3, 1 mM EDTA, 1% Triton X-100, 20 μM reduced cytochrome *c*-550, and the organelle preparation.

Determination of redox components

The amounts of redox proteins were determined by optical difference spectroscopy in a double-beam spectrophotometer. Reduced minus oxidized difference spectra of cytochrome aa_3 were obtained with mitochondria purified by sucrose-density centrifugation. For recording the spectra, the mitochondria were suspended in Tris-HCl buffer, pH 7.5 containing 1% Triton X-100, and the sample reduced with dithionite leaving the reference oxidized. For quantitation of cytochrome aa_3, a differential extinction coefficient (605–630 nm) of 13 mM cm^{-1} was used.

Assays for other parameters

The Cu content of *Scenedesmus* cells and pea leaves were measured with a Varian atomic-absorption spectrophotometer (Mod. AA 575) equipped with a carbon-rod atomizer. Before use, the leaves were dried (12 h, 120 °C), powdered and suspended in 2 N HNO_3. Intact *Scenedesmus* were suspended in 2 N HNO_3 and measured directly. Protein concentration was determined by the method of Bradford [17].

Results and Discussion

The standard Cu concentration of the medium for unrestricted formation of Cu-containing proteins in *Scenedesmus* is 1 μM [18]. A decrease of the Cu supply by 1/10 or 1/100 resulted in a strong depletion of the endogenous Cu accumulated by the cells (Fig. 1). In *Scenedesmus* the maximum Cu-sequestering capacity was almost 4-fold higher than in *Dunaliella,* another green alga [8]. However, when Cu is limiting the endogenous concentrations tend to be similar. A direct effect of this Cu depletion on the respiratory capacity could be observed. In mitochondria isolated from *Scenedesmus* cells with an optimum Cu supply the rate of respiratory NADH oxidation was highest and decreased with decreasing Cu concentrations. The lowest respiration values for *Scenedesmus* grown with no added Cu to the nutrient was about 40% of the respiration of mitochondria from Cu-supplemented control cells (Table I). Under these conditions, growth was about 60%.

In contrast to higher plants [19], only half of the respiratory activity was sensitive to comparably high KCN concentrations in *Scenedesmus* (Table I). This contribution is due to the cytochrome-oxidase reac-

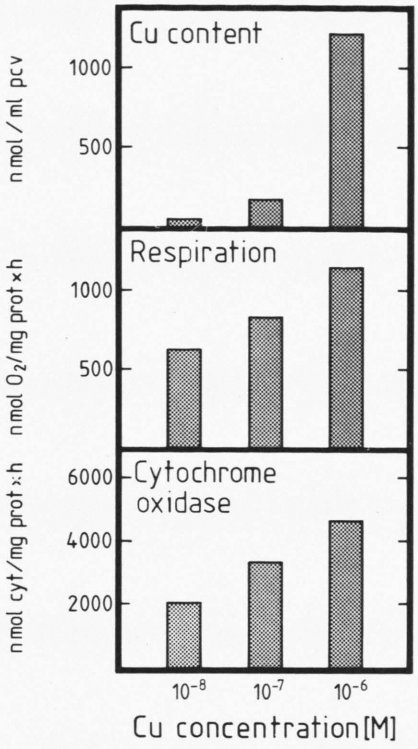

Fig. 1. Endogenous Cu content, respiratory NADH oxidation and cytochrome-oxidase activity in heterotrophically grown *Scenedesmus* cells supplemented with different Cu concentrations.

Table I. NADH-dependent respiration of mitochondria isolated from *Scenedesmus* cells grown under normal and Cu-deficient conditions.

	No Cu added	1 μM Cu
Respiration NADH → O_2 (nmol/mg prot × h)		
untreated	527	1349
+ KCN (0.5 mM)	287	767
+ SHAM (15 mM)	287	671
Contributions of different respiratory pathways [%]		
CN^--sensitive	46	43
SHAM-sensitive	46	50

tion which can be totally inhibited by cyanide. The other part of NADH-dependent oxygen uptake is sensitive to salicylhydroxamic acid (SHAM), an inhibitor of an alternative terminal oxidase [12]. This SHAM-sensitive respiratory oxygen uptake is retained after further purification of mitochondria on a sucrose gradient indicating that it is caused by an alternative branch of the respiratory pathway rather than by oxidizing enzymes located on contaminating microsomal membranes. The contributions of the cyanide-sensitive cytochrome oxidase and the SHAM-sensitive alternative oxidase on oxygen-dependent NADH oxidation were equal in *Scenedesmus* cultures. This is a similar contribution by the alternative oxidation pathway as reported for heterotrophic cultures of the alga *Euglena* [16].

Inhibition of cytochrome oxidation by growing sycamore cells with cyanide [20] or by growing yeast in low Cu media [21] activated the alternative respiratory pathway in both organisms. However, in *Scenedesmus* regardless whether the cells have been grown in high or low Cu media both respiratory pathways are operating and both pathways were inhibited by Cu deficiency to the same extent as the total respiratory activity. This result demonstrates that in *Scenedesmus* the alternative respiration pathway is unable to compensate for inhibited cytochrome oxidation during Cu deprivation. The Cu sensitivity of cyanide-insensitive NADH oxidation indicates a participation of a Cu-containing component in this alternative pathway. A stepwise decrease of the Cu supply from 10^{-6} M to 10^{-8} M by 1/10 resulted in total respiration rates which were 20 to 30% lower after each depletion step (Fig. 1). The same can be observed for the activity of cytochrome oxidase which was parallely decreased to the same extent.

The degree of Cu depletion in pea plants is dependent on the Cu supplementation of this medium and on the origin of the seeds. Seeds from Cu-deficient plants only contain 1/10 of the Cu which accumulates in seeds from optimal supplied plants [13]. For our experiments we used a Cu supplementation of 1 μM for the control plants and 0.03 μM for Cu-depleted plants in order to ensure the formation of a minimum amount of plastocyanin which is necessary for photosynthesis and unrestricted plant growth [13]. According to the growth conditions, the leaf Cu-content varied between 10 and 35 nmol/mg fresh weight (Table II). This resulted in a decrease of mitochondrial respiratory NADH oxidation by 20% in the Cu-depleted pea plants with a leaf Cu-content of 19.9 nmol/mg fresh weight and a decrease of 50% in case of strongly Cu-starved pea plants with a leaf Cu-content of 10.2. Cytochrome-oxidase activity measured with mitochondria from the two different

Cu-deficient cultures was lowered almost to the same extent as the overall respiration rate. In pea mitochondria not only the cytochrome-oxidase reaction but also the respiratory electron transfer from NADH to oxygen was completely inhibited by KCN. In this respect, the mitochondria from pea resemble the ones from wheat in which no alternative respiration was detected [19]. In pea mitochondria, the decrease of respiratory NADH oxidation during Cu deficiency could be attributed to the inhibited cytochrome-oxidase reaction in depleted and strongly depleted plants (Table II). Compared to the control, the relative decrease of NADH oxidation and cytochrome oxidation was very similar. This Cu-dependency of the cytochrome-oxidase reaction is typical for many higher and lower plants [4, 6–8]. However, in Cu-depleted sycamore cells, oxidative and phosphorylation activities were similar to Cu-supplied cells [5]. Although cytochrome aa_3-content was decreased, the higher turnover number of cytochrome oxidase in Cu-deficient mitochondria compensated for this loss.

Cytochrome aa_3, a component of the Cu-containing cytochrome-oxidase complex, was determined in isolated mitochondria from *Scenedesmus* and pea plants (Table III). In both organisms the amount of cytochrome aa_3 decreased concurrently with the Cu supply. Apparently, the formation of the cytochrome-oxidase complex is coordinated in pea and *Scenedesmus*. When single components such as Cu are missing, the synthesis of the other components is also prevented. Therefore, we could monitor a decreased synthesis of the cytochrome-oxidase complex by determination of the cytochrome aa_3-content.

Table III. Content of cytochrome aa_3 (nmol/mg prot) in control and Cu-deficient mitochondria of *Scenedesmus* and pea plants.

Cu status of the organism	*Scenedesmus*	*Pisum sativum*
optimal supplied	0.33	0.46
depleted	0.07	0.28/0.13*

* From pea cultures with strong Cu deficiency due to the use of Cu-depleted seeds.

Either for *Scenedesmus* or pea we found a very good relationship between the endogenous Cu in the cells and the cytochrome aa_3-content. This resembles very much the significant correlation between endogenous Cu pools and the formation of plastocyanin, another Cu-containing protein in pea chloroplasts [13]. For both organisms, we observed a stronger decrease of the cytochrome aa_3-content than of respiratory NADH oxidation during Cu deprivation. This was also reported for the green alga *Dunaliella* [8]. These results suggest that mitochondria of higher and lower plants contain a surplus of cytochrome aa_3 over the other respiratory components. This is in agreement with an accumulation of cytochrome aa_3 over *b*- or *c*-type cytochromes in *Candida* [22] and the conclusion drawn by Ducet and Rosenberg [23] that cytochrome aa_3 is in excess in plant mitochondria.

Table II. Cu content and respiration of mitochondria isolated from pea plants grown with high and low Cu supply.

	Control	Cu depleted	Strongly Cu depleted*
Cu content of leaves (nmol/g fresh weight)	35.8	19.9	10.2
Respiration with NADH as substrate** (μmol O_2/mg prot × h)	2.26	1.81	1.16
Cytochrome-oxidase activity (μmol cyt/mg prot × h)	8.56	6.30	3.81

* These plants were grown from Cu-depleted seeds in media containing 0.03 μM Cu^{2+}.
** The reaction is completely inhibited by 0.5 mM KCN.

Among the Cu-dependent reactions, respiration is one of the most important targets for Cu deficiency. Therefore, in higher or lower plants Cu-limited growth has a direct impact on the energy metabolism. Most likely, plant mitochondria possess a certain excess of Cu-containing cytochrome oxidase in order to alleviate temporary Cu shortages. Algae are able to operate an alternative respiratory pathway. However, this pathway cannot take over when cytochrome-oxidase activity is decreased in low Cu mitochondria because the alternative respiratory pathway itself is sensitive to Cu deprivation, to the same extent as cytochrome oxidase.

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