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CHLOROPLAST AND CYTOSOL ISOZYMES OF CuZn-SUPEROXIDE DISMUTASE: THEIR CHARACTERISTIC AMINO ACID SEQUENCES

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Isozymes of CuZn-superoxide dismutase (SOD) were purified from angiosperms (spinach and rice), fern (horsetail) and green alga (*Spirogyra*). Occurrence of CuZn-SOD was confirmed by its purification in the group of green algae which shows the phragmoplast type of cell division. Purified CuZn-SODs are divided to chloroplast and cytosol types by their cellular localization and immunological properties. Their amino acid compositions, absorption spectra, CD spectra, and sensitivity to hydrogen peroxide also are distinguished from each other. All organisms including *Spirogyra* contain both types of isozyme. Thus, the divergence of the two types of CuZn-SOD isozyme occurred immediately after its acquisition by the most evolved green algae.

Amino acid sequences of amino-terminal regions of CuZn-SOD isozymes from spinach, rice and horsetail were determined and compared with those of CuZn-SODs from other plants. The chloroplast and cytosol isozymes of CuZn-SOD show each characteristic sequences. Sequence differences among the cytosol CuZn-SODs are greater than those among the chloroplast CuZn-SODs. These observations indicate that each type of isozyme had independently evolved after the acquisition of CuZn-SOD.

KEY WORDS: Algae, amino acid sequence, chloroplasts, CuZn-superoxide dismutase, isoenzymes, molecular evolution.

INTRODUCTION

In plant cells, several isoforms of cyanide-sensitive CuZn-SOD have been usually found by separation of cell extracts with polyacrylamide gel electrophoresis, and have been purified from wheat,[1] pea,[2] maize[3] and tomato.[4] Some of them have been assigned to be chloroplast and cytosol enzymes from their cellular locations. On the other hand, it has been shown that, in photosynthetic organisms, CuZn-SOD is distributed in *Spermatophyta*, *Bryophyta*[5] and *Pteridophyta*, but not in prokaryotic and eukaryotic algae except for limited species of *Chlorophyta*.[6]

We have purified the isozymes of CuZn-SOD from the monocotyledon rice (*Oryza sativa*),[7] the dicotyledon spinach (*Spinacea oleracia*),[8] the fern horsetail (*Equisetum arvense*) and the green alga pond scum (*Spirogyra* sp.).[9] For spinach and rice enzymes the cellular location of the isozymes was deduced by distribution in chloroplasts and in leaf and non-photosynthetic tissues. Using a property of no immunological cross-reaction between the chloroplast and cytosol isozymes, we found the occurrence of both types of isozymes in the fern and alga. Further, amino acid sequences of amino terminal regions of the isozymes were determined. Different substitution rates of amino acid residues between chloroplast and cytosol CuZn-SODs suggest that each isozyme of CuZn-SOD in plants had independently evolved after the acquisition by the algae.

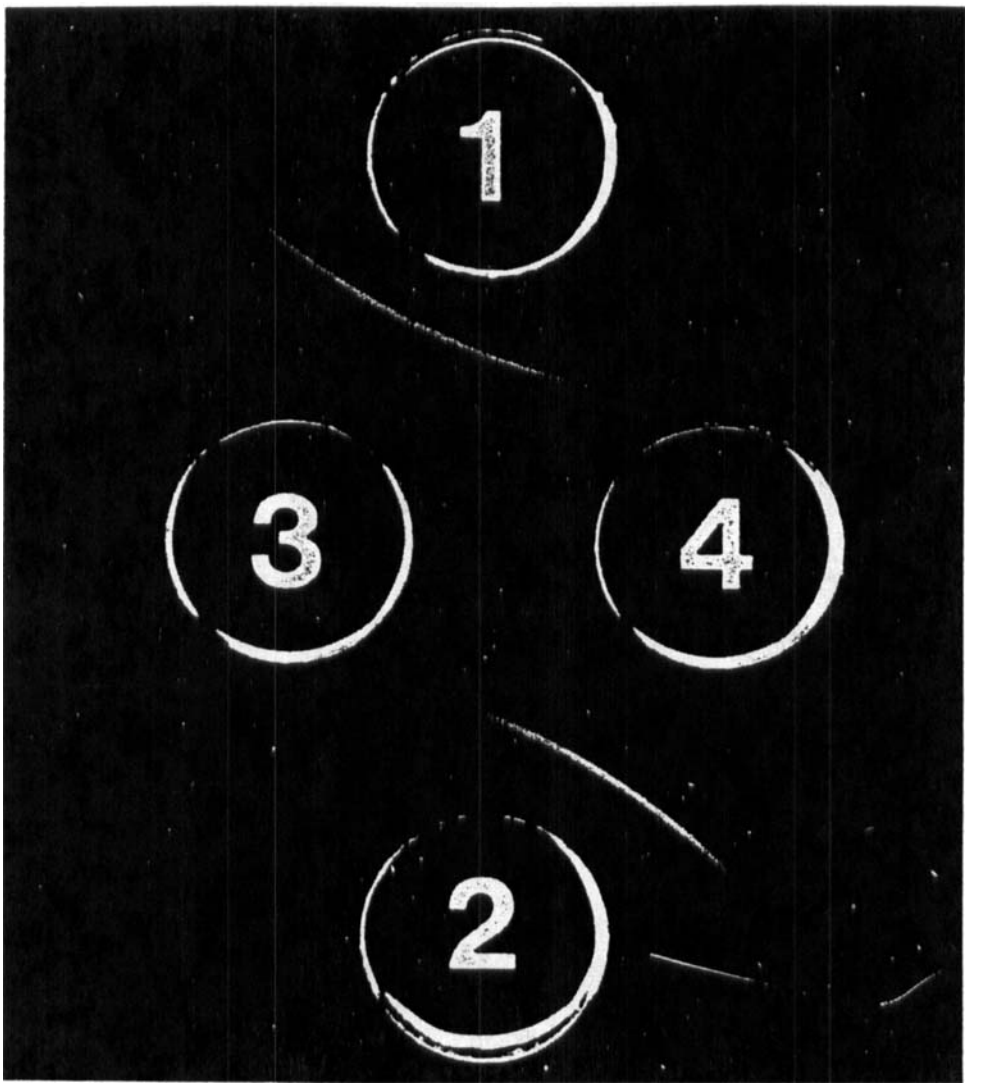

FIGURE 1 Ouchterlony double immunodiffusion of antibody against spinach CuZn-SOD I and antibody against spinach CuZn-SOD II with CuZn-SOD isozymes from spinach. 1, Antibody against spinach CuZn-SOD I (56 μg); 2, antibody against spinach Cu-Zn-SOD II (73 μg); 3, purified spinach CuZn-SOD I (1 μg); 4, purified spinach CuZn-SOD II (1 μg).

MATERIALS AND METHODS

Four isozymes of CuZn-SOD from rice, three isozymes from spinach, one isozyme from horsetail and one isozyme from pond scum were purified as described.[7-9] Antibodies against spinach CuZn-SODs I and II, rice CuZn-SOD I and horsetail CuZn-SOD were raised in rabbits. The immunoglobulin fractions were obtained by

passing the sera through a column of DEAE Affi-Gel Blue after ammonium sulfate precipitation at 50% saturation.

The amino acid sequences of the amino-terminal regions were determined using an Applied Biosystems gas-phase protein-peptide sequencer, model 477A, equipped with a PTH-amino acid analyzer, model 120A. The native CuZn-SODs were dialyzed against water and 0.5–1.0 nmol of each isozyme on a subunit basis was subjected to sequence analysis up to about the 60th residue. The sequence of the carboxyl-terminal region was determined by monitoring the liberation of amino acids by carboxypeptidase A.

RESULTS AND DISCUSSION

Distribution of Chloroplast and Cytosol Isozymes of CuZn-SOD in Photosynthetic Organisms

Rice CuZn-SOD I is a major enzyme in the leaves. Other isozymes (II, III and IV) were found in the seed embryos but only a minor isozyme in the leaves.[7] Spinach

TABLE I

Cross-reactivity between the antibodies raised against CuZn-SOD isozymes I and II from spinach, CuZn-SOD I from rice and CuZn-SOD from horsetail, and CuZn-SOD isozymes from spinach, rice, horsetail and *Spriogyra*. Sources: a) Fig. 1; b) present results (Fig. not shown); c) Ref.[7] and [9]; ND, not determined.

CuZn-SOD	Antibodies against			
	Spinach CuZn-SOD I (cytosol)	Spinach CuZn-SOD II (chloroplast)	Rice CuZn-SOD I (chloroplast)	Horsetail CuZn-SOD
Spinach				
CuZn-SOD I (Cytosol)	+ (a)	− (a)	− (b)	− (b)
CuZn-SOD II (Chloroplast)	− (a)	+ (a)	+ (b)	+ (c)
CuZn-SOD III	+ (b)	− (b)	ND	ND
Rice				
CuZn-SOD I	− (c)	+ (c)	+ (c)	+ (b)
CuZn-SOD II	+ (c)	− (c)	− (c)	− (b)
CuZn-SOD III	+ (b)	− (b)	− (b)	− (b)
CuZn-SOD IV	+ (c)	− (c)	− (c)	− (b)
Horsetail				
CuZn-SOD (purified)	− (c)	+ (c)	+ (c)	+ (c)
other isozymes	+ (c)	− (c)	ND	− (c)
Spirogyra				
CuZn-SOD (a mixture of three isozymes)	+ (c)	+ (c)	ND	+ (c)

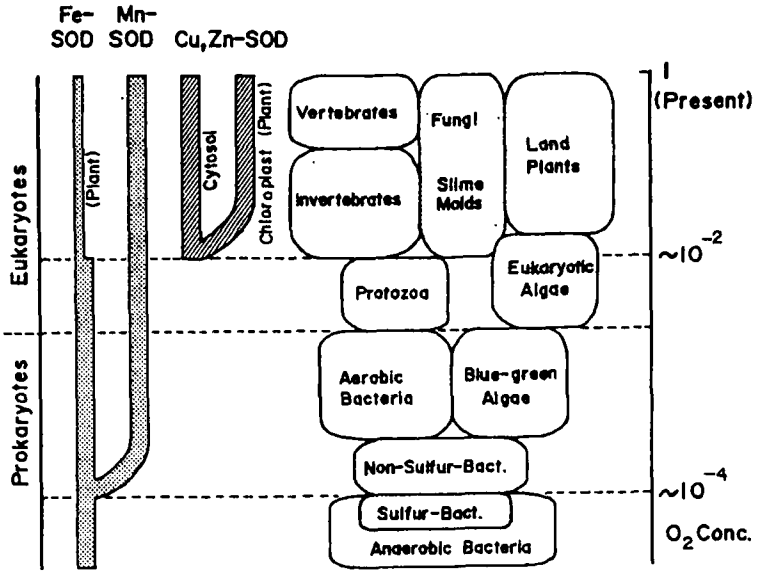

FIGURE 2 Phylogenic distribution of CuZn-, Mn- and Fe-SODs in organisms at various stages of evolution. Oxygen concentration shows the concentration of oxygen in the atmosphere, relative to the present comentration, when the indicated organisms appeared on the earth. Fe-SOD has been found in plants, but not in animals and fungi. Two types of isozyme of CuZn-SOD have been found in photosynthetic organisms (seed plants, ferns, mosses and the green alage which show a phragmoplast type of cell division), but such isozymes have not been found in animals and fungi except for extracellular SOD (For references on the isolation and characterization of three types of SOD from organisms at various stages of evolution, see [10]).

CuZn-SOD II was localized in chloroplasts, and other isozymes (I and III) were dominant in ungerminated seeds and in etiolated seedlings.[8] Thus, rice isozyme I and spinach isozyme II are the chloroplast enzyme, and the remaining isozymes from both plants seem to be the cytosol enzyme. The antibodies against the chloroplast CuZn-SODs from rice and spinach showed the cross-reaction with the chloroplast isozymes from the two plants, but not with the cytosol isozymes. On the contrary, the antibody against the cytosol isozyme from spinach (isozyme I) did not show any cross-reaction with the chloroplast isozymes (Figure 1 and Table I). Thus, using the antibodies against chloroplast and cytosol isozymes, we can distinguish the both types of isozyme in plants. Table 1 summarizes the cross-reaciton of CuZn-SOD isozymes from spinach, rice, horsetail and pond scum with the antibodies. The results indicate the occurrence of both chloroplast and cytosol isozymes in horsetail and pond scum in addition to rice and spinach.

Henry and Hall[6] showed the occurrence of cyanide-sensitive SOD in a group of green algae which show the phragmoplast type of cell division and contain glycolate oxidase, such as *Nitella*, *Spirogyra* and *Chara*. We confirmed the occurrence of CuZn-SOD by purification and showed that it has the similar properties to those of CuZn-SOD from other organisms.[9] Because of the absence of CuZn-SOD in other eukaryotic algae and prokaryotic algae (cyanobacteria),[5,6] photosynthetic organisms appear to acquire CuZn-SOD at the last evolutionary stage of green algae. Distribution of the three types of SOD, depending on the prosthetic metals, is schematically shown in Figure 2 (for review see[10]).

In this figure, the occurrence of CuZn-SOD in several bacteria is not included since it is still remained unsolved whether the bacterial CuZn-SOD gene was derived by the transfer from the host fish where the bacteria are symbionts.[11-13] The present results indicate that the divergence of chloroplast and cytosol types of CuZn-SOD started immediately after the acquisition of CuZn-SOD by the most evolved green algae, *Charales* and *Conjugales*.[9]

Properties of Chloroplast and Cytosol CuZn-SODs

Chloroplast and cytosol CuZn-SODs from plants showed the similar molecular weight, subunit structure, and metal contents of CuZn-SODs from mammalians and fungi. However, one cytosol isozyme from rice (CuZn-SOD IV) was a monomer having a molecular weight of the subunit of the dimer. Specific activity of the monomeric enzyme was the same as the dimeric enzyme, and showed a resistance to heat.[7]

Though the molecular sizes of the chloroplast and cytsol CuZn-SODs are the same, the two types fo the isozyme can be distinguished from each other by amino acid composition, absorption spectra, CD spectra, sensitivity to hydrogen peroxide.[1,3,4,7-9] These results clearly indicate that the chloroplast and cytosol CuZn-SODs are different gene products and have distinctly different ligand environments of the prosthetic Cu ions.

Amino Acid Sequences of Chloroplast and Cytosol CuZn-SODs

About 50 residues of the amino-terminal region of spinach CuZn-SOD I, rice CuZn-SODs I, II, III and IV and horsetail CuZn-SOD were determined. Three residues of carboxyl-terminal region of spinach CuZn-SOD I were also sequenced.[8] Whole sequence of chloroplast isozyme of CuZn-SOD from spinach (isozyme II) has been determined.[14] In additon, CuZn-SODs from cabbage,[15] tomato (two isozymes),[16] pea,[17] maize[18] and petunia[19]have been sequenced either by Edman degradation or from the nucleotide sequences of corresponding cDNAs.

These sequences are summarized in Figure 3. The amino acid sequences of CuZn-SODs from monocotyledons, dicotyledons and fern can be classified into chloroplast and cytosol types, in accordance with their intracellular localization, tissue-specfic distribution and also with the immunological criteria discussed above.[8] The amino terminuses of the mammalian CuZn-SODs so far sequenced are acetylated, but the plant CuZn-SODs of both types are not acetylated, neither are the CuZn-SODs from fish, insect, fungi and *Photobacterium*.

Although the chloroplast and cytosol CuZn-SODs have the conserved residues (shaded box) in the amino-terminal regions, they have conserved residues of respective isozymes which are boxed in the figure. The same is also the case of the carboxyl-terminal region. Further, the cytosol CuZn-SODs are characterized by a lack of two residues in the amino-terminus and of one residue in the carboxyl-terminus compared with the chloroplast CuZn-SODs. Thus, the both types of isozyme have conserved the amino acid residues for catalytic and key structural functions, but each isozyme have respective characteristic conserved residues.

The sequence differences between chloroplast and cytosol CuZn-SODs are 51-61 for the total residues, and 20-29 for the amino-terminal resdiues (50 for chloroplast and 48 for cytosol enzymes). Similar differences are found between the chloroplast and cytosol CuZn-SODs from the same species; 24 for spinach, 22-25 for rice and 25

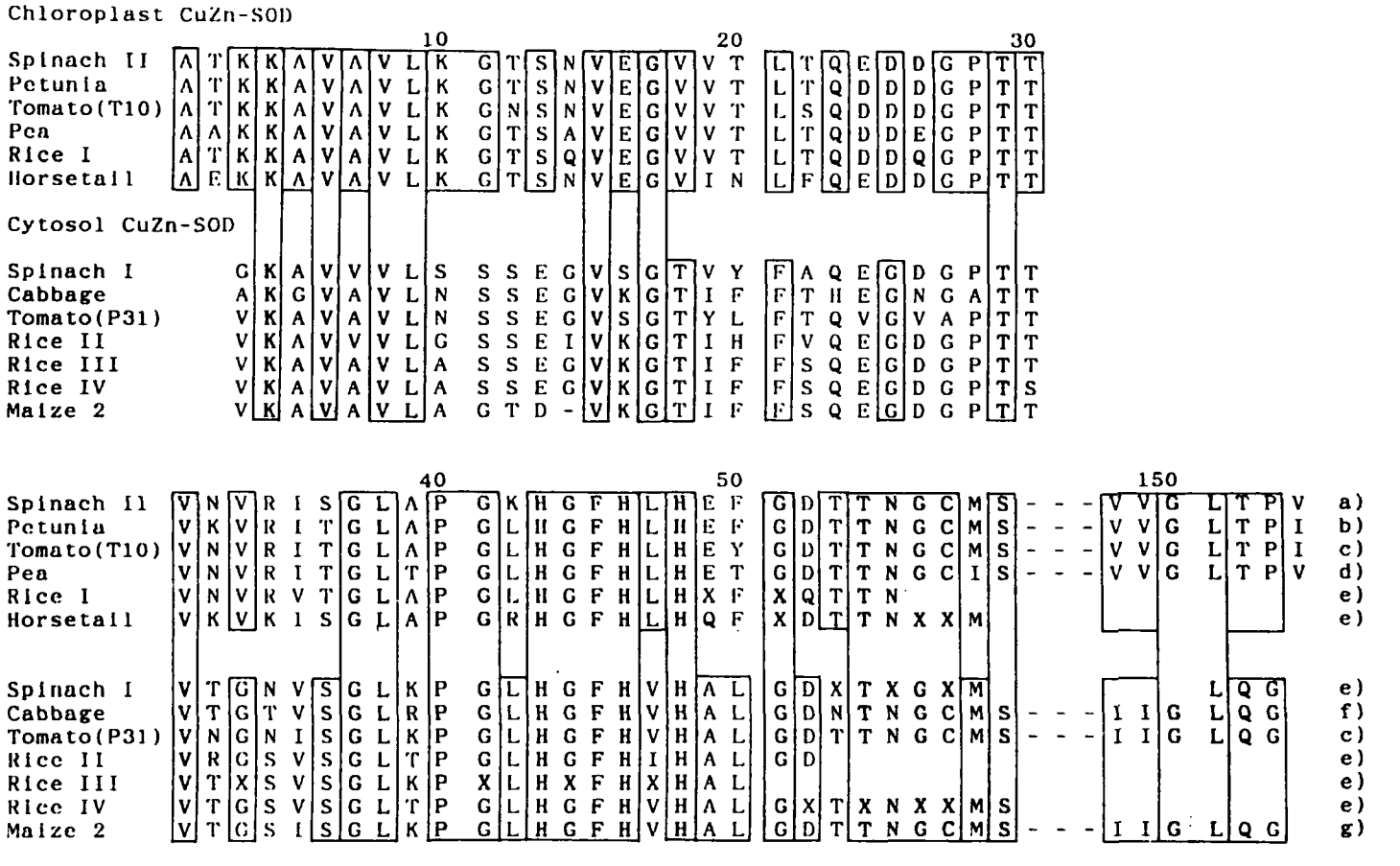

FIGURE 3 Amino acid sequences of the amino-terminal and carboxyl-terminal regions of chloroplast and cytosol isozymes of CuZn-SOD from spinach, rice and horsetail and their alignments with those of CuZn-SODs from other plants. The alignment for maximum similarity was made by visual inspection. The position number of the residues is based on the sequence of CuZn-SOD II from spinach (14). X: Unidentified residue. The amino acid residues shared by the chloroplast and cytosol CuZn-SODs are enclosed by shaded boxes, and those shared either by chloroplast CuZn-SODs or cytosol CuZn-SODs are enclosed by boxes. a) Ref. [14], b) Ref. [19], c) Ref. [16], d) Ref. [17], e) This work, f) Ref. [15], g) Ref. [18].

for tomato in the amino-terminal regions. On the other hand, the sequence differences among chloroplast CuZn-SODs from angiosperms are 12–19 for the total residues and 4–8 for the 50 amino-terminal residues, and those among cytosol CuZn-SODs from the angiosperms 28–33 for the total residues and 11–15 for the 48 amino-terminal residues. Thus, generally, the sequence differences among the cytosol enzymes from various plants are greater than those among the chloroplast enzymes. This is also the case when the sequence differences are compared between the chloroplast and cytosol enzymes from rice (isozyme II), spinach and tomato. The sequence differences of the chloroplast enzymes are all 6, but those of the cytosol enzyme are 11, 11 and 15. Distinct difference between the chloroplast and cytosol CuZn-SODs indicates that the rate of amino acid substitution of the cytosol CuZn-SOD is higher than that of the chloroplast CuZn-SOD.

A low substitution of amino acid residues of the chloroplast CuZn-SOD during the evolution is probably due to a high production rate of superoxide in chloroplasts. Chloroplasts photoproduce the superoxide anion radicals through the autooxidation of the primary electron acceptor in photosystem I and of the reduced ferredoxin at a rate of $240\,\mu M\,s^{-1}$.[20] Such a high rate would not allow the mutational changes resulting in a loss of the ability to scavenge the superoxide anion radicals. However, the cytosol CuZn-SOD may be more receptive of mutational changes because of a low production rate of superoxide in non-photosynthetic tissues and in cytosol of leaf cells where the cytosol CuZn-SOD is localized.

Concluding remarks

In mammalian and fungal cells, the association of CuZn-SOD to cell organelles has been shown, but, except for extracellular SOD, the isolation and characterization of CuZn-SOD isozymes have not been reported. On the contrary, seed plants, mosses, ferns and the group of green algae contain chloroplast and cytosol isozymes of CuZn-SOD. The two types of isozymes are distinguished from each other with respect to their amino acid sequences. Greater sequence differences among cytosol CuZn-SODs than those among chloroplast CuZn-SODs from various plants suggest the mutation rate of cytosol CuZn-SODs is higher than that of chloroplast CuZn-SODs. If organelle or cytosol specific CuZn-SOD occur in other organisms than plants, it should be considered that the rate of molecular evolution of each isozyme may not be the same.

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