Soluble & Membrane-bound Superoxide Dismutases in a Blue-green alga (Spirulina) and Spinach

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SUMMARY: Soluble extracts of Spirulina contained three cyanide-insensitive superoxide dismutase activities. The major isoenzyme was purified and shown to contain iron. Soluble extracts of spinach leaves contained two distinct cyanide-sensitive (Cu/Zn) dismutase activities one of which occurred in the stroma of isolated chloroplasts. A cyanide-insensitive dismutase activity was associated with chloroplast lamellar membranes: this was solubilised and partially purified.By subfractionation of lamellae, a dismutase activity was shown to be bound to particles with a chlorophyll a/b ratio of about 2 (photosystem II). These results are discussed in terms of the possible *in vivo* functions of the different enzymes.

INTRODUCTION: Superoxide dismutase (SOD) has been studied in a wide range of organisms[1]. Dismutases isolated from eukaryotic sources[2-6] show little diversity in their properties having molecular weights of near 32,000 and containing two copper and two zinc atoms per molecule (activity is cyanide sensitive). In contrast, the two enzymes isolated from prokaryotes e.g. *E. coli*, contain either two manganese atoms or one iron atom per molecule[7,8] (both types are cyanide-insensitive). Similarly, SOD from *Streptococcus mutans* has been found to be a manganoenzyme[9]. SOD isolated from mitochondria of chicken liver and of yeast has also been shown to contain manganese[10]. In several cases, the occurrence of multiple forms of SOD within one cell type has been demonstrated although only in the cases of the distinct mitochondrial enzymes and those of *E. coli*[11] have different isoenzymes been demonstrated to perform different functions *in vivo*. On the other hand during the purification of SOD activity, great care must be taken to avoid the selective destruction of some of the isoenzymes by harsh treatment such as organic solvents[6] or heat[8] or indeed the generation of spurious active forms[4].

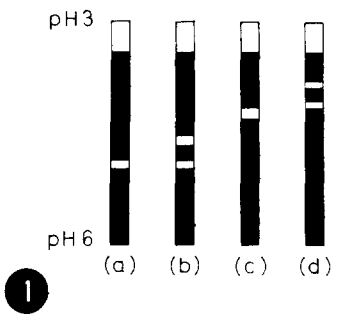
The univalent reduction of oxygen to superoxide anion by photosystem I of spinach chloroplasts has been reported by several groups. The superoxide so produced can initiate the oxidation of sulphite[12] or adrenaline[13], cause

the reduction of exogenous cytochrome c[14] or be itself reduced to peroxide by ascorbate[15,16]. This latter reaction may be of physiological importance[16] but is possibly suppressed by endogenous dismutase activity[15]. The generation of superoxide has been shown on autoxidation of purified ferredoxin[17] and of methyl viologen[18] and has been proposed as a possible mode of action for this and other bipyridyl herbicides[19].

In the present work we have studied the occurrence of multiple forms of SOD in the blue-green alga Spirulina and in spinach. The existence of one SOD in spinach has been reported[5]. We now have detected distinct chloroplast and cytoplasmic SOD activities in addition to a novel membrane-bound SOD in chloroplast lamellae.

MATERIALS AND METHODS: Fresh spinach was obtained from Covent Garden and frozen whole cells of Spirulina platensis were a gift from Mlle. G. Clement, Institut Francais du Petrole, Paris. Intact spinach chloroplasts ("Type A, complete"[20]) were prepared[21] by blending 100g of leaves in 250 ml. grinding medium. Each chloroplast preparation was washed briefly in 50 ml. of resuspending medium. Electrofocusing on a sucrose gradient and on polyacrylamide gels was carried out as previously described[22,23]. EPR spectra were obtained using a Varian E-4 spectrometer. Protein[24], chlorophyll[25] and chlorophyll a:b ratios[26] were measured as described. SOD activity was assayed during purification by the adrenochrome[27] or nitro blue tetrazolium (NBT) methods[28]. The activity of the purified spinach fractions was measured by the method of McCord and Fridovich[2].

Partial purification of superoxide dismutases: (a) Spinach leaf extract: leaves were ground up and filtered through muslin; the dismutases were purified by ammonium sulphate fractionation (40-80%) and DEAE-cellulose (Whatman DE23 + DE52) chromatography with linear NaCl gradients between 0 and 150mM. (b) Chloroplast stroma extract: "Type A" chloroplasts were hypotonically ruptured to release stroma. After centrifugation the clear supernatant was dialyzed and then fractionated by DE52 chromatography with NaCl (c) Spirulina: fresh



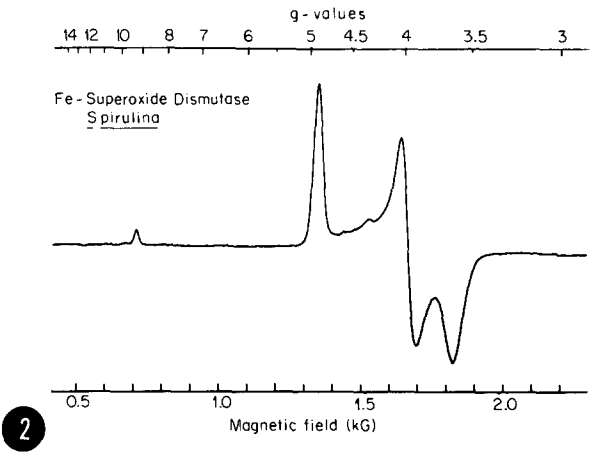


Fig. 1. Polyacrylamide gel isoelectric focusing of soluble superoxide dismutases (a) spinach chloroplast stroma (b) whole spinach leaf (c) Spirulina - major isoenzyme (d) Spirulina - minor isoenzymes.

Fig. 2. EPR spectrum of Fe-containing SOD from Spirulina. The settings for measurement were: microwave frequency 9.275 GH_z; power, 20mW; modulation amplitude, 10G; temperature, 77°K.

cells were disrupted by sonication; after centrifugation the supernatant was fractionated with ammonium sulphate (55-85%) and then chromatographed on DE52 with K phosphate buffer pH 7.8; the SOD containing fraction was further chromatographed on Whatman CM52 with K acetate buffer pH 5.5 to give major and minor fractions. The major dismutase activity was further purified by chromatography on Sephadex G75 with Tris KCl buffer pH 7.8. The Spirulina enzymes were inactive towards the adrenaline assay presumably due to the high pH^{29} and were monitored by the NBT method.

RESULTS AND DISCUSSION: The results obtained by gel isoelectric focusing with the soluble extracts from spinach and Spirulina are shown in Figure 1. The most acidic part of the gel could not be stained but it is unlikely that this affected the interpretation of the results. It is apparent that the spinach whole leaf extract contains two distinct soluble enzymes (both cyanide-sensitive) one of which can be equated with the enzyme from the chloroplast stroma. The patterns obtained were unaltered if an acetone precipitation was included in the pretreatment of the extracts. It is not possible from this study to say whether the "stromal" enzyme is confined to the stroma or whether

it also occurs in the cytoplasm. Spirulina contains one major and two distinct minor soluble SOD activities (all cyanide-insensitive) but the latter two are small quantitatively so their significance is unclear as yet.

Sucrose gradient isoelectric focusing was carried out on the partially purified enzymes from the chloroplast stroma and on the major Spirulina enzyme. The peaks of activity occurred at pH 4.60 and 4.35 respectively. These highly purified preparations were dialysed and concentrated and their EPR spectra recorded. The chloroplast stroma enzyme gave a signal characteristic of Cu^{++} similar to the various other copper-zinc superoxide dismutases which have been studied[1]. The EPR spectrum of the Spirulina enzyme (Figure 2) was that of high spin Fe^{3+} with nearly rhombic symmetry, similar though not identical to that of the ferrisuperoxide dismutase found in the periplasmic space of E.coli[8,11].

The high specific activity of SOD in the chloroplast stroma (6 units/mg. protein) suggests that the chloroplast is the principal site of superoxide production in the cell and/or that chloroplasts are more sensitive to superoxide. This is presumably due to the ferredoxin-catalysed Mehler reaction which appears to occur to a significant extent even in the presence of $NADP^+$ (Allen and Hall, unpublished).

The difference in metal content between the chloroplast and blue-green algal dismutases need not be used as an argument against the symbiotic theory of the origin of chloroplasts. The mitochondrial manganoenzyme is coded for in the cell nucleus [10] and it is only a short step from such a situation to one in which the original ferrisuperoxide dismutase of the prokaryotic symbiont is deleted and replaced by a nuclear coded host type copper-zinc SOD.

Chloroplast lamellar SOD

(i) Solubilisation and partial purification: Chloroplasts were incubated in KCl (3%w/v) and sodium cholate (5%w/v). Sodium dodecyl sulphate (1%w/v) was added to the supernatant after centrifugation. Ammonium sulphate fractionation (60-100%) was then performed. The solubilized lamellar SOD preparation was cyanide-insensitive. The solubilized preparation could be stored in liquid nitrogen without noticeable loss of activity. (ii) Effect of solubilized SOD on the

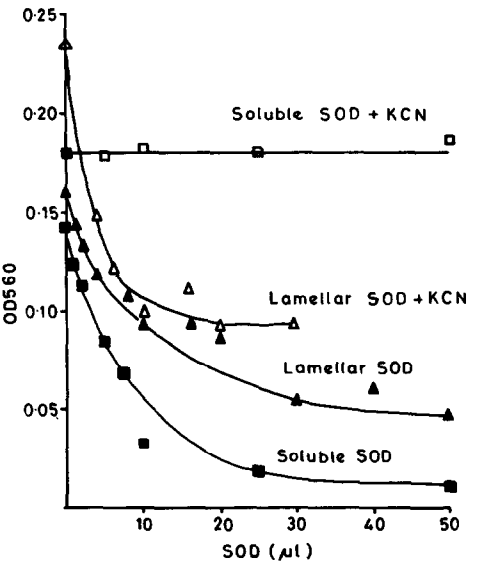


Fig. 3. Effects of soluble and lamellar spinach SOD on NBT assay system (aqueous phase).

NBT assay: The assay mixture was that of Beauchamp and Fridovich[28], omitting the cyanide. At the end of the period of illumination, 0.2 ml of 10% w/v bovine serum albumin was added to each assay in order to complex the blue formazan. The assay mixture was then shaken with 3.0 ml of ethyl acetate which extracted an unidentified colour produced by another component of the lamellar extract. The lamellar SOD preparation only suppressed 69% of the blue formazan formation compared with 93% by the soluble dismutases (Fig. 3); the possibly distinctive properties of lamellar SOD are being investigated further. (iii) Localisation in subchloroplast fragments: Chloroplast membranes were extracted with Triton X100 as described by Vernon et al[30] except that the Triton concentration was 5% and the time of incubation was 30 min. In order to remove the detergent rapidly to prevent inactivation of the SOD a four phase partition method[31] was applied to the extract after low speed centrifugation. The subchloroplast fragments were resuspended in water and applied to a discontinuous sucrose density gradient. This resulted in three major bands at 45, 50 and 55%^{w}/w sucrose (with average chlorophyll a:b ratios of 4.21 (possibly photosystem I particle) 2.44 and 2.27 (possibly

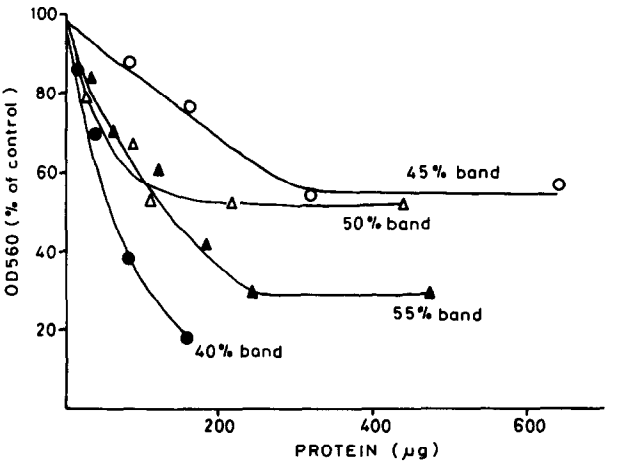


Fig. 4. Effects of spinach subchloroplast particles on NBT assay system.

photosystem II containing particles) respectively) as well as less dense minor bands between 20 and 40%. The amounts of material in any band varied from experiment to experiment. The bands at 45 and 50% suppressed colour formation in the NBT assay by up to a half. The 55% band consistently suppressed colour formation by up to 70% (similar to the solubilised preparation - see below) with a specific activity of 8 units per mg. protein. Two other types of particles appeared to have SOD activity - a small band at 30% (chl.^{a}/b = 2.84) had 13 units/mg.protein and a small band at (40% chl.^{a}/b = 2.08) had 20 units/mg. protein. This latter band was shown to inhibit blue formazan formation by more than 80% (Figure 4).

It seems likely that a complex interrelationship exists between the different particle types and these experiments are far from clear cut; however, the apparent association of the SOD activity with particles of low chlorophyll a:b ratio implies a role for the enzyme in photosystem II reactions. Two possible candidates are (a) the water splitting system and (b) the destruction of superoxide which might be produced by the semiquinone form of plastoquinone[32] or another component at the reducing side of photosystem II.

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