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PURIFICATION AND PHYSICOCHEMICAL PROPERTIES OF SUPEROXIDE DISMUTASE FROM TWO PHOTOSYNTHETIC MICROORGANISMS

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Summary

Superoxide dismutase (EC 1.15.1.1) has been isolated and characterised from the blue-green alga *Spirulina platensis* and from aerobically-grown *Rhodopseudomonas spheroides*, a purple, non-sulphur bacterium. The former enzyme contains 1 gatom of iron and the latter 1 gatom of manganese per mol; both enzymes have a molecular weight of 37 000—38 000, being composed of two non-covalently joined subunits of equal size.

Various spectral studies have been carried out including absorbance, circular dichroism and electron spin resonance. Catalytic activity has been studied as a function of pH and shows a decrease at alkaline pH values. The manganoenzyme is generally more stable to various potentially denaturing conditions and is resistant to inactivation by hydrogen peroxide. Amino acid compositions and N-terminal residue determinations are presented.

Introduction

Superoxide dismutases (EC 1.15.1.1) of the cuprein type, containing copper and zinc and inhibited by cyanide have been isolated from a wide range of eukaryotic organisms [1] and from one species of marine bacterium [2]. In contrast, the enzyme containing either iron or manganese as prosthetic metal ion and insensitive to inhibition by cyanide has been purified from a number of bacteria [3—7]. Manganese-containing superoxide dismutase has also been found in several eukaryotes, namely the fungus *Pleurotus olearius*, [8,9], chicken liver [10] and bakers yeast [11]; in the latter two cases, the enzyme is localised in the mitochondrial matrix. This finding, in conjunction with the

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striking homology existing between the N-terminal amino acid sequences of the chicken liver mitochondrial enzyme and those from *Escherichia coli* [12], prompted Fridovich [13] to a reconsideration of the symbiotic origin of mitochondria, in order to account for the distribution of the different types of superoxide dismutase.

However, certain deficiencies in this hypothesis were indicated by the discovery of cyanide-insensitive superoxide dismutase activity in crude extracts of various photosynthetic organisms including anaerobic bacteria [14,15] and eukaryotic algae [15,16]. In the light of these rather limited data, we have made a number of proposals based on a central role for photosynthetic organisms in the evolution of superoxide dismutases [15] and it is plainly desirable that representative dismutases should be isolated and characterized from this group of organisms in order to test the validity of these hypotheses. We have briefly reported the occurrence of a ferridismutase in *Spirulina* [17] while a similar enzyme from *Plectonema boryanum* has been studied by two other groups [16,18]. In the present paper, we describe physico-chemical studies of purified superoxide dismutases from *Spirulina platensis* and *Rhodopseudomonas spheroides.*

Materials and Methods

Rhodopseudomonas spheroides was obtained from the Microbiological Research Establishment, Porton Down, Wiltshire and *Spirulina platensis* was generously provided by Mlle G. Clement of the Institut Français du Petrole. Microgranular ion-exchangers (DE-52 and CM-52) were obtained from Whatman Biochemicals, Maidstone, Sephadex G-75 (superfine) from Pharmacia (G.B.) Ltd. and Carrier Ampholytes (pH 3—5) from LKB Produktor. Nitro-blue tetrazolium, xanthine oxidase, streptomycin sulphate and phenylmethane sulphonyl fluoride were Sigma products while cytochrome *c* and other molecular weight calibration proteins were obtained from Boehringer-Mannheim, 5,5′-dithio-bis (2-nitrobenzoic acid) was a product of the Aldrich Chemical Co.

Superoxide dismutase activity was monitored during purification, both in solution and on polyacrylamide gels, by the photochemical methods of Beauchamp and Fridovich [19] while the activity of the purified dismutases was studied by competition experiments in the system xanthine oxidase/cytochrome *c* as described [20]. Polyacrylamide gel electrophoresis was by a modification [21] of the method of Davis [22]; gel isoelectric focusing [21] and gel electrophoresis in the presence of sodium dodecyl sulphate [23] were performed as described. 10% acrylamide gels were used in all cases and protein was visualised with Coomassie Brilliant Blue R250. Absorbance spectra at room temperature and at 77°K were obtained with a Pye-Unicam SP800A spectrophotometer and a Shimadzu MPS-50L spectrophotometer with a low temperature accessory, respectively. Circular dichroism (CD) spectra were recorded on a Fica Spectropol 1 spectropolarimeter (Sofica, St. Denis, France) while electron spin resonance (ESR) spectra were studied with a Varian E4 spectrometer using either a liquid nitrogen insert dewar or a flow of cold helium gas to cool the samples. In the latter case, the temperature was monitored with a carbon resistance thermometer placed upstream of the sample. Iron and manganese

analyses were carried out by atomic absorption spectrophotometry using a Pye-Unicam SP1900 calibrated with standard salts of these metals (B.D.H. Ltd.). Protein was estimated both colorimetrically [24] and by a far ultraviolet absorption method [25] as modified by Weisiger and Fridovich [10]. Amino acid analyses were carried out using a Durrum model D 500 amino acid analyzer and N-terminal determination was by the dansyl chloride method [26].

Results

Isolation of the ferridismutase from Spirulina platensis

a. Preparation of soluble extract and ammonium sulphate fractionation. 500 g of frozen cells were suspended in 1 litre of extraction medium (50 mM potassium phosphate pH 7.8, 0.4 M sucrose, 10 mM NaCl) at 4°C and disrupted by passage through a French pressure cell at 20 000 lbs/inch^2. The suspension was made 10 mM in $MgCl_2$ and centrifuged for 2 h at 40 000 × *g*. The supernatant was stored on ice while the pellet was thoroughly resuspended by homogenisation in 200 ml of extraction medium and centrifuged as before. The combined soluble extracts were fractionated by ammonium sulphate; the solid salt was added with vigorous stirring over a 10-min period and stirring was continued for a further 10 min before centrifuging for 30 min at 40 000 × *g*. The addition of 0.208 g/ml (about 35% saturation) caused the precipitation of large quantities of green membrane fragments. An uncontrolled alteration in the concentration of these fragments in an extract would have a significant effect on the real concentration of salt in the supernatant, so that it was thought inadvisable to attempt too close a cut-off point at this first fractionation step. Thus, further additions of (1) 0.062 (35—45%), (2) 0.064 (45—55%), and (3) 0.215 g/ml (55—85% saturation) were made and all three precipitates were resuspended in a minimum volume of 5 mM potassium phosphate (pH 7.8), dialysed overnight against this buffer and clarified by centrifugation. Generally, only fractions (2) and (3) were active; the phycocyanin was removed from fraction (2) by collecting the precipitate which formed between 60 and 85% saturation with ammonium sulphate which was then dialysed and pooled with fraction (3).

b. Stepwise elution from DE-23 and CM-52. The combined red-brown extracts were applied to a column (10 cm × 5 cm diameter) of DE-23 pre-equilibrated with 5 mM potassium phosphate (pH 7.8). A pink fraction (*c*-type cytochrome) passed through unretarded, a blue-green fraction was eluted with 0.2 M phosphate and a brown fraction (ferredoxin) was eluted with 0.8 M NaCl. Only the blue-green fraction was active: again the 60—85% ammonium sulphate precipitate was collected and the resultant yellow-brown extract was resuspended in 5 mM potassium acetate (pH 5.5) and dialysed overnight against an excess of this buffer. Clarified by centrifugation, the extract was applied to a column (10 cm × 1 cm diamter) of CM-52 pre-equilibrated with the same buffer. Essentially all of the activity was in the unretarded fraction which was collected and dialysed against 5 mM potassium phosphate (pH 7.8). (A fraction could be eluted from the CM-52 with 0.2 M acetate which, on gel electrofocusing, gave two activity bands both distinct from the single band of the main fraction [17]. However, as this fraction accounted for only 0.1% of the

total activity, it is probably due to minor contaminants of the culture).

c. Gradient elution of isoenzyme I from DE-52. The isoenzyme I fraction was adsorbed onto a column (10 cm × 2.5 cm diamter) of DE-52 pre-equilibrated with 5 mM potassium phosphate (pH 7.8) and a 1000 ml linear gradient from 80 to 140 mM of this buffer was applied. The activity eluted between 90 and 100 mM phosphate, coincident with a prominent peak of 280 nm absorbance. The visible spectrum of this fraction was now recognisably that of an iron-containing dismutase except for a peak at 407 nm due to minor impurities.

d. Gel filtration on Sephadex G-75. The enzyme was dialysed against 50 mM Tris-HCl (pH 7.8), 0.1 M KCl and concentrated to a final volume of 2 ml by ultrafiltration over a PM-10 membrane (Amicon Corporation). 1 ml of this concentrated preparation was layered onto a bed of Sephadex G-75 (38 × 2.5 cm diameter) equilibrated with the same buffer. The activity eluted as a single symmetrical peak congruent with a similar peak of 280 nm absorbance. The total yield of protein from two such columns was 78 mg.

Isolation of the manganodismutase from aerobically grown Rps. spheroides

a. Preparation of soluble extract and ammonium sulphate fractionation. Initial experiments had shown the activity of crude extracts to be very labile but this problem was largely solved by the inclusion of phenylmethane sulphonyl fluoride (PMSF), a specific serine-protease inhibitor, in the extraction medium and dialysis buffer. 500 g of frozen cells were suspended in 1500 ml of extraction medium (50 mM potassium phosphate pH 7.8, 0.5 mM EDTA, 0.1 mM PMSF) at 4°C. The suspension was transferred to a jacketed Rosett cell with circulating iced water and sonicated using a Dawe soniprobe at full power for two or three periods of 5 min with intermediate cooling periods of 5 min. The cell sonicate was clarified by centrifugation for 30 min at 40 000 × *g* and the precipitate forming between 40 and 90% saturation of the soluble extract with ammonium sulphate was collected. This was resuspended in a minimum volume of dialysis buffer (50 mM potassium phosphate, pH 7.8, 0.1 mM EDTA, 0.01 mM PMSF) and dialysed overnight against an excess of this buffer. Streptomycin sulphate was added to the extract to 2.5% (w/v) and, after incubation for 30 min at 4°C, the copious precipitate was removed by centrifugation and discarded. Ammonium sulphate was added to the supernatant and the fraction precipitating between 60 and 90% saturation was resuspended in a minimum volume of 5 mM potassium phosphate (pH 7.8) and dialysed overnight against this buffer.

b. Stepwise elution from DE-52 and CM-52. The red-brown extract was adsorbed onto a column of DE-52 (20 cm × 4 cm diameter) pre-equilibrated with the same buffer. 50 mM phosphate eluted most of the activity (isoenzyme I and isoenzyme III) but subsequent washing with 100 mM phosphate eluted a minor active fraction (isoenzyme II). The major fraction was dialysed against 5 mM potassium acetate pH 5.5 clarified by centrifugation and applied to a column of CM-52 (10 cm × 1 cm diameter) pre-equilibrated with the same buffer. The brown-coloured material which passed through the column unretarded contained most of the activity (isoenzyme I) and it was collected and dialysed against 5 mM potassium phosphate (pH 7.8). 40 mM potassium acetate eluted another minor active fraction from the CM-52 column (isoenzyme III). As iso-

enzyme I accounted for 91% of the total activity at this stage (isoenzyme II, 3.5%; isoenzyme III, 5.5%), further attempts at purification were concentrated solely on this isoenzyme.

c. Gradient elution of isoenzyme I from DE 52. The isoenzyme I fraction was adsorbed onto a column (10 cm × 2.5 cm diameter) of DE 52 pre-equilibrated with 5 mM potassium phosphate (pH 7.8) and a 1000 ml linear gradient from 5 to 50 mM of this buffer was then applied. Three distinct coloured bands were resolved on the column, a leading red-purple band followed by brown and yellow bands. The activity eluted as a single peak in the same region as the red-purple band.

d. Gel filtration on Sephadex G-75. This was carried out as for the *Spirulina* enzyme, the yield of protein was 49.3 mg.

e. Superoxide dismutase isoenzymes in Rps. spheroides. Gel isoelectric focusing of a crude extract of aerobically-grown *Rps. spheroides* gave 3 distinct bands of activity which could be correlated with the fractions separable by ion exchange chromatography (Fig. 1). Notably, an extract prepared from anaerobically (photosynthetically) grown cells resulted in a different pattern of isoenzymes. It remains to be determined whether each minor isoenzyme has a specific function to perform in vivo.

Polyacrylamide gel electrophoresis

When analysed by gel electrophoresis, the *Spirulina* enzyme gave only a single protein band corresponding to the band of enzymatic activity; the *Rhodopseudomonas* enzyme had one inactive contaminant band accounting for about 5% of the total protein.

Molecular weight

The column of Sephadex G-75 used in the final purification step was calibrated with a mixture of proteins of known molecular weight: cytochrome *c* (13 500), chymotrypsinogen A (25 700) and ovalbumin (43 000). A plot of log (molecular weight) against elution volume indicated a molecular weight of 37 400 for both superoxide dismutases.

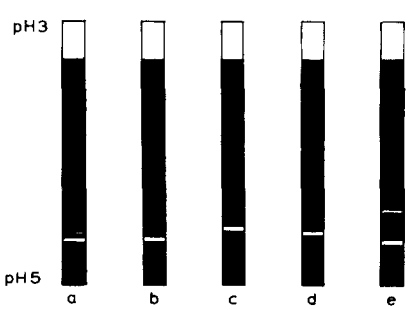

Fig. 1. Polyacrylamide gel isoelectric focusing of *Rps. spheroides* isozymes. (a) crude extract of aerobically-grown cells; (b) isoenzyme I; (c) isoenzyme II; (d) isoenzyme III; (e) crude extract of anaerobically-grown cells.

Subunit composition

The purified dismutases were subjected to gel electrophoresis in the presence of sodium dodecyl sulphate, with and without 2-mercaptoethanol. Both enzymes gave a single band in either case and a comparison of the mobilities with those of molecular weight standards (lysozyme, 14 300; chymotrypsinogen A 25 700; ovalbumin 43 000) gave the following values of subunit molecular weight: (a) without mercaptoethanol: *Spirulina* 18 100; *Rhodopseudomonas* 18 300; (b) with mercaptoethanol: *Spirulina* 18 400; *Rhodopseudomonas* 18 400. Thus, these enzymes conform to the general pattern of two, non-covalently joined subunits of equal size.

Metal analyses

Duplicate samples of each protein were analysed for iron and manganese content by atomic absorption spectroscopy. The values obtained were: 1.00 and 0.97 gatom Fe per mol of the *Spirulina* enzyme and 1.08 and 1.12 gatom Mn per mole for the *Rhodopseudomonas* enzyme. Protein was quantitated using the $A_{280}^{1\%}$ values as described below.

Absorbance spectra

The absorbance spectra in the ultraviolet region were fairly typical of proteins with a peak at 282 nm and a shoulder at about 290 nm. Absolute protein concentration was determined in two independent ways: (a) a far-ultraviolet absorption method [25,10] gave values for $A_{280}^{1\%}$ of 14.1 for the *Spirulina* and 16.3 for the *Rhodopseudomonas* enzyme: (b) a colorimetric method [24] gave corresponding values of 14.0 and 16.0. The spectrum of each enzyme in the visible region was similar to those previously reported for iron and manganese-containing dismutases. The values for the molar extinction coefficients were 2180 $M^{-1} \cdot cm^{-1}$ at 350 nm for the *Spirulina* enzyme and 542 $M^{-1} \cdot cm^{-1}$ at 475 nm for the *Rhodopseudomonas* enzyme. This latter enzyme did not exhibit the shoulder at about 600 nm which is commonly found in the spectra of manganodismutases, however, the spectrum at 77°K was resolved into three peaks, at about 420, 480 and 550 nm (Fig. 2). The spectrum of the *Spirulina* enzyme was unchanged in form at the lower temperature.

Circular dichroism

The CD spectra of the native enzymes in the visible region are shown in Figs. 3 and 4. The *Spirulina* enzyme exhibits a single negative CD band centred at 395 nm but the *Rhodopseudomonas* enzyme shows four negative CD bands between 300 and 600 nm, two of which correspond quite well with peaks in the low-temperature visible spectrum. The CD spectrum is slightly different from that of the *E. coli* manganodismutase [27], however, the positions of the bands at 542 nm and 435 nm, if not their relative intensities, are roughly the same in both enzymes. Keele et al. [27] attribute these bands to transitions of the *d*-electrons of a high-spin Mn(III) ion.

Electron spin resonance

As previously reported [17], the ESR spectrum of the *Spirulina* dismutase at 77°K is that of high spin Fe^{3+} of rhombic symmetry. Spectra recorded under

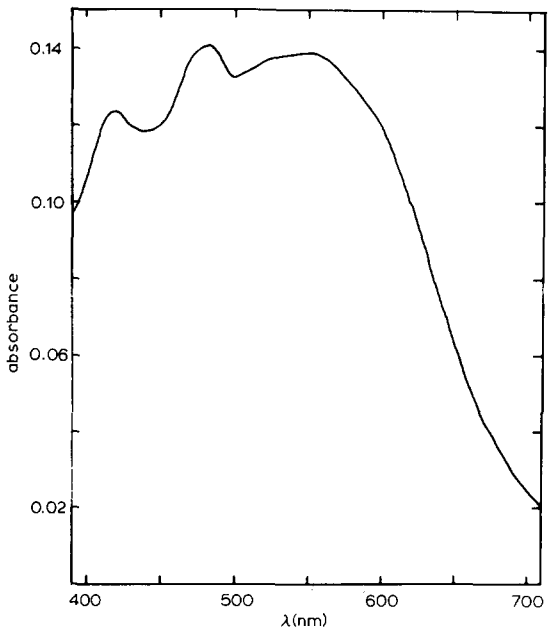

Fig. 2. Absorption spectrum of *Rps. spheroides* superoxide dismutase in the visible region at 77°K. 0.4 mM enzyme in 50 mM Tris-HCl (pH 7.8), 0.1 M KCl; path length 2 mm.

liquid helium conditions are now presented in Fig. 5. The three resonances around $g = 4.3$ can be ascribed to transitions in the middle Kramers doublet ($g_z = 4.88$; $g_x = 3.95$; $g_y = 3.60$). At 77°K a weak resonance at $g = 9.2$ is observed; in the spectra recorded at lower temperature, a second resonance has appeared in this region at $g = 9.9$, the intensity of which increases as the temperature decreases. The two possible resonances at this low field position are g_y of the lowest doublet and g_z of the highest doublet. The latter would nor-

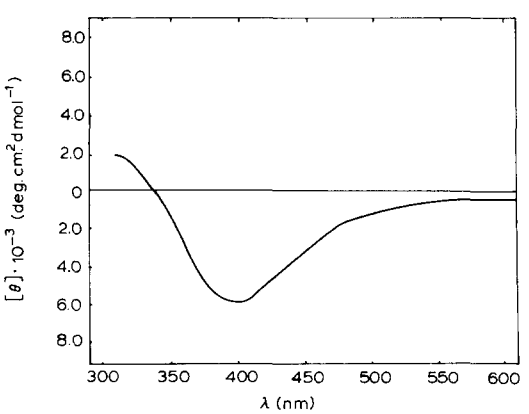

Fig. 3. Circular dichroism spectrum of *Spirulina platensis* superoxide dismutase.

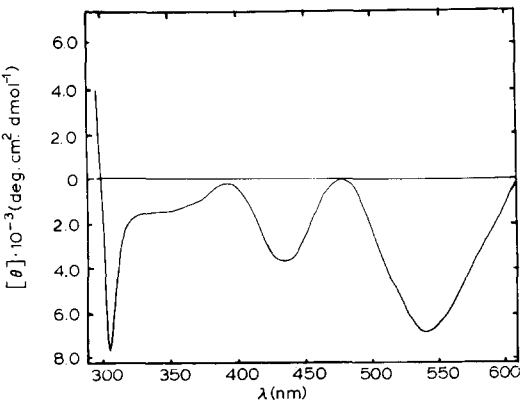

Fig. 4. Circular dichroism spectrum of *Rps. spheroides* superoxide dismutase.

mally be expected to have the higher g-value [28]. Nevertheless, in this case the temperature behaviour of the $g = 9.9$ signal is more like that expected of the lowest doublet. Experiments at even lower temperatures (around 1°K) may settle this point. With the exception of this last feature, and the absence of any signal at $g = 1.98$, the spectra are generally similar to those reported for the iron-containing dismutases of *Plectonema boryanum* [16] and *Photobacterium leiognathi* [7]. The *E. coli* enzyme, by comparison, appears to have a more nearly rhombic iron environment [4,28]. No ESR signals were observed for the *Rhodopseudomonas* enzyme at 77°K and 20°K.

Rate constants from competition experiments

Values for the second order rate constants for superoxide dismutation were

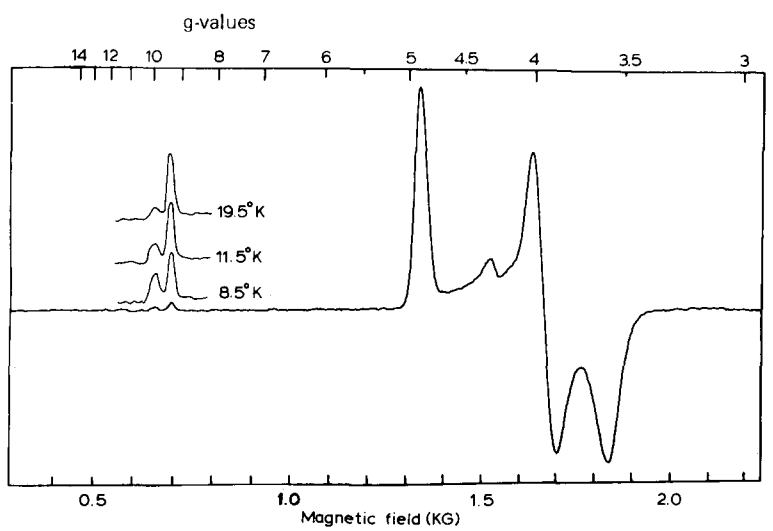

Fig. 5. ESR spectrum of *Spirulina platensis* superoxide dismutase at 8.5°K. 0.25 mM enzyme in 50 mM Tris-HCl (pH 7.8), 0.1 M KCl with the following instrument settings: time constant 0.1 s; modulation amplitude 10 G; power 20 mW; microwave frequency 9.195 GHz. The expanded spectra in the g 9—10 region are at a five-times greater gain.

TABLE I

THE CATALYTIC ACTIVITY OF THE SUPEROXIDE DISMUTASES AS A FUNCTION OF pH

Enzyme	pH 6.0		pH 7.8		pH 8.5		pH 10.2	
	1 unit (nM)	$k \times 10^{-9}$ ($M^{-1} \cdot s^{-1}$)	1 unit (nM)	$k \times 10^{-9}$ ($M^{-1} \cdot s^{-1}$)	1 unit (nM)	$k \times 10^{-9}$ ($M^{-1} \cdot s^{-1}$)	1 unit (nM)	$k \times 10^{-9}$ ($M^{-1} \cdot s^{-1}$)
Spirulina	7.57	1.29	3.70	1.60	3.98	0.28	15.93	0.009
Rhodopseudomonas	7.58	1.29	3.43	1.72	2.40	0.46	4.79	0.031

obtained by measuring the amount of purified dismutase required to compete equally with cytochrome *c* for superoxide generated by xanthine oxidase (Table I). The specific activities at pH 7.8 are 2410 units per mg for the *Spirulina* and 2600 units per mg for the *Rhodopseudomonas* enzyme. These experiments were based on a similar study of the iron- and manganes-containing dismutases from *E. coli* [20]. The only significant differences in the results are: (i) the *E. coli* enzymes have higher rate constants at pH 6.0 than at pH 7.8 and (ii) the rate constant of the *Spirulina* enzyme at pH 10.2 is about one third of that of the *E. coli* and *Rhodopseudomonas* enzymes. The pH optima reported for iron-containing dismutases from *Photobacterium* spp. [7] are around pH 9, but these workers used a different assay system.

Stability of the superoxide dismutase activity

In this series of experiments, superoxide dismutase activity was measured by the photochemical assay system.

a. As a function of pH. Each dismutase, at a concentration of 250 units per ml, was incubated for 30 min at room temperature in buffers in the pH range 3.0 to 11.9. The activity was then measured normally and expressed as a percentage of the pH 7.0 sample. Both dismutases were rapidly inactivated at pH values above 10.8 but, while the *Rhodopseudomonas* enzyme retained 70% of its activity after 30 min at pH 3.0, the *Spirulina* enzyme was very unstable below pH 5.0.

b. As a function of temperature. The thermal stabilities (Figs. 6a and b) can be compared with the findings of Puget and Michelson [7], both enzymes are more stable than the ferridismutases of *Photobacterium* but less stable than the manganodismutase of *Bacillus stearothermophilus.* Plots of log (activity) against time at 70°C gave straight lines and half-lives of 12.75 min and 6.5 min were calculated for the *Rhodopseudomonas* and *Spirulina* enzymes, respectively.

c. Towards dimethyl sulphoxide. The dismutases, at concentrations of 250 units per ml in 5 mM Tris-HCl (pH 7.8), 10 mM KCl were incubated for 1 h on ice in various concentrations of dimethyl sulphoxide, then assayed normally. The *Spirulina* enzyme was stable in up to 55% and the *Rhodopseudomonas* enzyme in up to 70% (v/v). This implies a higher degree of internal stabilisation in the latter enzyme since dimethyl sulphoxide is considered to be effective in disrupting hydrophobic forces.

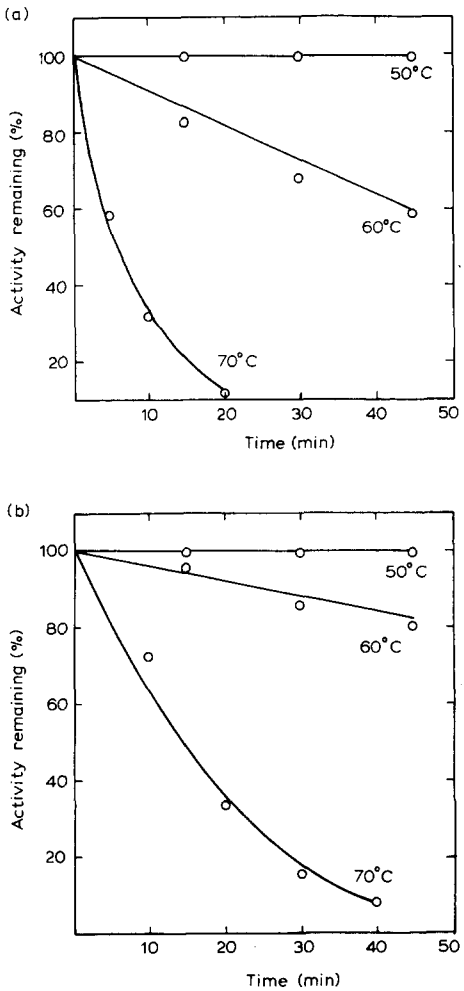

Fig. 6. Stability of superoxide dismutases as a function of temperature: (a) *Spirulina platensis*; (b) *Rps. spheroides*. 450 units per ml in 50 mM Tris-HCl (pH 7.8), 0.1 M KCl.

d. Towards hydrogen peroxide. The *Rhodopseudomonas* enzyme was unaffected by incubation with 5 mM hydrogen peroxide over a period of 30 min but the *Spirulina* enzyme was rapidly inactivated. The second order rate constant for inactivation was 0.88 $M^{-1} \cdot s^{-1}$ compared with 3.1 $M^{-1} \cdot s^{-1}$ for bovine erythrocuprein [29]. Asada et al. [16] similarly found that the iron- but not the manganese-containing dismutase from *Plectonema boryanum* was inactivated by peroxide. This property may not be a universal attribute of manganodismutases, since Keele et al. [27] have reported loss of manganese from the *E. coli* enzyme on exposure to peroxide: these differences may relate to the nature of the metal binding sites since peroxide inactivation involves the destruction of a specific histidine residue in erythrocuprein [29].

The *Rhodopseudomonas* enzyme was not inhibited by the presence of 30 mM hydrogen peroxide in the assay.

Amino acid analyses

The amino acid compositions of the two dismutases are presented in Table II. The proteins were hydrolysed in vacuo in 6 M HCl at 110°C for 24 h (there is thus some uncertainty over the exact values for Ser, Thr, Val, Leu and Ile). Cysteine was determined as cysteic acid after oxidation with performic acid [30] and tryptophan was estimated from the ultraviolet absorption spectrum in 6 M guanidine hydrochloride [31]. The compositions of the two enzymes are largely similar to one another and to other prokaryotic dismutases, perhaps the most notable feature being the probable absence of arginine in the *Spirulina* enzyme.

In view of the discrepancy between the data of Misra and Keele [18] and of Asada et al. [16] on the cysteine content of the *Plectonema ferridismutase*, it was decided to check the cysteine content of the dismutases using Ellman's reagent [32]. Neither enzyme reacted in the native state, suggesting that there are no exposed sulphydryl groups, but, even in the presence of 2% sodium dodecyl sulphate, there was only a very slow rate of reaction, unless the proteins had also been heated for 15 min at 90°C under a nitrogen atmosphere. After this treatment, reaction with Ellman's reagent was essentially instantaneous and values of 1.29 and 2.01 mol cysteine per mol subunit were obtained for the *Spirulina* and *Rhodopseudomonas* enzymes, respectively.

N-terminal residue analysis gave only N-terminal alanine for both enzymes;

TABLE II

AMINO ACID COMPOSITIONS

Amino acid	*Spirulina platensis*		*Rhodopseudomonas spheroides*	
	Moles per mole of subunit	Residues per subunit	Moles per mole of subunit	Residues per subunit
Lysine	10.08	10	14.65	15
Histidine	6.07	6	7.60	8
Arginine	0.2	0	2.59	3
Aspartic acid	26.26	26	19.26	19
Threonine	9.85	10	7.96	8
Serine	11.88	12	10.98	11
Glutamic acid	16.91	17	22.29	22
Proline	7.94	8	6.61	7
Glycine	12.78	13	18.46	19
Alanine	25.32	25	18.03	18
Valine	8.76	9	11.54	12
Methionine	2.94	3	4.08	4
Isoleucine	6.77	7	4.47	5
Leucine	15.79	16	14.51	15
Tyrosine	4.59	5	6.69	7
Phenylalanine	14.04	14	10.27	10
Cysteic acid	1.13	1	2.11	2
Tryptophan	3.23	3	3.86	4
Total residues		185		189

this compares with the serine of the *E. coli* enzymes [12], the lysine of the mitochondrial enzyme [12] and the proline of *Bacillus stearothermophilus* [6].

Discussion

Two points of interest to emerge from this study are: (i) the use of circular dichroism and liquid nitrogen temperature absorption spectroscopy to reveal transitions which are not detectable in room temperature visible spectra of the manganodismutases, further knowledge of the valence state and binding site of this rather intractable metal ion must await the application of more sophisticated techniques such as electron spectroscopy; (ii) the manganodismutase is consistently more stable than the ferridismutase to low pH, high temperature, dimethyl sulphoxide or hydrogen peroxide. The notable stability of the former enzyme may be caused by an interprotomerial active site, with the metal ion securely "locked-in" between the subunits and released only with their dissociation. This would also explain the commonly obtained stoichiometry of 1 gatom Mn per mol and warrants further investigation. In the case of the ferridismutases, reported iron contents vary from one, through intermediate values, to two Fe per molecule, suggesting that one or two binding sites may be occupied, dependent perhaps on conditions of cell growth or enzyme isolation.

It is probable that atmospheric oxygen was initially derived from oxygenic photosynthesis by blue-green algal-like cells. Some of the oxygen so produced would inescapably become reduced by electron transport components of low redox potential, as has been found in studies of green-plant chloroplasts [33] and bacterial reaction centres [34]. Indeed, Patterson and Myers [35] have recorded in vivo production of hydrogen peroxide by blue-green algae while Abeliovich et al. [36] have noted that the amount of superoxide dismutase in the blue-green alga *Anacystis nidulans* was proportional to the concentration of oxygen in the growth medium.

These findings suggest that the primitive blue-green algae would have been the first organisms to require a superoxide dismutase. This leads to the question of whether all present-day aerobic bacteria had photosynthetic ancestors, whether this is true only in some cases or whether chemoheterotrophs evolved entirely independently of the facultatively aerobic phototrophs [37] (e.g. from primitive nitrate reducing bacteria [38]). If it is assumed that the independent evolution of respiration would be accompanied by the independent evolution of superoxide dismutase, then a knowledge of the amino acid sequences of these enzymes might provide some answers to this question. It has already been mentioned that both of the enzymes discussed in this work have N-terminal alanine and a study of the N-terminal sequences (cf. ref. 6) is now in progress.

Acknowledgements

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