Cyanide Insensitive Iron Superoxide Dismutase in *Euglena gracilis* Comparison of the Reliabilities of Different Test Systems for Superoxide Dismutases

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Z. Naturforsch. **34 c**, 374–380 (1979); received February 9, 1979

Fe^- Superoxide Dismutase, Superoxide Dismutase Test Systems, *Euglena gracilis*, Pulse Radiolysis

Two proteins (P_1 and P_2, with mol weights of 57,500 and 27,500, respectively) were isolated from *Euglena gracilis*. Both proteins show cyanide-insensitive superoxide dismutase activity in the "classical" superoxide dismutase assay, using xanthine-xanthine oxidase as $O_2^{\cdot -}$ generator. If $O_2^{\cdot -}$ is generated chemically (autoxidation of reduced anthraquinone), photochemically (illuminated riboflavine) or pulse radiolytically, only protein P_1 but not P_2 shows SOD activity. Protein P_1 contains 1 g atom (determined: 0.82) iron (no Mn or Cu) per mole protein and may thus be defined as iron-superoxide dismutase. Protein P_2, showing the spectral properties of a flavoprotein, exhibits the activities of ferredoxin-NADP-oxidoreductase and "diaphorase". The cyanide-insensitive SOD-activity of this "diaphorase" in the xanthine oxidase-assay for superoxide dismutase makes this classical and commonly used test unreliable for assaying cyanide insensitive SOD activities. The existence of the "prokaryote-type" of superoxide dismutase (Fe-SOD) in *Euglena gracilis* is exceptional for an eukaryotic, autotrophically grown organisms.

Introduction

The commonly applied methods for assaying superoxide dismutase (SOD) are based on the reactions of superoxide generation and photometric or polarographic measurements of differences after oxidation or reduction of certain compounds by superoxide ions. SOD competes for $O_2^{\cdot -}$ and thus changes the measurable effects. Only the not commonly available pulse radiolysis, where O_2^- is produced by radiation, allows a direct assay for SOD by comparing the differences of the decay kinetics of $O_2^{\cdot -}$ in the presence and absence of SOD (for reviews see [1] and [2]).

In most of the reports on SOD-activities in living tissues or microorganisms published since 1969, xanthine oxidase (with xanthine or hypoxanthine as substrates) served as $O_2^{\cdot -}$ generator, using different (*e. g.* cytochrome c, ref. [3]; nitrotetrazolium blue, ref. [4]; adrenaline, ref. [5] or hydroxylamine, ref. [6]) detector compounds. Besides cyanide sensitive (copper-zinc) and cyanide insensitive (mangano- and iron) enzymes, SOD activities have been observed with metallocomplexes [7–9] and enzymes different to SOD, as galactose oxidase [10] and cytochrome oxidase [11].

The present communication describes a possible pitfall of SOD determination using the classical [3] xanthine oxidase method: two proteins from Euglena gracilis with cyanide insensitive SOD activities (as determined by the xanthine oxidase method) exhibit weak or no SOD activities using pulse radiolysis or chemical or photochemical $O_2^{\cdot -}$ generation. The two proteins were characterized as (for eukaryotic algae unusual) iron SOD, which is more or less active in all applied test systems, and ferredoxin-NADP-oxidoreductase which only exhibits activities in the classical xanthine oxidase tests. Thus, certain reports on cyanide insensitive SOD using the xanthine oxidase method may need reevaluation.

Materials and Methods

SOD-active proteins from *E. gracilis* (cultured as described in ref. [12] or from 400 l cultures, generously donated by Prof. Soeder, Dortmund) were prepared as follows:

Frozen cells were thawed and treated with acetone (70% v/v). After centrifugation, the precipitate was

Abbreviations: SOD, superoxide dismutase (E.C. 1.15.1.1.); AQ, anthraquinone sulfonic acid.

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dissolved in 0.05 M Tris-HCl pH 8.0, dialyzed and stirred for 2 h with Triton X-100 (0.1% v/v). After a second acetone precipitation (80% v/v) and centrifugation, the pellet was dissolved in 0.05 M Tris-HCl pH 8.0, dialyzed and centrifugated at 20 000 $\times g$ for 30 min. The clear supernatant was precipitated with ammonium sufate and the fraction from 40 – 70% saturation was dialyzed and chromatographed on DE_{52} (Whatman, London) cellulose, pretreated with 0.05 M Tris-HCl pH 8.0. The column (10 × 2) was stepwise eluted with 0.05 M NaCl and 0.2 M NaCl in 0.05 M Tris-HCl pH 8.0. The 0.2 M NaCl-eluate showed SOD activity and was further purified by gel chromatography as outlined under "Results". Each purification step was assayed for SOD according to ref. [6].

Ferredoxin and ferredoxin-NADP-reductase were prepared from spinach [13], copper-zinc SOD from green peas [14, 15]; oxygen concentrations were monitored polarographically with a "Hansatech" oxygen electrode (Bachofer, Reutlingen). The following SOD assays were used:

Dianisidine photooxidation by illuminated riboflavin [16], cytochrome c reduction [3] or hydroxylamine oxidation [6] by xanthine-xanthine oxidase and hydroxylamine oxidation by autoxidation of reduced anthraquinone-sulfonic acid [17, 18]. Diaphorase activity with dichlorophenol-indophenol as electron acceptor was assayed according to ref. [19].

Molecular weights were determined by gel chromatography using cytochrome c (MW 11 700), chymotrypsinogen A (MW 25 000), albumine (MW 45 000), albumine (MW 68 000) and ferritin (MW 450 000) as standard proteins (Combithek, Boehringer).

The measurement of the absorption spectra were carried out on Zeiss PMQ III and DMR 10 spectrophotometers. Metal analysis was done with Perkin Elmer 403 atomic absorption spectrophotometer equipped with a HGA 76 graphit cuvette.

All chemicals used were of analytical grade. Sodium formate, hydroxylammoniumchloride, α-napthylamine, sulfanilic acid, and potassium cyanide were purchased from Merck (Darmstadt). Xanthine and xanthine oxidase were from Boehringer (Mannheim) and superoxide dismutase and diaphorase from Sigma (Munich).

For the pulse radiolysis experiments a 3 MeV Van de Graaf accelerator was used. The optical detection system was composed of a Osram XBO 450 W xenon lamp, a Zeiss MM 12 double monochromator, an EMI 9558 QB photomultiplier unit and a Tectronix 556 oscilloscope. Oscilloscope traces were photographed with a Polaroid camera. The rate constants were calculated with a Wang 2200 computer.

The orders of the reactions were determined by regression analysis. The reactions were monitored by absorption measurements at 250 nm. All rate constants represent the constants of second order reactions. In the cases of the spontaneous dismutation evaluation of the decay kinetics of superoxide radicals resulted in second order type reactions directly. When substrates reacted with O_2^-, the reactions showed pseudo first order type kinetics, from which with regard to the concentrations of substrate, the second order rate constants were determined.

The accelerator delivered electron pulses of 100 nsec duration at a dose of 1.85 krad/pulse. For the pulse radiolysis experiments the solutions were prepared with triply distilled and pyrolized water. The pH was adjusted with NaOH and no buffers were used. The solutions contained 0.1 M sodium formate and were saturated with oxygen in order to convert all primary water radicals into superoxide radicals.

Results

1) Preparation and characterization of proteins

Autotrophically grown *Euglena gracilis* (strain Z) cells contain cyanide-insensitive SOD-activity: after centrifugation of thawed *E. gracilis* cell-material (deep frozen and rethawed three times) CN^--insensitive SOD activity was found both in the supernatant and in the green precipitate (Fig. 1). Therefore, a combined detergent-organic solvent-treatment (see methods) was used to solubilize SOD active proteins. After partial purification (see methods) the SOD active protein fraction eluted with 0.2 M Cl^- from the DE_{52} column was further purified by gel chromatography on Sephadex G 75 (Pharmacia, Freiburg) in 0.065 M phosphate buffer pH 7.8.

Three major protein fractions were eluted from Sephadex G 75 according to the following molecular weights. This was confirmed by rechromatography of the isolated and concentrated (PM 10 and XM 50 filters of Amicon, Witten) proteins:

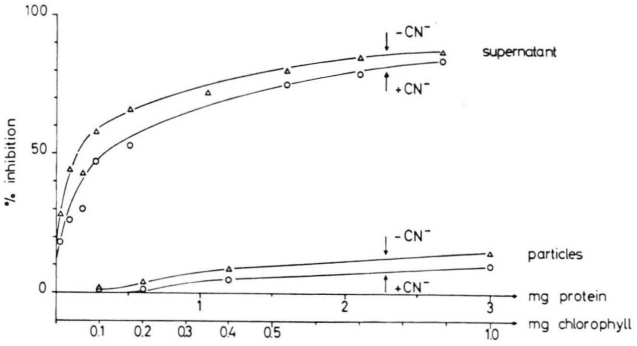

Fig. 1. Inhibition of hydroxylamine oxidation by cell free preparations from *Euglena gracilis*. Inhibition of nitrite formation from hydroxylamine was analyzed as described [6]. A 50% inhibition of nitrite formation is defined as one SOD unit [3, 6]. 10^{-3} M KCN was present in the assay system, as indicated.

peak 1 (P_1): MW = 57 500,
peak 2 (P_2): MW = 27 500 and
peak 3 (P_3): MW = 18 300.

With the hydroxylamine test for SOD [6] we found that protein 3 (P_3) had no SOD activity at all. Proteins No. 1 and No. 2 (P_1 and P_2) showed significant SOD activities, proportional to the protein content (absorption at 280 nm) in the assay system.

The optical absorption spectra of protein 1 and protein 2 are presented in Fig. 2. Protein 1 has a peak at 275 nm with a molar absorptivity of 61 087 $M^{-1}\,cm^{-1}$ and a shoulder at 287 nm. Towards the visible region the absorption is continuously decreasing. Protein 2 has absorption peaks at 275 and 380 nm, a prominent shoulder at 435 nm and a light shoulder at 480 nm. The molar extinction coefficient at 380 nm is 6718 $M^{-1}\,cm^{-1}$. Atomic absorption analysis of Cu, Mn and Fe content of the two proteins indicated the presence of 0.82 atoms Fe in protein 1 and 0.15 atoms Fe for protein 2. The content of Cu and Mn was negligible in both proteins. Due to the ratio of 2:1 of the molecular weights of protein 1 and protein 2 it might be taken into consideration that protein 2 could be the subunit of protein 1. This is very unlikly, however, due to the different optical absorption spectra and due to the discrepancy in iron contents.

2) Biochemical SOD-tests

If one unit of SOD is defined as 50% inhibition of nitrite formation from hydroxylamine [6], both proteins P_1 and P_2 exhibit reasonable CN^--insensitive SOD activities (Fig. 3). These activities are also found if cytochrome c reduction (the classical SOD-test, introduced by McCord and Fridovich, ref. [3]) instead of hydroxylamine oxidation is monitored in the presence of xanthine-xanthine oxidase as $O_2^{\cdot -}$ generator (Fig. 4), although the latter test (cytochrome c reduction) seems to be less sensitive as compared to nitrite formation from hydroxylamine.

If xanthine/xanthine oxidase reaction as the superoxide generating system is replaced by either anthraquinone autoxidation [17, 18] or by illuminat-

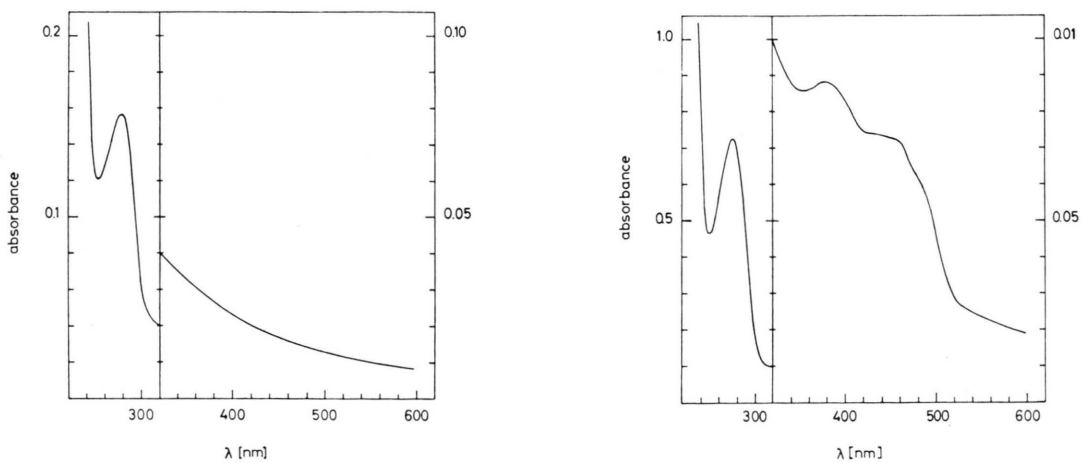

Fig. 2. Absorption spectra of the "SOD-active" proteins P_1 (left) and P_2. The concentration of P_1 is 7.92 mg in 10 ml buffer, the concentration of P_2 is 5.37 mg protein in 10 ml buffer. For experimental details see Materials and Methods.

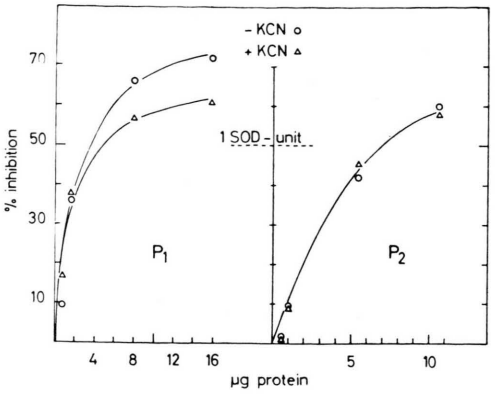

Fig. 3. Cyanide-insensitive SOD activities of the proteins P_1 and P_2, as measured with the hydroxylamine method. The reaction conditions were essentially as described in ref. [6] and in Fig. 1. 50% inhibition of nitrite formation from hydroxylamine are defined as 1 SOD unit.

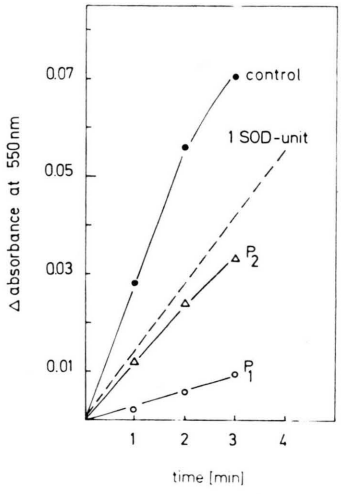

Fig. 4. Inhibition of cytochrome *c* reduction by proteins P_1 and P_2. The reaction conditions were essentially as described by McCord and Fridovich [3]. 40 µg P_1 and 27 µg P_2 were present in the test-cuvettes as indicated. One SOD unit is defined as the decrease of the rate of cytochrome *c* reduction by 50% [3].

ed riboflavine, only the protein P_1 with a molecular weight of 57 500 shows significant SOD activities while the protein with a MW of 27 500 (P_2) is inactive (Fig. 5 and 6).

3) Biochemical characterization of the proteins P_1 and P_2

The protein P_1 exhibits SOD activities in both xanthine oxidase-dependent and -independent test systems and could therefore biochemically be characterized as a cyanide insensitive iron superoxide dismutase (Fe – SOD) with 1 g atom Fe per mol apoprotein (assuming a MW of 57 500). The presence of an iron-containing SOD in *Euglena gracilis*

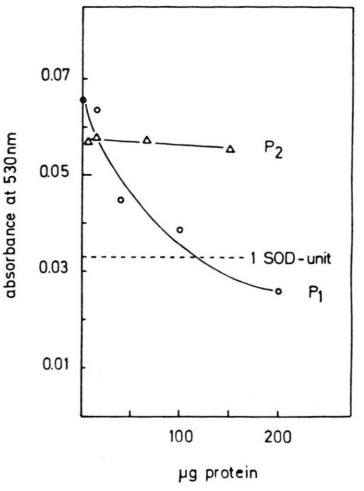

Fig. 5. Influence of the proteins P_1 and P_2 on hydroxylamine oxidation during autoxidation of reduced anthraquinone sulfonic acid (AQ). The reaction mixture contained in 3 ml: (see also ref. [18]) Air-saturated Tris-HCl buffer pH 7.8 (160 µmol), NADP (5 µmol), glucose-6-phosphate (10 µmol), glucose-6-phosphate dehydrogenase (20 µg, Boehringer) AQ (0.2 µmol) KCN (1 µmol), NH_2OH (1 µmol), saturating amounts of ferredoxin-NADP-reductase [13, 18] and the indicated amounts of P_1 or P_2. The reaction was conducted for 20 min at 20 °C; 0.2 ml of the reaction mixture were analyzed for nitrite (with sulfanilic acid + α-naphthylamine).

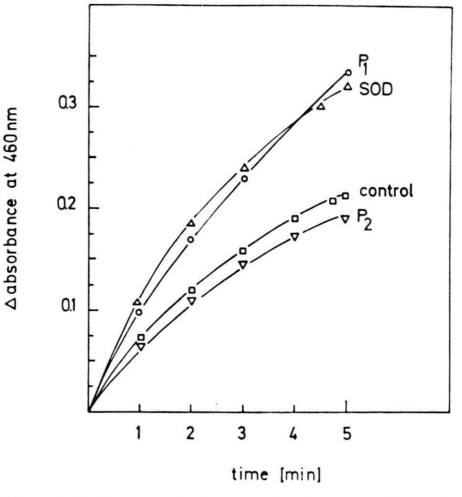

Fig. 6. Influence of the proteins P_1 and P_2 on photooxidation of dianisidine. The test system contained in 3 ml: 2×10^{-4} M dianisidine (Sigma), 1.3×10^{-5} M riboflavine, 0.01 M phosphate buffer pH 7.5, 40 µg P_1 or 27 µg P_2 or 85 units SOD [14, 15]. The reaction mixture was illuminated with 100 W photolamp (*cf.* ref. [16]).

was briefly mentioned very recently by Asada and Kanematsu [22] as unpublished results of the above authors. The protein P_2 exhibits SOD activity only in test systems depending on xanthine oxidase as $O_2^{\cdot -}$ generator. Neither the xanthine oxidase reaction itself (oxygen uptake in the presence of the substrat xanthine) nor the coupled test reactions for $O_2^{\cdot -}$ (*e. g.* hydroxylamine oxidation) are influenced in the presence of the protein P_2. Since the protein P_2 exhibits spectral characteristics (Fig. 2) resembling flavoproteins [20] we investigated P_2 for diaphorase activities [19]. As shown in Fig. 7, in contrast to protein P_1 which only has one prominent absorption peak at ca. 280 nm, the protein P_2 exhibits significant DCPIP-reduction at the expense of $NADPH + H^+$, while P_1 has only very low activity in this respect.

Protein P_2 but not P_1 is also active with $NADPH + H^+$ as electron donor and AQ as autoxidizable ($E'_0 = -0.2$ V) electron acceptor, the product of oxygen reduction being H_2O_2 as demonstrated by reduction of the rate of oxygen uptake by 50% in the presence of catalase (Fig. 8).

Ferredoxin ($E'_0 = -0.43$ V), from thermodynamic reasons, in the presence of P_2 (but not with P_1) stimulated oxygen uptake by only ca. 15% as compared to AQ. Only $NADPH + H^+$ but not $NADH + H^+$ can act as electron donor for P_2 (Fig. 9). The protein P_2 therefore has the activity of a ferredoxin-NADP-oxidoreductase [20]. Commercial diaphorase from Clostridium kluyveri (Sigma, Prod. Nr. D-2381) also exhibits SOD-activity in the xanthine-oxidase dependent hydroxylamine oxidation. If present in the hydroxylamine test system (*cf.* Fig. 1 and 3), ca. 5 diaphorase units are equivalent to one SOD unit.

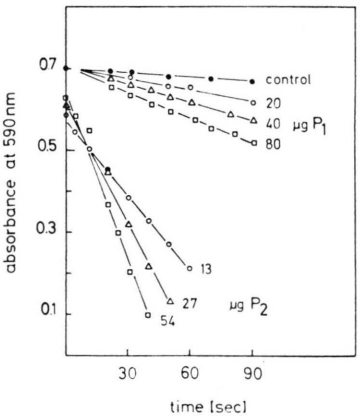

Fig. 7. Comparison of the catalysis of dichlorophenolindophenol reduction by proteins P_1 and P_2. The reaction mixture contained in 3 ml: DCPIP (0.033 nmol); glucose-6-phosphate (10 µmol); glucose-6-phosphate dehydrogenase (25 µg); NADP (5 µmol); phosphate buffer pH 7.6 (120 µmol) and the indicated amounts of proteins P_1 or P_2 (see also ref. [19]).

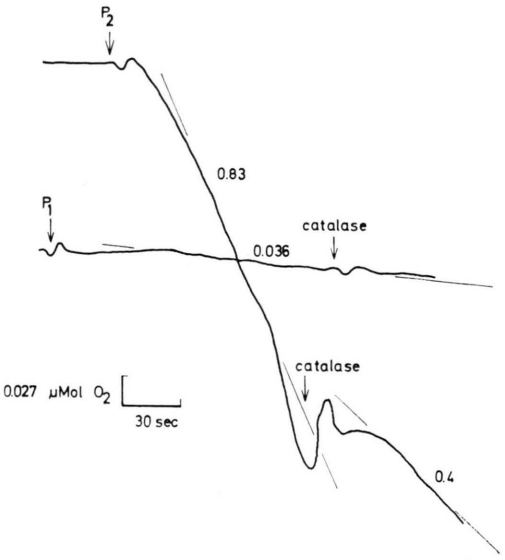

Fig. 8. Anthraquinone reduction by protein P_2 as indicated by oxygen uptake. Oxygen uptake and evolution were analyzed polarographically with $NADPH + H^+$ (see Figs 5 and 7) as electron donor, 0.2 µmol AQ and either 40 µg protein P_1 or 27 µg protein P_2 in the reaction mixtures; catalase (360 units, Boehringer) was added as indicated.

4) Pulse radiolysis experiments

With pulse radiolysis, the reaction of the protein to be tested with superoxide radicals can be measured directly by observation of the kinetics of optical absorption of the superoxide anion. In the experiments described here, the concentration of superoxide radicals at the end of the electron pulse was 1.6×10^{-5} M throughout. At pH 7.2 a reaction rate constant of protein 1 with superoxide of $K = 8.13 \pm 0.36 \times 10^7\ M^{-1}\ sec^{-1}$ was determined. In the presence of protein 2 or commercially available diaphorase the measured rate constants were identical with that of the spontaneous dismutation of superoxide at this pH. This indicates that a reaction, if at all, occured with protein 2 or with commercial (Sigma) diaphorase with a rate constant below $10^5\ M^{-1}\ sec^{-1}$.

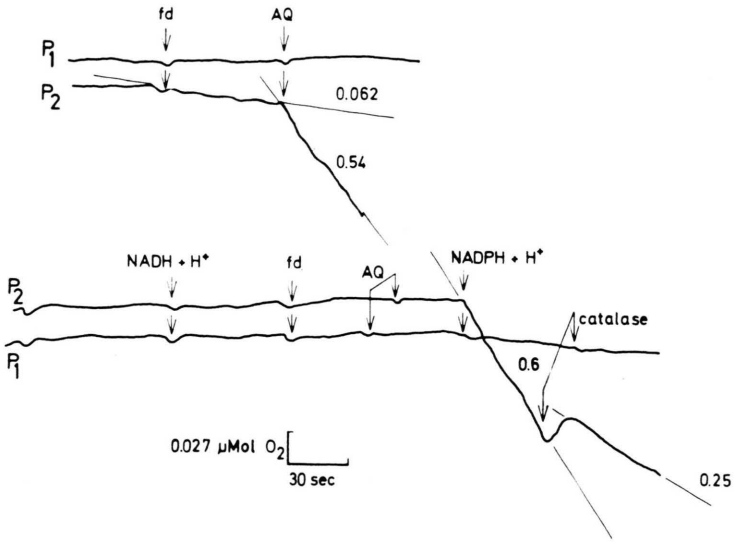

Fig. 9. Comparison of NADH+H$^+$ and NADPH+H$^+$ as electron donators and ferredoxin and AQ as electron acceptors of proteins P_1 and P_2. Reaction conditions were as outlined for Figs 5 and 8. 20 nmol ferredoxin, 5 μmol NADH+H$^+$ or NADPH +H$^+$, 0.2 μmol AQ and 360 units catalase were added as indicated; either 40 μg protein P_1 or 27 μg protein P_2 were present in the test vessels.

For commercially available Cu-Zn superoxide dismutase (Sigma), a reaction rate constant of $K = 1.6 \pm 0.64 \times 10^9$ M^{-1} sec^{-1} and for the penicillamine copper complex, which acts as a low molecular weight "artificial superioxide dismutase" [8, 9] $K = 1.54 \pm 0.5 \times 10^9$ M^{-1} sec^{-1} was determined during the same set of experiments. The reaction rate constants of CuZn-, Mn- and Fe-superoxide dismutases with O_2^- are in the range of $10^8 - 10^9$ M^{-1} sec^{-1} [1, 2]. Therefore unknown proteins should react with superoxide radicals with rate constants around 10^8 to 10^9 M^{-1} sec^{-1}, if they should be characterized as superoxide dismutases with a role in biological systems.

Discussion

Superoxide dismutases seem to be obligatory for the life with oxygen [1, 2]. Three types of enzymes have been described : cyanide-sensitive copper-zinc enzymes only in eukaryotes, cyanide-insensitive mangano enzymes mainly in eukaryotic algae and in mitochondria of eukaryotes and cyanide-insensitive iron enzymes only in prokaryotes [22]. The presence of the type of SOD (Cu-Zn, Mn or Fe) in different cell compartments of eukaryotic organisms and in eukaryotic or prokaryotic microorganisms has been taken as indicator of the states of phylogenetic development [21, 22] and/or for ecologic-physiological studies [1]. Most of the reports on the identification and quantitative determination of SOD in crude or purified extracts from animal or plant tissues or from microorganisms were based on enzymatic or chemical reactions as generators for superoxide radicals. Examples for these generators are xanthine oxidase with xanthine, hypoxanthine or purine as substrates, autoxidation of pyrogallol or anthraquinone, riboflavin photooxidation or the oxidation of NADH by phenazine methosulphate, to mention just a few, where a second reaction serves as superoxide radical detector. Examples of detectors are cytochrome c reduction or nitrite formation from hydroxylammonium chloride. The superoxide anion generators mentioned above produce radicals at a constant, but low rate. In the absence of superoxide dismutase, the steady state concentration of radicals is determined by the equilibrium of the generation rate and the reaction rate with the detector. If SOD is present, it competes with the detector for superoxide, resulting in a lowered steady state concentration of the radical. If proteins are tested for superoxide dismutase activity, they are added to the assay system, but in many cases it is very difficult to estimate, whether at all or to what extent they act upon the superoxide generator and/or the detector. The determination of the rate constant of the reaction between the protein of interest and the superoxide by biochemical assays thus can only be achieved by competition reactions. In this case the assay system consists of the superoxide generator, the detector (where the reaction rate constant with superoxide has to be known) and the competitor.

The proteins P_1 and P_2 isolated from *E. gracilis* cells represent examples for the kind of problems which have to be envisaged, assaying for superoxide dismutase:

a) both proteins act as SOD when tested in the classical systems, using xanthine/xanthine oxidase as $O_2^{\cdot -}$ generator,

b) only protein P_1 turns out to be "a real" SOD, exhibiting more or less activity in all applied test systems, including pulse radiolysis. Thus, pulse radiolysis (in addition to the mentioned biochemical methods not including xanthine oxidase) seems to represent the most reliable method for assaying SOD activities.

The kind of interation of P_2 with the xanthine oxidase system is unclear: one might speculate that P_2 (as a flavoprotein with diaphorase character, coupling with autoxidizable quinones) may drain off electrons from the site of univalent oxygen reduction in xanthineoxidase, thus enhancing two electron oxygen reduction and avoiding the formation of $O_2^{\cdot -}$.

E. gracilis with protein P_1 as an iron containing superoxide dismutase (though its reaction rate constant with $O_2^{\cdot -}$ according the pulse radiolysis results is more than one of magnitude lower as compared to Cu-Zn enzymes or the "artificial SOD", copper-penicillamine) [8, 9] to our knowledge represents the first example of an eukaryotic organism described containing only the iron enzyme. Asada and Kanematsu recently mentioned unpublished results of their own (*cf.* ref. [22]) where Euglena is found to contain iron-SOD. Our finding that the protein P_1 contains 1 g atom Fe/mol apoprotein is in agreement with the reports on iron-SOD from prokaryots [23, 24] *e. g.* cyanobacteria. This result may support speculations, concerning the "evolutionary hypothesis" (ref. [21]), but it is, on the other hand, very unusual for *Euglena gracilis*, an organism, which also contains chlorophyll b.

The authors wish to thank Miss Chr. Fuchs and Mrs. E. Paur for expert technical assistance. Special thanks go to Dr. L. Gürtler for valuable help with the chromatography experiments, to Dr. P. Schrammel for performing the atomic absorption measurements and to Prof. Schulte-Frohlinde and his coworkers, Inst. f. Strahlenchemie, Mühlheim, for their extraordinary help with the pulse radiolysis experiments.

This work was supported by the Deutsche Forschungsgemeinschaft and the KleinwanzlebenerSaatzucht AG., KWS-Einbeck/Hann.

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