CHEMICAL MODIFICATION STUDIES ON A FERRI-SUPEROXIDE DISMUTASE FROM MARINE BACTERIA

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1. Introduction

Superoxide dismutases (SODs) are metallo-enzymes whose function is to catalyse the dismutation of superoxide radical anion [1] to oxygen and hydrogen peroxide. In the eucaryotic enzymes one finds either Cu^{2+} and Zn^{2+}, or Mn^{2+}, whereas in procaryotes Mn^{2+} and Fe^{3+} enzymes have been reported [2]. It is assumed that the metal plays an essential role in enzymatic activity [2,3].

The use of specific chemical reactions to modify amino acid residues in proteins is a method which has been of great value in studies of the mechanism of enzyme action and in the identification of amino acid residues involved in the catalytic or in other ligand binding sites of enzymes [4–6]. In the present communication we report on specific modification of histidine, tryptophan, tyrosine, and carboxyl groups in ferri-superoxide dismutase from the marine bacteria *Photobacterium leiognathi* [7,8] as a means of identifying amino acids involved in enzymatic activity and in metal binding.

2. Materials and methods

The ferri-SOD from *Ph. leiognathi*, prepared as in [9] was supplied by Dr A. M. Michelson as a suspension in saturated ammonium sulphate solution and was stored at −20°C. Before use it was dialysed for 16 h at 4°C against sodium phosphate buffer 0.1 M, pH 7.8, containing 10^{-4} M EDTA. Cytochrome *c*, xanthine oxidase and hypoxanthine were from Boehringer, Mannheim, FRG.

The apoenzyme was prepared by dialysis at 4°C for 48 h against 50 mM sodium acetate buffer, pH 3.8, containing 1 mM EDTA and 0.1 mM β-mercaptoethanol. A white precipitate formed which subsequently redissolved on dialysis successively against 10 mM and 5 mM sodium phosphate buffer, pH 7.8. Holoenzyme was reconstituted by incubation for 6 h at 4°C in 5 mM phosphate buffer, pH 7.8, containing 0.5 mM ferrous sulphate followed by dialysis against the same phosphate buffer to remove the iron in excess.

The homogeneity of the SOD was established using electrophoresis in 5% polyacrylamide gel in the Tris–asparagine buffer system of Maurer [10]. Subunit molecular weight determination was by SDS–gel electrophoresis [11]. Amino acid analysis was carried out after hydrolysis for 16 h in vacuo at 110°C with 6 N HCl on a Locarte amino acid analyser (Locarte Co., London, England). Tryptophan was determined separately in 6 M guanidine hydrochloride, pH 6.5, at 280 nm and 288 nm [12].

The activity of the enzyme was determined using hypoxanthine (18 μM)/xanthine oxidase (0.1 μM) to generate O_2^- and the reduction of cytochrome *c* (43 μM) at 550 nm to detect the O_2^- in sodium phosphate buffer 100 mM, pH 7.8, containing 0.1 mM EDTA.

Chemical modification was carried out as follows: histidine (and tyrosine) were modified by diazonium 1-H-tetrazole [13] and histidine by diethyl pyrocarbonate [14]. The number of residues modified was determined spectrophotometrically at 480 nm and 570 nm for diazonium 1-H-tetrazole using a Beckman-DB GT spectrophotometer (Analis, Namur,

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Belgium) and at 230 nm for diethyl pyrocarbonate using a Cary 16 spectrophotometer (Cary, Palo-Alto, USA). Tryptophan was modified with 1-hydroxy-5 nitrobenzyl bromide [15] and the number of residues modified determined at 420 nm. Tyrosine was determined with tetranitromethane as described in [16]. Carboxyl residues were blocked with glycineamide after activation with the water-soluble carbodiimide 1-ethyl-3-(3 dimethylaminepropyl)-carbodiimide, at pH 5.2, and the number of residues modified determined by amino acid analysis after dialysis and acid hydrolysis [17].

3. Results and discussion

The ferri-SOD from *Ph. leiognathi* has an isoelectric point of 4.4, mol. wt 40 660 (as estimated from the sedimentation constant) and contains 1.6 g atoms of iron/molecule [9]. The preparation on which the chemical modification experiments were carried out gave a single band on polyacrylamide gel electrophoresis in SDS corresponding to mol. wt 18 900 ± 590, in good agreement with the value of 20 000 reported by Puget and Michelson [9]. We assume that the enzyme is a dimer containing one iron atom/subunit. In Tris–asparagine buffer, pH 7.3, we observed two bands on polyacrylamide gel electrophoresis which we presume correspond to different forms of aggregation of the enzyme.

The amino acid composition was determined and is given in table 1, as residues of each amino acid/subunit. For comparison, the composition reported previously for the ferri-SOD of *Ph. sepia* is given [3]; it may be noted that there is good agreement except for a few amino acids such as leucine, valine etc. The table also gives the composition of other ferri-SODs from *Escherichia coli, Bacillus megaterium* and *Pseudomonas ovalis* [9–18]. There is a marked similarity in the compositions of the different bacterial ferri-SODs. The *Ph. leiognathi* enzyme has a rather high content of non-polar amino acids (51%). On the basis of its low isoelectric point of 4.4 [9] and its content of basic amino acids, we calculate that no more than 13–14 of the Glx and Asx are present as Glu and Asp, the rest being in the amide form.

The enzyme activity of the SOD preparation was measured using the hypoxanthine/xanthine oxidase system to generate O_2^- and the reduction of cytochrome *c* to detect the superoxide. One unit of native SOD

Table 1
Amino acid composition of ferri-superoxide dismutases from bacteria

Amino acid	*Ph. leiognathi*		*Ph. sepia*	*B. megaterium*	*E. coli*	*Ps. ovalis*
	Residues/subunit	Residues/mol enzyme				
Asx	20.39 ± 0.54	40.8	40	48.8	44.8	35.6
Thr	20.29 ± 0.49	20.6	26	18.9	25.8	24.3
Ser	9.29 ± 0.39	18.6	15	14.6	19.6	26.8
Glx	18.55 ± 0.44	37.1	30	37.4	31.6	32.3
Pro	7.57 ± 0.41	15.1	17	22.4	17.6	18.1
Gly	13.02 ± 0.55	26.0	25	28.0	31.8	35.5
Ala	19.84 ± 0.30	39.7	40	33.0	52.8	33.1
Val	10.99 ± 0.39	21.9	16	23.0	22.0	18.4
Met	0.43 ± 0.05	0.9	6	3.5	0	0
Ileu	6.64 ± 0.22	13.3	14	19.0	16.4	10.8
Leu	20.94 ± 1.47	41.9	27	21.9	29.0	28.3
Tyr	7.14 ± 0.19	14.3	11	13.5	12.8	12.5
Phe	10.22 ± 0.26	20.4	20	15.0	19.8	21.4
His	5.73 ± 0.16	11.5	12	15.0	11.4	13.6
Lys	9.60 ± 0.40	19.2	20	25.7	20.4	19.9
Arg	2.65 ± 0.13	5.3	4	9.5	8.0	3.7
Trp	5.77	11.6	–	7.3	7.8	18.2

The data for *Photobacterium sepia* is from [9] and for *Bacillus megaterium, Escherichia coli, Pseudomonas ovalis* from [18]. The composition given is as residues/molecule of dimeric enzyme

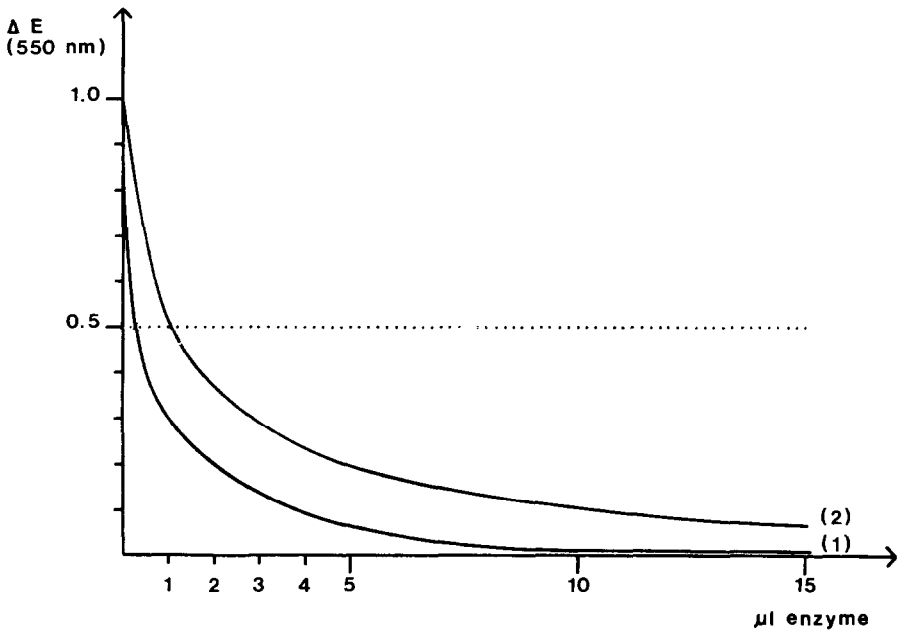

Fig.1. Dosage of SOD activity for native holo-SOD (1) and reconstituted apo-SOD (2). Conditions are described in Materials and methods.

corresponded to 0.45 µg protein (fig.1). Reconstitution of the enzyme from apoenzyme and iron, prepared as described above, gave an activity of one unit/1.58 µg protein (fig.1) or 28% of the activity of native holoenzyme.

Chemical modification was undertaken with a view to identifying the role of histidine, tyrosine and tryptophan residues and carboxyl-groups both in the enzyme activity (holoenzyme modification) and in the iron binding site (apoenzyme modification). Table 2 summarises the results obtained with diazonium 1-H-tetrazole and diethyl pyrocarbonate for histidine,

Table 2
Results of chemical modifications

Reagent	Experimental conditions	Amino acid modified	Number of residues/subunit Holoenzyme	Apoenzyme
Diazonium 1-H-tetrazole	pH 8.0, 10.0	Bisazohistidine	1.85	1.80
		Monoazotyrosine	1.50	2.35
		Bisazotyrosine	1.45	1.90
Diethyl pyrocarbonate	pH 5.3	Histidine	2.30	2.35
1-Hydroxy-5-nitro benzyl bromide	pH 5.5	Tryptophan	2.60	3.45
Glycinamide	pH 5.2	Carboxyl	5.35	4.95
Tetranitromethane	pH 8.0	Tyrosine	3.63	4.32

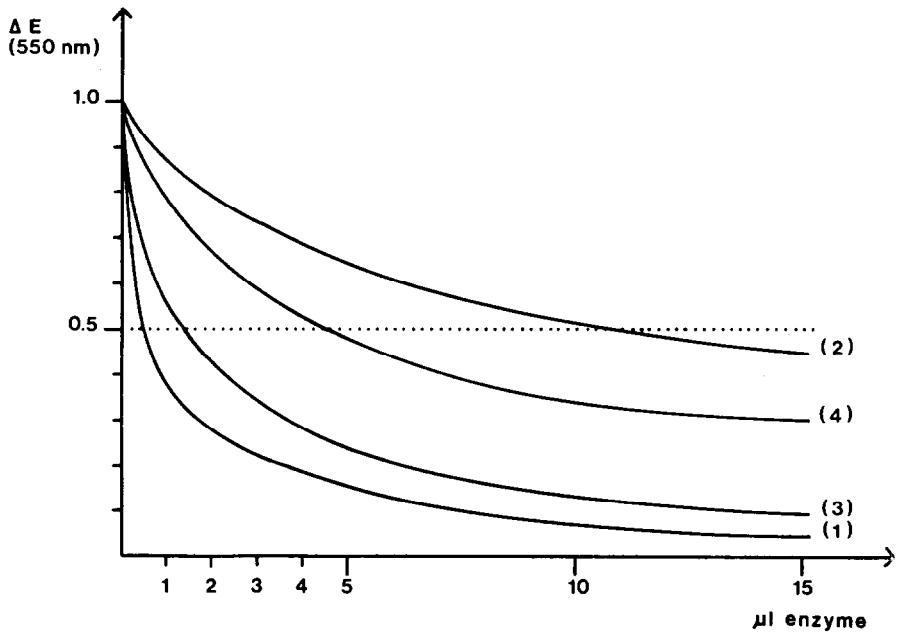

Fig.2. Dosage of SOD activity for chemically modified SODs. Tetranitromethane-modified holoenzyme (1). Reconstituted tetranitromethane-modified apoenzyme (2). Glycineamide-modified holoenzyme (3). Reconstituted glycineamide-modified apoenzyme (4).

diazonium 1-H-tetrazole and tetranitromethane for tyrosine, hydroxy-nitrobenzyl-bromide for tryptophan and glycineamide in presence of a water soluble carbodiimide for carboxyl groups.

It appears that 2 histidine residues can be modified out of the total of 6 with diazonium–1-H-tetrazole and with diethyl pyrocarbonate both in the holoenzyme and in the apoenzyme. From this we conclude that histidine is either not a ligand of the iron or else that those histidines which are liganded to the iron remain buried and/or unreactive in the apoenzyme. The histidine-modified enzyme (fig.2) has completely lost its catalytic activity, and even when the iron is added back the modified apoenzyme does not recover its activity. This is consistent with a role for one or two histidine residues in the catalytic activity of the enzyme. It should be noted [19] that in the bovine Cu–Zn-SOD, seven histidines have been identified as metal ligands. The reaction of the bovine Cu–Zn enzyme with diethyl pyrocarbonate [20] results in modification of 1 histidine residue/subunit in the holoenzyme and 4 histidines/subunit in the apoenzyme. It was concluded that 3 histidines become exposed when the Cu is removed. A similar result (3.6 histidine residues/modified subunit) was found for the bovine apo-SOD by photoxidation [21].

We selected to use both diethyl pyrocarbonate and diazonium tetrazole because of the well known reactions of the latter with tyrosine. Although diethyl pyrocarbonate is also known to react with tyrosines we were able to conclude that it was the histidine modification that was the cause of inactivation by specific tyrosine modification with tetranitromethane. Of the 7 tyrosine/subunit, 3.6 are modified in the holoenzyme and 4.3 in the apoenzyme with tetranitromethane; somewhat less are modified by diazonium tetrazole (table 2). We cannot conclude with certainty if the one extra tyrosine/subunit modified in the apoenzyme is a ligand for the metal. The modified holoenzyme retains 63% of its activity (fig.2) suggesting that the tyrosines that have been modified are not essential for activity. In contrast, the tetranitromethane-modified apoenzyme regained only 2.6% of its activity compared with the native

enzyme and 9.2% compared with the reconstituted apoenzyme. This suggests that there is one tyrosine/subunit involved as a ligand to the metal and that its modification in the apoenzyme affects the fixation of the iron so that the enzymic activity is impaired.

Tryptophan also seems to be a likely metal ligand (table 2). Of the six tryptophan residues in the protein one additional residue/subunit is modified specifically in the apoenzyme compared with the holoenzyme. The total loss of activity on modification of 2.6 tryptophan residues/subunit in the holoenzyme suggests that tryptophan residues may also be important for the enzymatic activity. The final, potential iron-ligand that we investigated was the carboxyl group. Here there was no evidence for a role in metal binding (table 2) with 5 carboxyls of the total of 13–14 modified/subunit in the holo- and apoenzyme. The glycineamide-modified SOD still retained some activity (21%) and the same level of activity was also found in the reconstituted apoenzyme (23% compared with reconstituted apoenzyme). The partial loss of activity may be due to conformational changes induced by the modification.

We conclude from this study that in the ferri-SOD from *Ph. leiognathi* histidine and tryptophan residues are probably involved in the catalytic activity and that one tyrosine and one tryptophan residue/subunit may be metal ligands. Neither carboxyl groups nor tyrosine residues seem to be involved in the catalytic site.

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