Copper–zinc superoxide dismutase in *Haemophilus* species

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(Received 26 September 1991; revised 21 November 1991; accepted 12 December 1991)

Copper-zinc superoxide dismutases ([Cu,Zn]-SODs) are ubiquitous in eukaryotes but have rarely been found in prokaryotes. A gene for [Cu,Zn]-SOD (*sodC*) has recently been cloned from *Haemophilus influenzae* type b and *H. parainfluenzae*, so other *Haemophilus* and related species were screened for the presence of [Cu,Zn]-SODs by visualization of bands of SOD activity in non-denaturing polyacrylamide gels and by gene probing. Strains of *H. aphrophilus*, *H. paraphrophilus*, *H. haemolyticus*, *H. paraphrohaemolyticus*, some non-typable *H. influenzae*, *H. haemoglobinophilus* (*canis*) and *H. parasuis* were all found to have [Cu,Zn]-SOD activity (inhibited by 2 mM-cyanide) in polyacrylamide gels. In a Southern blot analysis, DNA from *H. aphrophilus*, *H. paraphrophilus*, *H. haemolyticus* and [Cu,Zn]-SOD-containing non-typable *H. influenzae* – but not the other species – hybridized to a 360 nucleotide DNA probe containing the 5'-part of *sodC* cloned from *H. influenzae* type b. Bacterial [Cu,Zn]-SODs are more prevalent than has previously been recognized.

Introduction

Superoxide dismutases (SODs) (EC 1.15.1.1) are metalloenzymes catalysing the conversion of superoxide radicals into hydrogen peroxide and oxygen (McCord & Fridovich, 1969). They are ubiquitous, forming an important defence against oxygen toxicity in oxygen-consuming organisms. There are two families of SODs differing in the metal(s) found at their active sites: manganese or iron, and copper/zinc. SODs containing manganese ([Mn]-SODs) occur in prokaryotes and mitochondria, while those containing iron ([Fe]-SODs) are found in prokaryotes and lower eukaryotes. Copper and zinc containing SODs ([Cu,Zn]-SODs) are principally found in eukaryotes but a few have been found in some bacteria. [Fe]- and [Mn]-SODs form one family and [Cu,Zn]-SODs another on the basis of protein sequence similarities, the two families having evolved independently under the selective pressure of oxygen toxicity (Grace, 1990).

After the first description of a bacterial [Cu,Zn]-SOD, in the fish symbiont *Photobacterium leiognathi* (Puget & Michelson, 1974), numerous bacterial species have been investigated (Britton *et al.*, 1978; Bang *et al.*, 1978;

reviewed in Hassan, 1989). Only a small number have been found to possess [Cu,Zn]-SOD: *Caulobacter crescentus* (Steinman, 1982b), *Paracoccus denitrificans* (Vignais *et al.*, 1982), *Pseudomonas diminuta*, *Ps. maltophilia* (Steinman, 1985) and *Brucella abortus* (Beck *et al.*, 1990). Recently, the [Cu,Zn]-SOD gene *sodC*, encoding a protein very similar to the known bacterial [Cu,Zn]-SODs (Steinman, 1982a; Steinman & Ely, 1990; Beck *et al.*, 1990), has been found in *Haemophilus influenzae* type b and *H. parainfluenzae* (Kroll *et al.*, 1991). In this work, *Haemophilus* and related species have been screened for [Cu,Zn]-SOD by staining whole cell extracts separated in non-denaturing polyacrylamide gels, and by Southern hybridization to a *sodC* gene probe cloned from *H. influenzae* type b.

Methods

Strains and culture conditions. *H. influenzae* NCTC 8468 and strains identified by RM numbers were obtained from the Departmental Culture Collection. Two further *H. influenzae* strains were kindly provided by Dr P. Williams, University of Nottingham, UK. *H. aegyptius* strains were kindly provided by Dr G. M. Carlone (Centers for Disease Control, Atlanta, USA); strain Hae7 was a Brazilian purpuric fever isolate, and strain Hae5 (NCTC 8135) a conjunctivitis control (Brenner *et al.*, 1988). *H. parasuis* 857 and *H. parasuis* 870 were kindly provided by Dr J. Musser (Pennsylvania State University, USA). *Tylorella equigenitalis* was kindly provided by Dr A. Williams (Neurophysiology Unit, AFRC/MRC, Edinburgh, UK). *Pseudomonas*

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Abbreviation: SOD, superoxide dismutase.

diminuta NCTC 8545 (ATCC 11568) and *Ps. maltophilia* NCTC 10257 (ATCC 13637) were obtained from the National Culture Type Collection, Colindale, London, UK. All other strains were obtained from the culture collection of the Public Health Laboratory Service, Oxford, UK. With the exception of the pseudomonads, strains were grown in Brain Heart Infusion broth supplemented with NAD ($2 \mu\text{g ml}^{-1}$) and haemin ($10 \mu\text{g ml}^{-1}$) at 37°C on an orbital shaker (200 r.p.m.) until late exponential phase, harvested and stored at -20°C until required. *H. aphrophilus*, *H. paraphrophilus*, *H. parasuis* and *H. paraphrohaemolyticus* strains were additionally sparged with 5% (v/v) carbon dioxide and sealed prior to incubation, because these strains were listed by Kilian (1976) as having enhanced growth in the presence of carbon dioxide. *Ps. diminuta* and *Ps. maltophilia* were grown to stationary phase at 30°C as described by Steinman (1985).

PAGE conditions, SOD visualization and M_r determination. Thawed cells were suspended in 1 ml 50 mM-Tris/HCl (pH 7.8)–25 mM-benzamidine, sonicated for 1 min at 0°C , centrifuged (13 500 g, 10 min) and the supernatants used directly or stored at -20°C . Measurements of protein concentration were made using the method of Bradford (1976) modified by Stoscheck (1990), using bovine serum albumin as standard.

The PAGE conditions used were 4.5% (w/v, acrylamide) stacking gel (pH 8.3) and 10% separating gel (pH 8.9) using the buffer system of Davis (1964) except that the pH of the upper buffer was raised to 8.9 with 10 M-NaOH. SOD activity in PAGE gels was visualized by the modification of the Beauchamp & Fridovich (1971) method as described by Steinman (1985). When used as inhibitors hydrogen peroxide or potassium cyanide were added to the riboflavin-TEMED solution to final concentrations of 5 mM and 2 mM respectively. [Cu,Zn]-SODs are characteristically inactivated by cyanide, [Fe]-SODs by hydrogen peroxide, but [Mn]-SODs are resistant to both (Crapo *et al.*, 1978; Dunlap & Steinman, 1986). Bovine erythrocyte [Cu,Zn]-SOD (Sigma) and a sonicate of *Escherichia coli* strain DH5 α containing [Fe]-, [Mn]- and hybrid [Fe,Mn]-SODs were used as appropriate controls.

The M_r values of [Cu,Zn]-SODs were estimated from the dependence of their electrophoretic mobility in PAGE gels upon acrylamide concentration (Hedrick & Smith, 1968). Gels were prepared containing concentrations of acrylamide between 6% and 14% (w/v), and *Haemophilus* extracts were compared with [Cu,Zn]-SOD standards of known M_r as follows: *Ps. maltophilia*, 16 000 and 20 000 (Steinman, 1985); bovine erythrocyte [Cu,Zn]-SOD (Sigma), 31 200 (McCord & Fridovich, 1969); and *Ps. diminuta*, 45 000 (Steinman, 1985). Sonicates of the *Pseudomonas* strains were used as the source of the [Cu,Zn]-SODs.

Preparation and analysis of genomic DNA. DNA was prepared from 3 ml broth cultures as previously described (Moxon *et al.*, 1984). Standard methods were used for restriction digestion and Southern blotting with washing to ~80% stringency (Maniatis *et al.*, 1982). Southern blots were probed with the *Haemophilus* DNA insert of pJSK114. This consists of a 360 nucleotide HindIII–NcoI fragment, the 5'-part of the *H. influenzae* type b [Cu,Zn]-SOD gene *sodC* (Kroll *et al.*, 1991).

Results

SOD visualization in PAGE gels

Thirty strains of bacteria predominantly from the genus *Haemophilus* were screened for the presence of [Cu,Zn]-SODs. PAGE was chosen as the method for detecting enzyme activity since this has been shown to be more reliable than solution assay (Steinman, 1985). The

presence or absence of SODs and their sensitivity to 2 mM-cyanide and 5 mM-hydrogen peroxide are shown in Table 1. All strains contained a single band of SOD activity not inhibited by hydrogen peroxide or cyanide, thus characteristic of a [Mn]-SOD (Crapo *et al.*, 1978). *T. equigenitalis* had two such bands. As this result might have been caused by the presence of catalase co-migrating with the SOD, duplicate gels carrying extracts of *H. influenzae* and *H. aegyptius* were stained for this enzyme (Gregory & Fridovich, 1974). This demonstrated that catalase was present in all these strains except NCTC 8468 and RM107, but that it migrated differently from SOD (data not shown) and was therefore not responsible for the resistance of the putative [Mn]-SOD to hydrogen peroxide. In strains of *H. parainfluenzae*, some non-typable *H. influenzae*, *H. aphrophilus*, *H. paraphrophilus*, *H. haemolyticus*, *H. paraphrohaemolyticus*, *H. haemoglobinophilus* and *H. parasuis*, a second band of SOD activity was seen. This activity, of lower mobility than the putative [Mn]-SOD, was inhibited by cyanide but not by hydrogen peroxide, characteristics that identify it as due to [Cu,Zn]-SOD (Dunlap & Steinman, 1986). Tracks from duplicate PAGE gels ($\pm$ cyanide) containing extracts from ten examples with a cyanide-sensitive band are shown in Fig. 1.

The M_r values of some of these [Cu,Zn]-SODs were estimated by the method of Hedrick & Smith (1968) as follows: *H. aphrophilus* NCTC 5906, 42 000; *H. aphrophilus* 1291, 39 000; *H. paraphrophilus* NCTC 10557, 41 000; *H. haemoglobinophilus* NCTC 1659, 41 500; *H. paraphrophilus* 1041, 33 000; *H. parasuis* 857, 34 000; and *H. parasuis* 870, 16 000.

Southern hybridization studies with the *sodC* probe pJSK114

Southern-blotted *Eco*RI digests of chromosomal DNA were probed with the *Haemophilus* DNA insert of pJSK114. In the case of non-influenzae strains of *Haemophilus*, the probe only hybridized to DNA from organisms that showed [Cu,Zn]-SOD activity in PAGE gels (Table 1). A single band of hybridization was seen with *H. haemolyticus*, *H. aphrophilus*, *H. paraphrophilus*, *H. parainfluenzae* and *H. paraphrohaemolyticus* (Fig. 2). However, *H. haemoglobinophilus* and *H. parasuis* strains, which had activity in PAGE gels, had no hybridization signals at the 80% stringency of filter-washing employed in this study (*H. haemoglobinophilus*, Fig. 2; *H. parasuis*, data not shown). A newly prepared full-length gene probe for *sodC* from *H. parainfluenzae* 1391 in addition hybridized to chromosomal DNA from *H. haemoglobinophilus* NCTC 1659. The paradoxical observation of pJSK114 hybridization in capsulate *H. influenzae* strains lacking [Cu,Zn]-SOD activity has been discussed at length elsewhere (Kroll *et al.*, 1991).

Table 1. Cyanide and hydrogen peroxide sensitivity of SODs in PAGE gels, and hybridization of chromosomal DNA to the *Haemophilus influenzae* type b *sodC* probe cloned in pJSK114

I and II correspond to phylogenetic division I or II of capsulate *H. influenzae* (Musser *et al.*, 1988). +/– indicates presence or absence of activity or probe hybridization.

Strain	SOD not inhibited by 5 mM-peroxide or 2 mM-cyanide	SOD inhibited by 2 mM-cyanide ([Cu,Zn]-SOD)	Hybridization to insert of pJSK114
<i>Haemophilus influenzae</i> RM7109 (type a, I)	+	–	–*
<i>Haemophilus influenzae</i> RM107 (type a, II)	+	–	+*
<i>Haemophilus influenzae</i> RM153 (type b, I)	+	–	–*
<i>Haemophilus influenzae</i> NCTC 8468 (type b, II)	+	–	+*
<i>Haemophilus influenzae</i> RM8032 (type c, I)	+	–	–*
<i>Haemophilus influenzae</i> RM6137 (type d, I)	+	–	–*
<i>Haemophilus influenzae</i> RM6157 (type e)	+	–	+*
<i>Haemophilus influenzae</i> RM7283 (type f, II)	+	–	+*
<i>Haemophilus parainfluenzae</i> 1391	+	+	+*
<i>Haemophilus influenzae</i> RM8019 (non-typable)	+	–	–
<i>Haemophilus influenzae</i> RM8093 (non-typable)	+	–	–
<i>Haemophilus influenzae</i> 11P5 (non-typable)	+	+	+
<i>Haemophilus influenzae</i> 26E (non-typable)	+	+	+
<i>Haemophilus aegyptius</i> Hae5 NCTC 8135	+	–	–
<i>Haemophilus aegyptius</i> Hae7	+	–	–
<i>Haemophilus aphrophilus</i> NCTC 5906	+	+	+
<i>Haemophilus aphrophilus</i> 1291	+	+	+
<i>Haemophilus paraphrophilus</i> NCTC 10557	+	+	+
<i>Haemophilus paraphrophilus</i> 1041	+	+	+
<i>Haemophilus haemolyticus</i> NCTC 10659	+	+	+
<i>Haemophilus paraphrohaemolyticus</i> NCTC 10670	+	+	+
<i>Haemophilus haemoglobinophilus</i> NCTC 1659	+	+	–
<i>Haemophilus parasuis</i> NCTC 4557	+	+	–
<i>Haemophilus parasuis</i> 857	+	+	–
<i>Haemophilus parasuis</i> 870	+	+	–
<i>Haemophilus avium</i> NCTC 11297	+	–	–
<i>Haemophilus paragallinarum</i> NCTC 11296	+	–	–
<i>Haemophilus segnis</i> NCTC 10977	+	–	–
<i>Eikenella corrodens</i> NCTC 10596	+	–	–
<i>Taylorella equigenitalis</i>	+†	–	–

* Data from Kroll *et al.* (1991).

† Two SOD bands.

Discussion

All the *Haemophilus* and related species examined in this study possess a SOD with activity resistant to cyanide and hydrogen peroxide, typical of the [Mn]-containing enzyme. A wide range of R_F values (0.25–0.83 in 10% PAGE gels) was obtained for these enzymes (data not shown) suggesting structural heterogeneity as reported before for [Mn]-SODs (Steinman, 1983). In addition, strains of *H. parainfluenzae*, some non-typable *H. influenzae*, *H. aphrophilus*, *H. paraphrophilus*, *H. haemolyticus*, *H. paraphrohaemolyticus*, *H. haemoglobinophilus* and *H. parasuis* were found to possess a second enzyme with activity characteristic of [Cu,Zn]-SOD. This study has thus considerably extended the known number of [Cu,Zn]-SODs in bacteria. In fact it has been argued before that the reported rarity of bacterial [Cu,Zn]-SODs

may be more apparent than real, as although a considerable range of micro-organisms has been examined, in many cases only one strain has been investigated, and in any case, the molecular heterogeneity of these enzymes and in particular their high isoelectric point makes their detection difficult without particular attention to gel conditions (Steinman, 1985). The M_r determinations presented here suggest a degree of molecular heterogeneity within [Cu,Zn]-SODs of *Haemophilus* species. With the exception of [Cu,Zn]-SOD from *H. parasuis* 870, all the other *Haemophilus* [Cu,Zn]-SODs had estimated M_r values greater than the 31 500 and 29 200 reported for the enzymes from *P. leiognathi* (Steinman, 1982a) and *C. crescentus* (Steinman & Ely, 1990) respectively, although none were as high as the 45 000 of *Ps. diminuta* (Steinman, 1985). The apparent high M_r of [Cu,Zn]-SOD in this last species was

Fig. 1. Tracks from duplicate non-denaturing PAGE gels (a untreated, b with 2 mM-cyanide) stained to reveal SOD activity. Tracks contain sonicates ($\sim 60 \mu\text{g}$ protein) from representative strains as follows: 1, *H. haemolyticus*; 2, *H. haemolyticus* NCTC 5906; 3, *H. aphrophilus* NCTC 5906; 4, *H. aphrophilus* 1291; 5, *H. paraprothophilus* NCTC 10557; 6, *H. paraprothophilus* NCTC 10557; 7, *H. parainfluenzae* 1391; 8, *H. paraprothophilus* 857; 9, *H. parasuis* 857; 10, *H. parasuis* 870. In each case the upper band (faint, and therefore marked with an asterisk, in the case of *H. paraprothophilus*) is identified as $[\text{Cu,Zn}]$ -SOD by its cyanide sensitivity. Track 7 has been reproduced previously (Kroll *et al.*, 1991).

Fig. 2. Southern hybridization of *sodC* DNA to different *Haemophilus* species. (a) Electrophoretically separated *Eco*RI-digested DNA visualized in an agarose gel by staining with ethidium bromide. (b): Southern blot of the same gel. Tracks contain DNA from strains as follows: 1, *H. influenzae* type b NCTC 8468; 2, *H. haemolyticus*; 3, *H. haemoglobinophilus*; 4, *H. aphrophilus* NCTC 5906; 5, *H. paraphrophilus* 1041; 6, *H. paraphrophilus* NCTC 10557; 7, *H. parainfluenzae* 1391. DNA fragment size markers (kb) are indicated on the right. The estimated size of the single hybridization band of *H. paraphrophilus* NCTC 10557 is 6.8 kb.

attributed to asymmetry of the protein altering its mobility in PAGE gels, and the same explanation may apply here. Native [Cu,Zn]-SODs are generally dimers composed of identical subunits (Steinman, 1983). The low M_r (16000) estimated for the *H. parasuis* 870 enzyme may be that of the monomeric subunit, found here due to instability of the enzyme at pH 8.9 and reassociation at pH 7.8 upon SOD visualization, as has been suggested for the *Ps. maltophilia* enzyme (Steinman, 1985).

DNA hybridization using the *H. influenzae* type b *sodC* probe pJSK114 suggests that in each of these *Haemophilus* species, [Cu,Zn]-SOD activity is encoded by an homologous gene. Where the probe failed to hybridize to chromosomal DNA despite the presence of [Cu,Zn]-SOD activity, it may be that sequence divergence greater than the limit of 20% reliably detectable in these experiments was responsible. Such divergence has been found in a gene homologous to *sodC* that has now been cloned from *Actinobacillus pleuropneumoniae* (unpublished results).

Visualization of enzyme activity in PAGE gels has been considered the method of choice for screening for [Cu,Zn]-SODs, as it is simple and more accurate than solution assay (Steinman, 1985). The use of [Cu,Zn]-SOD gene probes like pJSK114 can greatly extend the scope of investigations, particularly where SOD activity is too low

to be detected as in strains with modified or inactivated enzyme. Kroll *et al.* (1991) found that a CAT→TAT transition in the structural gene, mutating a critical histidine to tyrosine, was most likely responsible for the lack of [Cu,Zn]-SOD activity in *H. influenzae* type b. [Cu,Zn]-SOD activity may not be detected where experiments are carried out under non-permissive conditions where expression of the enzyme is environmentally regulated. Such environmental regulation of bacterial [Cu,Zn]-SOD expression has been shown in the case of *P. leiognathi* (Kobayashi *et al.*, 1991).

DNA probes like pJSK114 will be important tools to facilitate the cloning of homologous [Cu,Zn]-SOD genes from other species in order to study the role of this enzyme in the biology of oro-pharyngeal commensals and pathogens.

This work was supported by grants to J.S.K. from the Wellcome Trust and the British Lung Foundation.

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