Role of prokaryotic Cu,Zn superoxide dismutase in pathogenesis

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Abstract

Several bacterial pathogens possess *sodC* genes that encode periplasmic or membrane-associated Cu,Zn superoxide dismutases. Since professional phagocytes generate large amounts of reactive oxygen species to control the growth of invading micro-organisms, Cu,Zn superoxide dismutase might protect infectious bacteria from oxy-radical damage and facilitate their survival within the host. This idea has gained support from studies showing that *sodC*-null mutants of different bacteria are less virulent than their parental wild-type strains, and from the discovery that, despite apparent dispensability for growth under laboratory conditions, various pathogens (including several highly virulent *Salmonella* strains) possess multiple copies of *sodC*. Our studies indicate that Cu,Zn superoxide dismutase effectively protects bacteria from phagocytic killing, and that the role in infection of the redundant *sodC* genes may vary in distinct *Salmonella enterica* serovars. More unexpectedly, we have found that Cu,Zn superoxide dismutase also modulates bacterial survival within epithelial cells, where bacterial killing appears to be mediated by an NAD(P)H oxidase resembling the enzyme complex typical of phagocytes. Finally, a striking feature of Cu,Zn superoxide dismutases from bacterial pathogens is their apparent ability to exploit the structural versatility of the enzyme to modulate its function. In fact, several enzyme variants exhibit unique properties that may lead to the acquisition of novel specialized functions distinct from superoxide dismutation.

Cu,ZnSOD (Cu,Zn superoxide dismutase) and bacterial virulence

Until relatively recently, Cu,ZnSOD was considered to be an almost exclusively eukaryotic enzyme, and its presence in bacteria, originally identified in a very small number of micro-organisms, was thought to be an exception rather than a rule. The identification of genes encoding Cu,ZnSOD (*sodC*) in several Gram-negative pathogens in the mid 1990s [1], and the complete or near-complete sequencing of a large number of bacterial genomes, have modified this assumption, and established that Cu,ZnSODs are widespread throughout the eubacterial world. The exact role of Cu,ZnSOD in bacteria is not yet well understood, but all prokaryotic Cu,ZnSODs are located in the periplasm or anchored to the outer membrane [2,3]. In agreement with the view that the superoxide anion is unable to cross membranes, Cu,ZnSOD does not protect bacteria from superoxide generated intracellularly [4]. Moreover, with the notable exception of *Legionella pneumophila*, where the absence of *sodC* induces considerable loss of viability during the stationary phase [5], *sodC*-negative mutants are phenotypically indistinguishable from their wild-type strains under laboratory conditions, although they are sensitive to extracellular sources of oxygen radicals [6–9].

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Abbreviation used: SOD, superoxide dismutase.
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In apparent contrast with the dispensability of *sodC* for aerobic growth of micro-organisms cultivated *in vitro*, various bacteria (including *Mycobacterium avium*, enterohaemorrhagic *Escherichia coli* O157:H7, several *Salmonella enterica* strains and the radiation- and oxidative stress-resistant bacterium *Deinococcus radiodurans*) possess redundant copies of this gene. These observations, and the absence of known periplasmic sources of oxygen radicals in most of the bacteria possessing *sodC* genes, have led to the hypothesis that the most likely function of Cu,ZnSOD is to protect bacteria from exogenous sources of superoxide [4]. Such a role could be particularly advantageous for bacterial pathogens facing attack from inflammatory cells [10]. In fact, in their effort to control the growth of invading bacteria, professional phagocytes generate large amounts of reactive oxygen species, whose production is primed by the single-electron reduction of molecular oxygen to superoxide by a transmembrane NADPH oxidase complex. This defence mechanism, known as the respiratory burst, has a key role in the control of bacterial infections, as shown by clinical observations of patients affected by chronic granulomatous disease [11]. The hypothesis that Cu,ZnSOD plays a role in pathogenesis has gained support from studies showing that *sodC*-null mutants of several (although not all) pathogens exert attenuated virulence in animal models [6,7,9,12–16].

The contribution of Cu,ZnSOD to bacterial pathogenicity is likely to be correlated with the modality of the interaction

between bacteria and the phagocytes of their natural host, being greater in micro-organisms capable of an intracellular lifestyle [17]. Therefore most studies on the role of Cu,ZnSOD in bacterial pathogenicity have focused on facultative intracellular bacteria, and in particular on those belonging to the *Salmonella* genus. Interest in the role of Cu,ZnSOD in *Salmonella* has been significantly enhanced by the observation that, in addition to the chromosomal *sodC* gene (*sodC2*) common to all Salmonellae, some highly virulent strains belonging to different serotypes contain a second *sodC* gene (*sodC1*) [15] which is located on the λ-like bacteriophage Gifsy-2 [16] and encodes the Cu,ZnSOD with the highest catalytic rate ever found in a natural enzyme of this class [18]. Moreover, an additional *sodC* gene (*sodC3*), carried by the prophage Fels1, has been identified in *Salmonella typhimurium* strain LT2 [19]. *sodC* genes contribute to *Salmonella* virulence, but studies carried out so far have not established whether *sodC1* and *sodC2* have additive roles or if their contribution to bacterial pathogenicity is the same in all *S. enterica* serotypes. A few studies have suggested that *sodC1sodC2* double mutants of *S. typhimurium* are less virulent than *sodC1* or *sodC2* single mutants [15,20]. However, a recent investigation suggested that most the contribution of Cu,ZnSOD to the murine salmonellosis induced by *S. typhimurium* should be ascribed to *sodC1* [21]. The differential contributions of *sodC1* and *sodC2* to the pathogenicity of *S. typhimurium* correlate with the differential accumulation of the two proteins in intracellular environments, where *sodC1* is up-regulated and *sodC2* is expressed at negligible levels [21]. Studies to elucidate the molecular mechanism underlying the differential regulation of the two *sodC* genes in *S. typhimurium* are in progress, but recent results obtained with a *Salmonella choleraesuis* strain indicate that the relative roles of the two *Salmonella sodC* genes might differ in distinct *Salmonella* strains and/or serovars ([9]; S. Ammendola, P. Pasquali, J.S. Kroll, P.R. Langford, G. Rotilio, P. Valenti and A. Battistoni, unpublished work).

Role of Cu,ZnSOD in bacterial survival within professional phagocytes

Several groups have investigated the role of Cu,ZnSODs in the resistance of facultative intracellular bacteria against the oxidative attack of phagocytes by comparing the differential survival of *sodC*-knockout mutants and wild-type cells. Some studies have failed to identify a contribution of *sodC* to the intracellular survival of *Legionella pneumophila* [5] and *Brucella abortus* [12,22], while contradictory results have been obtained by two different groups investigating the role of *sodC* in *Mycobacterium tuberculosis* [23,24]. However, the hypothesis that Cu,ZnSOD is involved in the mechanisms that facilitate the intracellular growth of *M. tuberculosis* is supported by the observation that *sodC* is up-regulated upon entry into human macrophages [3], and is repressed during mycobacterial non-replicating persistence in mice [25]. Conversely, *sodC*-negative mutants of *Neisseria meningitidis* [26] and of various serovars of *S. enterica* [7,9,20] have been shown to be killed more readily than their isogenic wild-type strains in phagocytic cells. Several hypotheses could explain such conflicting results, including variations in the robustness of the respiratory burst elicited by macrophages of different sources [24], the effects of different activation procedures on macrophage oxygen radical production, and the levels of Cu,ZnSOD activity expressed by different bacteria (which can rely on a large number of factors, including the availability of the enzyme metal cofactors).

To demonstrate the ability of a periplasmic Cu,ZnSOD to protect bacteria from the respiratory burst, we initially tested the resistance to intracellular killing within macrophages of non-pathogenic *E. coli* strains engineered to produce various amounts of Cu,ZnSOD [27]. These experiments demonstrated that wild-type cells expressing very low levels of the enzyme were much more sensitive to phagocytic killing than *E. coli* strains overproducing the enzyme. Moreover, some proportionality between the amount of Cu,ZnSOD expressed and bacterial survival was observed. This experimental approach also proved to be useful for testing the effects of mutations in Cu,ZnSOD on its anti-phagocyte activity [28].

The importance of carefully evaluating the experimental conditions used to assess the role of *sodC* in intracellular survival is exemplified by recent studies carried out in our laboratory (S. Ammendola P. Pasquali, J.S. Kroll, P.R. Langford, G. Rotilio, P. Valenti and A. Battistoni, unpublished work). We tested the intracellular survival of wild-type and *sodC*-negative strains of a *S. choleraesuis* strain in the murine macrophage-like cell line J774. We observed that opsonized *sodC1*-, *sodC2*- and *sodC1sodC2*-negative strains were equally impaired in their ability to survive within J774 cells, as already observed in porcine and murine alveolar macrophages [9]. However, when bacterial killing experiments were carried out with non-opsonized bacteria, differences in the survival of the mutant strains were observed. Non-opsonized *sodC1* mutants were as resistant as wild-type cells to intracellular killing, whereas the *sodC1sodC2* mutant strain was even more impaired than the *sodC2* mutant. The differential resistance of opsonized and non-opsonized Salmonellae to macrophage-mediated killing suggests that *sodC* genes could play different roles depending on the pathways leading to the internalization of bacteria into macrophages, as opsonization induces significant rearrangements in the molecular mechanisms underlying phagocytosis [29]. Alternatively, the increased intracellular survival of non-opsonized bacteria might suggest that opsonization affects the expression of *sodC* genes within macrophages, or activates bacterial killing pathways that are differentially dependent upon the production of oxygen radicals.

Role of Cu,ZnSOD in bacterial survival within epithelial cells

Several facultative intracellular bacteria can penetrate epithelial cells located at mucosal surfaces. Although the intracellular environment of such cells is usually considered

to be a favourable niche where the bacteria can multiply and evade the host's immune response, bacterial entry into intestinal cells induces inflammatory pathways that initiate events required for the clearance of the infecting micro-organism [30]. In this context, the discovery that a large number of non-phagocytic cells, including intestinal epithelial cell lines, can actively generate the superoxide anion by enzymic complexes resembling phagocytic NADPH oxidase [31,32] is of considerable interest. Production of oxygen radicals might be involved in the response to bacterial invasion, either by inducing cytokine production or by causing oxidative damage to intracellular bacteria. In support of this latter possibility, we have demonstrated that increased expression of the *sodC* gene enhances the survival of invasive *E. coli* strains within HeLa and Caco-2 cells, suggesting that production of oxygen radicals is involved in the mechanisms of bacterial killing in epithelial cells [33]. Pretreatment of HeLa cells with inhibitors of NADPH oxidase significantly enhances the intracellular survival of wild-type invasive *E. coli* cells [34]. In contrast, these drugs have no effect on the intracellular survival of an invasive *E. coli* strain engineered to overexpress Cu,ZnSOD (A. Battistoni, M. Ajello, S. Ammendola, G. Rotilio and P. Valenti, unpublished work). These results support the hypothesis that superoxide generation by an NAD(P)H oxidase-like complex can limit bacterial survival within epithelial cells.

To extend our observations on the possible role of *sodC* in bacterial survival in epithelial cells, we have studied the ability of wild-type and *sodC* mutants of *S. choleraesuis* to enter and survive within Caco-2 cells. All the strains tested entered epithelial cells with comparable efficiency. However, inactivation of *sodC2* significantly decreased bacterial viability at 48 h post-infection, and a further decrease in survival was observed for the double mutant, suggesting that *sodC1* also contributes, although to a lesser extent, to the survival of *Salmonella* within Caco-2 cells. These results highlight the importance of periplasmic Cu,ZnSOD in infections by facultative intracellular bacteria, and suggest that the role of this enzyme in bacterial pathogenesis should not be considered as being limited to the confines of the bacterium–phagocyte interaction.

Structural versatility of Cu,ZnSODs from bacterial pathogens

While all eukaryotic Cu,ZnSODs conform to a single structural model that appears to have been strictly preserved throughout evolution, analysis of amino acid sequences of Cu,ZnSODs from different bacterial species suggests much greater variation, so that individual enzyme variants may exhibit unique properties [35,36]. The most obvious differences between bacterial Cu,ZnSODs include insertions and deletions in some of the major loops protruding from the β-barrel, which could plausibly result in differences in active-site channel architecture and subunit assembly, and substitutions of some of the conserved metal ligands that are expected to significantly affect enzyme activity [36]. The functional implications of these variations are still to be explored, but it is likely that such differences may lead to modulation of enzyme activity in different bacteria. To evaluate this possibility, we have undertaken the characterization of novel variants of Cu,ZnSODs from pathogenic bacteria that show unique sequence features, indicative of significant structural differences not only with respect to eukaryotic Cu,ZnSOD, but also with respect to already characterized prokaryotic enzymes.

An interesting example is provided by the Cu,ZnSODs from *Haemophilus* spp. [28,37]. All of these enzymes are characterized by histidine-rich N-terminal extensions, whose sequence composition resembles that typical of several metal binding sites found in proteins able to bind transition metals. The presence of such N-terminal extensions modulates the antioxidant properties of periplasmic Cu,ZnSOD and facilitates bacterial survival within phagocytes. We have demonstrated by spectroscopic analyses and immobilized-metal affinity chromatography experiments that such histidine-rich domains bind the active-site metal ions with elevated affinities [28]. Under conditions of limited copper availability, the metal ion is bound initially at the N-terminal region and subsequently transferred to an active site, possibly by intermolecular pathways of transfer from the N-terminal domain of an enzyme subunit to an active site located on a distinct dimeric molecule [28]. Our results suggest that the histidine-rich N-terminal region constitutes a metal binding domain that can favour the uptake of the prosthetic metals in an environment where the concentration of free metal ions is low. This discovery is of particular interest in the light of recent findings showing that the availability of metal cofactors is also limited in media supplemented with copper and zinc, and that Cu,ZnSOD should compete with periplasmic metal transporting systems to obtain the cofactors essential for its catalytic activity (A. Battistoni and G. Berducci, unpublished work).

A further example of the structural flexibility of bacterial Cu,ZnSOD was provided by the demonstration that the enzyme from *Haemophilus ducreyi*, the causative agent of the genital ulcerative disease known as chancroid, is able to bind a single 6-co-ordinated haem moiety in the crevice below the dimer interface [37]. Histidine ligands provide the two haem-iron axial ligands, while several amino acid substitutions account for the formation of a high-affinity haem binding pocket in this specific enzyme variant. *H. ducreyi* is unable to synthesize haem, and must obtain this cofactor from host haemoglobin. It is likely that periplasmic Cu,ZnSOD plays a role in haem metabolism in *H. ducreyi*, possibly preventing damage due to oxyradicals formed upon the reaction of haem iron with oxygen.

In conclusion, our studies suggest that the structural flexibility of prokaryotic Cu,ZnSODs allows the acquisition of novel specialized functions in addition to superoxide dismutation, and adapt the enzyme to the requirements of bacteria colonizing hostile environments.

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