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INVITED REVIEW

THE MOLECULAR GENETICS OF SUPEROXIDE DISMUTASE IN *E. COLI*. AN APPROACH TO UNDERSTANDING THE BIOLOGICAL ROLE AND REGULATION OF SODS IN RELATION TO OTHER ELEMENTS OF THE DEFENCE SYSTEM AGAINST OXYGEN TOXICITY

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Superoxide-mediated oxidative stress initiates a chain of events resulting in numerous cellular injuries. We have used genetics and *E. coli* to investigate the role and regulation of superoxide dismutase (SOD) and its relationship with the other constituents of the oxygen toxicity defence system. Structural SOD genes have been cloned and sequenced, permitting us to refine structural analysis and to isolate SOD-deficient mutants. The conditional oxygen sensitivity of these mutants, together with their increased mutation rate, demonstrated the essential biological role of SOD. Furthermore the complementation of SOD-lacking *E. coli* deficiencies by introducing a plasmid containing the gene encoding the human SOD supported the proposal that superoxide dismutation is the physiological function of SOD. Regulation of the MnSOD, through which the global SOD level in *E. coli* is modulated, has been studied using operon and protein fusions with the lactose operon, and led to the conclusion of a multicontrol of MnSOD. Isolation and characterization of regulation mutants are in progress, with the aim of identifying effectors and targets involved in the response to superoxide-mediated oxidative stress.

KEY WORDS: Superoxide dismutase, *E. coli*, mutants, oxidative stress, mutagenesis, regulation.

INTRODUCTION

Although superoxide dismutase (SOD) is found in almost all aerobic organisms, [1,2] its essentiality and its role *in vivo* have been questioned for several years.[3,4] Genetics and *E. coli*, which remains the preferred organism studied by geneticists, were the appropriate tools to answer such a question.

SOD is only one of many constituents of the defence system against oxygen toxicity.[5] The system includes enzymatic and non-enzymatic factors which act sequentially or together. Thus, superoxide-mediated oxidative stress induces SOD, which, as it decreases the intracellular superoxide (O_2^-) level, increases the intracellular hydrogen peroxide (H_2O_2) concentration. This in turn induces catalase which eliminates H_2O_2, the excess of superoxide and hydrogen peroxide resulting in formation of hydroxyl radicals ($OH^\cdot$) which damage DNA.[5] In the absence of an efficient DNA repair process, this is deleterious to the cell.[6] Add to this very schematic description of

oxidative stress, the fact that enzymes are often sensitive to high concentrations of their substrate (thus, catalase is sensitive to H_2O_2) and that, although superoxide radicals generally constitute a threat to the cell, there are physiological situations in which they play a positive role,[7] it is clear that the equilibrium between these factors must be very subtle and rigorously controlled for cells to survive. Molecular genetics and *E. coli* provide convenient and powerful tools with which to untangle this complex system. This paper reviews the work in progress in our laboratory or in which we have substantially participated. It includes studies of various elements of the superoxide-mediated oxidative stress in *E. coli*, with emphasis on superoxide dismutase.

RESULTS AND DISCUSSION

E. coli, like many gram-negative bacteria, possesses two superoxide dismutases (SOD): an iron-containing SOD (FeSOD) and a manganese-containing SOD (MnSOD).[1] The isoenzymes are dimeric proteins with two active sites. The monomer folding of the iron protein is very similar to the folding of the manganese protein, so that an active hybrid protein can be formed from one subunit of each isoenzyme. Both enzymes appear to be located in the same cellular compartment, the cytoplasm. The maintenance, in spite of their relatedness, of those two isoenzymes in the cell during the course of evolution is presumably due to differences in their regulation. Under normal aerobic growth conditions, FeSOD and MnSOD have similar activities. However, whereas the activity of FeSOD appears to be oxygen-independent, MnSOD is not synthesized under anaerobic conditions and is over-produced in response to superoxide-mediated stress.[1]

Attempts to obtain SOD-defective mutants directly have been unsuccessful; we therefore adopted the strategy of cloning the SOD genes and constructing mutants from the cloned genes.

Cloning of the MnSOD and FeSOD Genes from E. coli

The choice of the tool for cloning was guided by two factors: (i) we had, at that stage, no way of selecting a clone carrying a SOD^+ gene, so that we could only recognize it by SOD over-production. We therefore needed a tool which minimized the number of clones to be screened. (ii) We did not know whether a too drastic increase in the intracellular level of SOD could be dangerous for the cells. Consequently, we chose a small plasmid for cloning, which permitted cloning of large DNA inserts (about 40 kb), with a relatively small number of hybrid cosmid copies.

About 400 clones from the *E. coli* cosmid library were screened for SOD over-production, as detected in crude extracts by SOD enzymatic assays or immunoprecipitation with specific antibodies, and two clones, one over-producing MnSOD[8] and one over-producing FeSOD[9] were isolated. Further subcloning, electrophoretic analysis and immunoprecipitation with specific antibodies, were used to demonstrate that the MnSOD (*sodA*) and FeSOD (*sodB*) structural genes had been cloned.

The *sodA* and *sodB* genes were mapped on the *E. coli* chromosome, and found to be at 88.3 minutes (*sodA*) and 36.2 minutes (*sodB*). Over-production of SOD from a sod^+ gene carried by a multicopy plasmid led to no obvious phenotype in wild type *E. coli* under normal growth conditions.

The sequence of *E. coli sod*$^+$ genes has been further established, by Takeda and Avila[10] for the *sodA* gene and in our laboratory for the *sodB* gene.[11]

Nucleotide Sequences and Structural Analysis

X-ray crystal structures of FeSODs and MnSODs show that they share a common polypeptide form which is completely unlike that of Cu/ZnSOD.[12] The determination of the *E. coli* FeSOD gene nucleotide sequence allowed us, in collaboration with W. Stallings and M. Ludwig, to refine the three dimensional model of the enzyme and to interpret details in the metal-ligand cluster and its environment.[11] Comparison with MnSOD structure established the indentity of the residues which serve as metal ligands and also indicated that the environments of the metal ligands were remarkably similar, with only one residue difference at three positions. This is puzzling since FeSOD and MnSOD from *E. coli* display metal selectivity both *in vitro* and *in vivo*.[1] Site-directed mutagenesis at the residues which differ might allow elucidation of the metal binding selectivity of these enzymes at the molecular level and help to understand its biological significance.

Mutants Lacking SOD Activity: Isolation and Properties

Rationale for obtaining mutants Mutations were made in the structural cloned *sod*$^+$ genes by insertion of Mu transposons. Several Mu transposons were used, conferring various antibiotic resistances. Random insertions were performed on plasmids overexpressing SOD, and plasmids carrying an insertion into the *sod*$^+$ gene were recognized as those in which the SOD level returned to the wild-type SOD level. The location of the insertion in the structural *sod*$^+$ gene was confirmed by restriction mapping.

Mutations were then introduced into the chromosome by exchange between the mutated *sod* allele carried by the plasmid and the corresponding chromosomal wild-type allele.

The resulting mutants were shown to lack the corresponding enzyme by visualization on electrophoresis gels, by measurements of enzyme activity, and by immunoblot analysis.[13,14]

Properties of mutants lacking SOD activity The use of transposons conferring different antibiotic resistances for the insertional mutagenesis, allowed each of the mutations (*sodA* and *sodB*) to be readily transferred to a wide variety of genetic backgrounds. This also permitted construction of a double mutant completely lacking SOD activity.

As it was widely held that a total lack of SOD might be lethal for cells exposed to oxygen, we attempted to construct the double mutant under both anaerobic and aerobic conditions. Both constructions succeeded; showing that, at least in certain conditions, *E. coli* can survive without superoxide dismutase.

However, physiological studies of the SOD-lacking mutants revealed a conditional sensitivity to oxygen, which renders the evolutionary survival of such organisms very unlikely in natural conditions.

SOD-lacking mutants behave as wild-type strain in anaerobiosis. These mutants grow poorly in rich medium in aerobiosis: slow growth and low saturation level suggest that the cells are seriously damaged. They are hypersensitive to oxygen and intracellular superoxide generators, such as paraquat or plumbagin. They are also

hypersensitive to hydrogen peroxide, presumably because the accumulation of superoxide radicals in SOD deficient cells favors the formation of hydroxyl radicals upon addition of hydrogen peroxide.[5]

The SOD-mutants do not grow in minimal medium unless all 20 amino acids are provided. The molecular nature of the defect(s) responsible for this phenotype is unknown. However, although all twenty amino acids are necessary to restore normal growth, some, such as the branched amino acids, are more effective than others, suggesting that there are targets with differential sensitivities to superoxide radicals, or their derivatives, in amino acid biosynthesis. It is noteworthy that only 10% of the SOD activity of a wild-type strain – expressed from a plasmid carrying the *sodA*$^+$ gene under foreign promotor control (*ptac*) – is sufficient to restore growth on minimal medium (unpublished results). New mutants that are able to grow aerobically on minimal medium, but which still lack SOD activity have been found.[15] Their phenotype could fit with a diminished ability of the cells to produce intracellular superoxide. Indeed, preliminary experiments show an attenuated phenotype (for instance, a slightly reduced sensitivity to paraquat or to H_2O_2). The intracellular superoxide concentration in these mutants might not reach the critical threshold below which growth on minimal medium is impaired. Finally, SOD-deficient strains of *E. coli* show greatly enhanced mutation rates during aerobic growth.[16]

All the deleterious effects observed in SOD-lacking mutants were oxygen-dependent and were reversed by SOD expressed from a plasmid carrying a *sod*$^+$ gene. This was shown both with plasmids carrying *E. coli* MnSOD or FeSOD genes, and with plasmids carrying the human copper-zinc SOD gene.[17] Recently cloned SOD genes from other species such as plants[18] have also been shown to complement *E. coli* SOD deficiency.

The physiological properties of the single mutants suggest that MnSOD and FeSOD have similar functions *in vivo*. The FeSOD would provide a constant standby defence, while MnSOD would adjust the response to an oxidative challenge (proposed by Fridovich[2]). Indeed, the two mutants, which have about the same SOD activity, show no difference in growth in rich or minimal medium under aerobic conditions. The growth of the *sodA* mutant, which is unable to increase its SOD activity in response to an oxygen challenge, is drastically impaired under oxidative stress, whereas the *sodB* mutant is only moderately affected. These results show that:

i) an excess intracellular flux of superoxide radicals not accompanied by an increase in SOD is deleterious to *E. coli* in various ways, including impairment of amino acid biosynthesis, and DNA damage. This supports the idea that superoxide must be eliminated very rapidly to insure maintenance of aerobic cells and that SOD plays this essential role.

ii) unrelated eukaryotic CuZnSOD can satisfactorily replace *E. coli* SOD in preventing damage caused by oxidative stress, providing evidence that the superoxide dismutation is the important *in vivo* function of these two different protein types.

iii) the presence of two SOD enzymes in *E. coli* is probably more related to adaptative than to specialized functions.

Potential Uses of SOD-Deficient Mutants

Invaluable information can often be obtained from assays in a genetic SOD-lacking background on any biological process which presuppose a role for O_2^- or SOD.[19] The

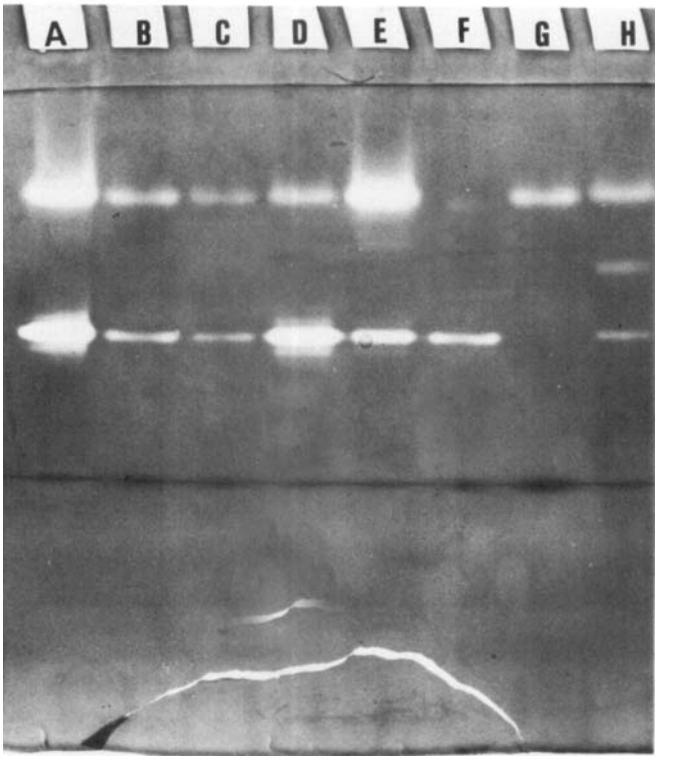

FIGURE 1 Hybrid SOD. Cultures of QC772 (*sodA*) and QC773 (*sodB*) strains were inoculated from fresh single colonies and grown to an OD of 1, chilled on ice, and mixed, in various proportions, for protein extraction. Crude extracts, prepared as previously described,[14] were loaded on a non-denaturing 7% polyacrylamide gel, run under 25 mA constant current, and stained for SOD activity. Lane H: wild-type strain, lane G: *sodB* mutant, lane F: *sodA* mutant. Lanes A, B, C, D and E were loaded with extracts of mixed cultures of *sodA* and *sodB* in the ratio 1 to 1 (A, B, C), 9 to 1 (D), 1 to 9 (E). Loaded protein amounts were: 70, 15, 10, 50, 50, 7.5, 7.5, 5 μg corresponding to A, B, C, D, E, F, G, H lanes, respectively.

complementation of SOD-negative mutant deficiencies by the expression of SOD originating from a different organism provides a tool for screening cDNA libraries for SOD genes in other species. The most convenient character to screen for is the complementation for growth on minimal medium. Selection of SOD-negative mutants able to grow on minimal medium can be avoided by adding 10^{-10} to 10^{-8} M paraquat to the minimal medium.

Since Dougherty *et al.*[20] discovered the "third *E. coli* SOD", the process of formation of this hybrid has been questioned. The absence of hybrid protein in crude extracts from single *sod* mutants cultures verified that the hybrid was formed from two different subunits encoded by each *sod* gene.[14] We (Touati and Carlioz) examined the formation of the hybrid protein in crude extracts from mixed single mutant cultures. As shown in Figure 1, no hybrid SOD was detected under conditions which reveal hybrid protein from wild-type *E. coli* cultures. This established that the hybrid protein formation is not an artefact of protein extraction, but is formed *in vivo*. A possible role

of this protein in modulating, by its formation, the whole cell SOD activity should not be neglected.

Superoxide and Mutagenesis

As mentioned above, SOD-deficient mutants exhibit an increased oxygen-dependent spontaneous mutation rate.[16] Under conditions of oxidative stress (e.g. 100% oxygen, challenge with intracellular superoxide generators, H_2O_2 or near UV irradiation[21]) mutagenesis is further increased. The mutagenesis does not result from induction by the DNA lesions of the repair-induced SOS response, but seems to be specific to DNA oxidative lesions. Indeed, it is largely dependent on a functional exonuclease III (encoded by the *xth* gene) which acts on DNA damaged by oxidizing agents. However, the repair mechanism which converts the oxidative DNA lesion to a mutation is unknown. We are now establishing the mutation spectrum of SOD-lacking mutants in collaboration with Abe Eisenstark. Its comparison with the spectra for spontaneous mutations or ionizing radiation-induced mutations could help to evaluate the contribution of superoxide radicals to these mutagenic processes.

Hydroxyl radicals are the only active oxygen species which have been proved to act directly on DNA *in vitro*. The question is whether the increased mutagenesis mediated by the elevated intracellular flux of O_2^- results from a direct action of O_2^- on DNA, or, as is more likely,[5] reflects an increase in hydroxyl radical formation due to the excess of O_2^-. There are a few observations in favor of the latter hypothesis. Both H_2O_2 and near UV (probably by generating H_2O_2) induced a large increase in mutations over and above the increase that occurs normally in the SOD minus mutants. This increase is also dependent on a functional exonuclease III, suggesting that the additional DNA lesions induced by H_2O_2 in SOD minus cells are similar to the preexisting ones. The exonuclease III-dependent increase above the spontaneous mutagenesis rate in SOD-lacking mutants, produced by elevated O_2^- or H_2O_2 intracellular concentrations, supports the hypothesis that both increasing mutagenesis result from DNA lesion produced by the same agent, probably the hydroxyl radical.

The SOD-lacking mutants constitute a unique tool for specifically assessing the role of superoxide radicals in the mutagenic activity of chemical agents or radiation. We

TABLE I

Spontaneous Tet^S to Tet^R forward mutations in SOD-lacking *E. coli*. Strains to be tested were transformed with the plasmid pPY98, a derivative of pBR322 in which the *tet* gene is under control of the *mnt*-regulated *ant* promoter of P22. Cells containing the wild type plasmid are Amp^R, Tet^S. Mutations in the *mnt* gene or its operator confer tetracycline resistance to the cell. Cultures were inoculated from single tetracycline-sensitive colonies, grown to early stationary phase and plated on LB plates containing 4 μg/ml tetracycline and 25 μg/ml ampicillin. Tetracycline-resistant colonies were counted after 17–18 h incubation at 37°C (further incubation did not modify the number of Tet^R colonies)

Strain	Tet^R colonies/plate[a]
GC 4468/pPY98	42
GC 4468 *sodA sodB*/pPY98	705
GC 4468 or GC 4468 *sodA sodB*[b]	0

(a) numbers are given for 10^9 cells per plate.
(b) plated without ampicillin.

have described several mutagenicity tests and the corresponding set of strains required to perform them.[22] We presently favour a test described by Lucchesi *et al.*,[23] in which forward mutations in a gene borne by a plasmid, confer tetracycline resistance to the strain (Table I). This test is easier to handle than earlier ones, but its use is somewhat restricted.

Regulation of MnSOD: the Oxidative Stress Response

Superoxide-mediated induction of MnSOD was first shown by Hassan and Fridovich.[24] It now appears that there is a coordinated global response to superoxide-mediated stress, including induction of defence and repair enzymes. It has been shown that numerous proteins are transiently over-produced after a challenge with an intracellular superoxide generator, or permanently over-produced in strains lacking SOD.[25,26] The functions of these proteins have not been identified, except for the repair enzyme endonuclease IV which is encoded by the *nfo* gene. Like exonuclease III, endonuclease IV recognizes oxidative lesions on DNA and cleaves DNA 5′ to AP sites.[27] This global response is independent of the other known stress regulatory responses, heat shock, hydrogen peroxide-mediated stress or the SOS response, which are controlled by the *htpR*, *oxyR* and *recA* gene products respectively. There may thus be specific regulatory response to superoxide radical-mediated stress, although there may be coordination with other responses, as some induced proteins are common to different stresses.

Any study of MnSOD regulation should therefore bear in mind that MnSOD is one element of a global response and that factors acting on MnSOD synthesis might act on several other superoxide-inducible functions, and thus contribute to the strain phenotype.

Our initial studies on MnSOD regulation were designed to establish the level at which regulation occurred.[28,29] We constructed tools to dissociate transcriptional, post-transcriptional and post-translational regulatory events. These tools constituted protein and operon fusions with the *lac* operon. In the former the *lacz* gene is fused to a fragment of the MnSOD structural gene *sodA* and is under the MnSOD transcriptional control; in the latter the MnSOD gene is transcribed from the foreign *tac* promoter, insensitive to oxygen. These tools, together with a plasmid battery carrying all or part of the *sodA* DNA region, were used to investigate the response to MnSOD inducers, the effect of metals (iron or manganese) on MnSOD activity, and to attempt to determine the type of control(s) of MnSOD expression. The data obtained suggested multicontrol of MnSOD, including: (i) transcriptional regulation via Fe^{2+}, compatible with the hypothesis of Hassan[30] (based on physiological data) of an iron repressor protein active when iron is in its Fe^{2+} state; (ii) superoxide-mediated activation of transcription; (iii) autogenous regulation; and (iv) post-translational modulation of the enzyme activity by manganese and iron, depending on the intracellular concentration of manganese ions.

Our present approach entails the isolation and characterization of regulation mutants to identify the effectors of MnSOD expression (trans-acting factors) and the DNA targets of these effectors in the MnSOD regulatory region (cis acting mutants). We have isolated (with H. Hassan) several regulation mutants and are in the process of characterizing, cloning and sequencing them. As expected from our previous studies, the mutants in the regulatory region define several DNA targets and the transcriptional regulation depends on several genetically-unlinked effectors.

CONCLUDING REMARKS

When we started this research we naively thought that we could focus on SOD and clearly establish its role in biological processes. As the work progressed, it became obvious that, although SOD plays an essential role in the defence mechanisms against oxygen toxicity, the balance between the different elements of the defence system might be even more crucial. We are becoming more and more convinced that many of the deleterious effects induced by an increase in superoxide radicals are due to their contribution to hydroxyl radical formation. This is very probable for DNA lesions, but might also be true for the membrane effects, since the disturbances of membrane transport after challenge with H_2O_2 or O_2^- are apparently identical.[31] Therefore, the intracellular H_2O_2/O_2^- ratio became critical in the cell. It is perhaps significant that the gene encoding the repair enzyme exonuclease III, on which the O_2^--mediated mutagenesis is largely dependent, is regulated by the same gene product (*katF*) as is the hydroperoxidase II gene, *katE*.[32] The biological significance of this coregulation is still unclear, but this shared regulation strengthens the idea of a coordinated regulatory system.

Clearly, an understanding of the biological role of superoxide dismutase implies an overview of all molecular elements interacting to protect the cells from the oxygen derivatives or to repair the damage they may cause. Only this knowledge will enable us to act on one or another of the elements without running the risk of completely disturbing the cell equilibrium.

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