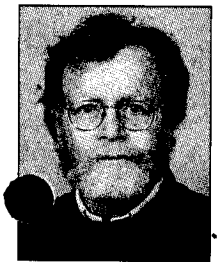
Catalase—an "Old" Enzyme That Continues To Surprise Us

The catalases are diverse in structure and play a variety of roles in microbial cells

Peter C. Loewen, Martin G. Klotz, and Daniel J. Hassett



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Catalase has been studied longer than any other enzyme, with the first biochemical characterization reported in 1900. This early attention arose from its striking phenotype of frothing oxygen evolution in the presence of its substrate H_2O_2. Summarized in standard notation, the reaction is deceptively simple:

$$2\ H_2O_2 \rightarrow 2\ H_2O + O_2 \quad (1)$$

Because of its prevalence in many mammalian tissues, particularly blood, the enzyme proved easy to isolate, facilitating its purification to provide sufficient protein for detailed biochemical studies. The heme chromophore provided a convenient tool for workers intent on studying the reaction mechanism, eventually leading to the definition of two distinct stages in the reaction pathway. The first step involves heme iron oxidation to form Compound I, an oxyferryl species with charge delocalized on the heme (reaction 2). The second step involves the reduction of Compound I using a second molecule of peroxide as the electron donor (reaction 3).

$$\text{Heme-Fe}^{III} + H_2O_2 \rightarrow \text{Heme-Fe}^{V}{=}O\ (\text{Cpd I}) + H_2O \quad (2)$$

$$\text{Heme-Fe}^{V}{=}O + H_2O_2 \rightarrow \text{Heme-Fe}^{III} + H_2O + O_2 \quad (3)$$

Although the enzyme proved easy to crystallize, its large size complicated an accurate solution of the crystal structure. The protein sequence of bovine liver catalase was determined in 1969, providing the impetus for the eventual crystal structure determination in 1981. With the reaction mechanism defined, the sequence known and the crystal structure defined, what more was there to learn about the enzyme? The answer has been quite a bit!

Diversity of Catalase Structure

At the structure and sequence level, catalases are not restricted to one protein type. Rather, catalases from different organisms have a broad range of subunit sizes, a variety of quaternary structures, at least two different prosthetic groups, and even substantially different sequences. Because of this diversity in sequence and function, catalases are assigned to one of three main groups (see table).

The first of these three groups, the monofunctional catalases, are produced by many bacteria, plants, fungi, and animals, and include the well-characterized bovine liver catalase. Typically, the monofunctional catalases display minor peroxidase activity, and target molecules are limited to small organic substrates. This group of enzymes can be further subdivided on the basis of subunit size into catalases with small (55- to 69-kDa) and large (75- to 84-kDa) subunits. Associated with the size differential is a difference in heme prosthetic group, with heme *b* being present in small-subunit enzymes and heme *d* being present in large-subunit enzymes (table). Generally, the monofunctional catalases are active as tetramers, but dimers, hexamers, and even an unusual heterotrimer structure (from *Pseudomonas aeruginosa*) are found.

The second group—the catalase-peroxidases—thus far identified only in bacteria and fungi, resemble plant and fungal peroxidases in sequence but have larger subunits (~80 kDa) than do those enzymes. Because the catalase-peroxidases contain two similar, fused domains—one

be integrated within a common experimental background. The second sense of the word "standard" is that the system should be a benchmark against which new results are measured. If a result becomes a "standard," then future work would include this result as part of the description of the system.

I am working with a cell line that may be a candidate for serving as a standard cell line with which to study the mammalian division cycle. This suggestion is put forward not as an ultimate solution to all the problems in this field, but as an example of the characteristics which make this cell line a good candidate for serving as such a standard system.

The particular cells I nominate for a standard cell line are those of the mouse lymphocytic L-1210 line. These cells grow in suspension culture just like bacteria. They do not have to be cultivated in a CO_2 atmosphere and grow well in the well-buffered L-15 (Liebovitz) medium. Strain L-1210 has a doubling time of 11.5 hours (measured in the laboratory using time-lapse videomicroscopy). Thus, experiments on growth can be done easily and quickly. This strain can be studied both by time-lapse videography, as well as by the mammalian "baby machine" that Charles Helmstetter at the Florida Institute of Technology, Melbourne, has recently developed. The new method, adapted from the successful bacterial "baby machine" also developed by Helmstetter, produces extremely well-synchronized mammalian cells. Using such a cell "baby machine," mammalian cells can readily be synchronized for three successive cycles. The growth of L-1210 cells is very robust; they are not sensitive to contact inhibition as are other adherent mammalian cells.

The ease of growth and manipulation of strain L-1210 makes it well suited for the study of the mammalian division cycle. I suggest that the basic division cycle mechanisms are conserved in all mammalian cells, and therefore that this simple system can be used to study the general phenomenon of the basic division cycle. Even though there are many biological phenomena that cannot be studied in such a cell line—for example, lipid accumulation, thyroxine synthesis, and antibody production—L-1210 remains a good candidate for the study of the mammalian cell cycle. Other strains that appear to be suitable candidates for a standard system are the human leukemic lines MOLT-4 and U937.

I hope that the problems described here regarding the diversity and nonreproducibility of phenomena in the study of the mammalian cell cycle lead to a more rigorous reexamination of the basic systems being used by cell biologists. As the bacterial examples—T-even phage growth, β-galactosidase synthesis, and steady-state bacterial growth—show, when a large number of investigators agree to study a system that is simple, reproducible, and controllable, rapid advances in understanding are possible.

SUGGESTED READING

Cairns, J., G. S. Stent, and J. D. Watson. 1966. Phage and the origins of molecular biology. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Cooper, S. 1991. Bacterial growth and division. Academic Press, San Diego, Calif.

Cooper, S. 1993. The origin and meaning of the Schaechter-Maaloe-Kjeldgaard experiments. J. Gen. Microbiol. **139**:1117–1124.

Cooper, S. 1998. Mammalian cells are not synchronized in G1 phase by starvation or inhibition: considerations of the fundamental concept of G1-phase synchronization. Cell Prolif. **31**:9–16.

Cooper, S. 1997. DNA replication: the 30th anniversary of the bacterial model and the 'baby machine.' Trends Biochem. Sci. **22**:490–494.

Cooper, S. 1999. The continuum model and G1-control of the mammalian cell cycle, p. 27–39. *In* L. Meijer, S. Guidet, and M. Philippe (ed.), Progress in cell cycle research, vol. 4. Plenum Press, New York.

Judson, H. F. 1979. The eighth day of creation. Simon and Schuster, New York.

Schaechter, M., F. C. Neidhardt, J. L. Ingraham, and N. O. Kjeldgaard. 1984. The molecular biology of bacterial growth. Jones and Bartlett, Boston.

Categories of catalases

Class	Example	Subunit	Multimer	Heme
Monofunctional catalases				
Small subunit	Bovine liver	57,500	Tetramer	*b*
Large subunit	*Escherichia coli* HPII	84,172	Tetramer	*d*
Catalase-peroxidases	*Mycobacterium tuberculosis* KatG	80,029	Dimer	*b*
Non-heme catalases	*Lactobacillus plantarum*	28,300	Hexamer	–

retaining activity and the other inactive—the genes encoding this group of enzymes appear to have evolved through a duplication event. Enzymes in this group bear no resemblance to the monofunctional catalases in sequence, but the catalase-peroxidases do contain heme *b* and are active as dimers or tetramers.

In *Mycobacterium tuberculosis*, the catalase-peroxidase, KatG, has gained notoriety as the mediator of isoniazid antibiotic activity. The enzyme uses isoniazid as a peroxidatic substrate creating an activated form of the drug (either isonicotinic acyl anion or isonicotinic acyl radical) that is covalently attached to the nicotinamide adenine dinucleotide ring within the active site of InhA, an enzyme essential for mycolic acid biosynthesis. Mutation of *katG*, encoding the enzyme, leads to isoniazid resistance and is found commonly among clinical isolates.

The third group, consisting of non-heme catalases, currently contains the smallest number of examples, with only three enzymes from different bacterial species characterized and an equal number sequenced. Activity is derived from a manganese-rich reaction center rather than a heme group. This lack of a heme distinguished these enzymes from other catalases and led to them being called "pseudo-catalases."

Catalases are not essential for growth and survival of microbial cells under common laboratory conditions, but they play important roles in a number of environmental situations

Catalases Play Diverse but Not Always Essential Roles in Microbial Cells

Catalases are not essential for growth and survival of microbial cells under common laboratory conditions, but these enzymes play important roles in a number of environmental situations. For example, in *Vibrio fisheri*, KatA is required for bacterial colonization of the squid light organ (*ASM News*, November 1998, p. 639); *Pseudomonas putida* mutants deficient in catalase are less able to colonize root surfaces; and catalase is implicated in protecting cells against damage by high-intensity light in plants. In an instance of a symbiotic relationship based at least in part on catalases, the catalase-deficient nematode *Onchocerca volvulus* utilizes the catalase from an obligate endobacterium in the *Rickettsiales* family (*Wolbachia* sp.) to detoxify peroxides.

Catalases sometimes play an important role in bacterial virulence—typically, by combatting the microbiocidal "oxidative burst" of human phagocytes, in which a membrane-bound NADPH oxidase generates copious quantities of the one-electron reduction product of molecular oxygen, superoxide (O_2^-). The low pH within the phagolysosomes results in virtually all of the O_2^- being converted to H_2O_2 (nearly 100 mM), which is lethal to bacteria lacking catalase.

For instance, the important nosocomial pathogen *Staphyloccocus aureus* depends on catalase for intraperitoneal survival in mice. Killing by polymorphonuclear phagocytes of the soil-dwelling *Nocardia asteroides* and *Nocardia* sp., which can cause pulmonary infections of immunocompromised hosts, is reduced when catalase levels are increased. A homozygous null catalase CAT1 mutant of the yeast *Candida albicans*, also a pathogen of the immunocompromised, is dramatically more sensitive to damage by human neutrophils and less virulent in mice with disseminated candidiasis. The catalase activity

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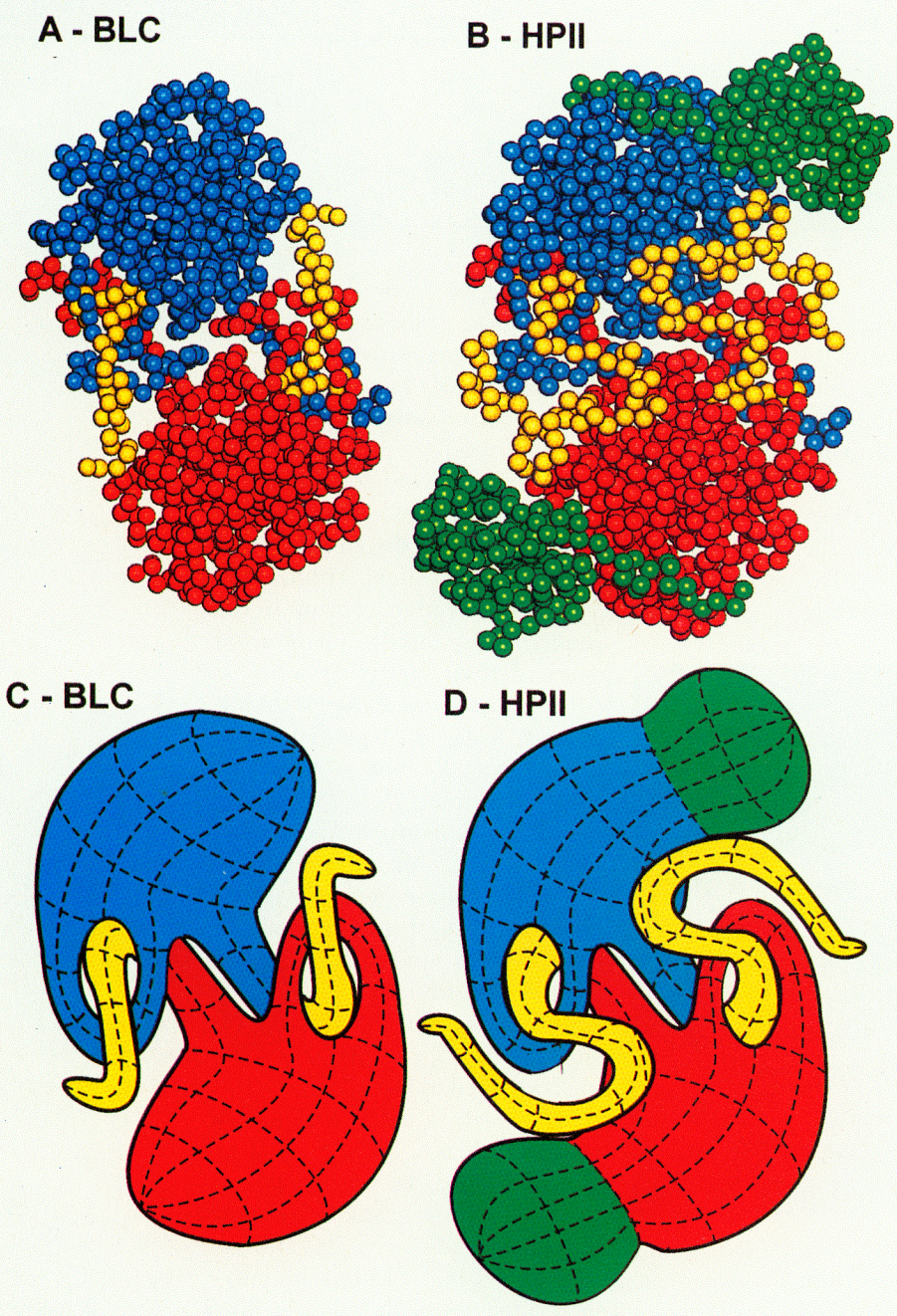
FIGURE 1



Comparison of the structures of the intertwined dimers of monofunctional catalases with small (A - BLC) and large (B-HPII) subunits. The core of the subunits with a common structure and similar sequence is shown in either blue or red. The C-terminal extension on HPII is shown in green in B. The yellow segments are the N-terminal portions of both enzymes that are fed through a loop on the adjacent subunit, 25 residues in the case of BLC and 80 residues in the case of HPII. Panels C and D contain a cartoon to illustrate the interwoven structure.

of *Pseudomonas aeruginosa*, an opportunistic pathogen principally as a chronic colonizer of the airways of patients afflicted with cystic fibrosis (CF), has been correlated with the levels of pyocyanin in CF sputa. Finally, in addition to its notoriety as the activator of isoniazid as an antibiotic, the catalase-peroxidase, KatG, is essential for virulent *M. tuberculosis* H37rv to persist in spleens of mice and guinea pigs.

However, although widespread, the need for catalase in bacterial virulence is by no means universal. In *Salmonella typhimurium*, for instance, virulence in mice is not affected by the elimination of catalase, whereas DNA repair processes are essential for virulence. Similarly, DNA repair systems in *Escherichia coli* including *recA* (RecA protein), *recB* (RecBCD enzyme), *xth* (exonuclease III), *polA* (DNA polymerase), and *dps* (DNA binding protein from stationary phase) are significantly more important for survival against H_2O_2 than is catalase.

The intracellular location of catalases, whether periplasmic or cytoplasmic, apparently has an impact on their physiological roles. A preponderance of catalases and catalase-peroxidases are cytoplasmic, but others apparently are periplasmic, including *V. fisheri* KatA, *Brucella abortus* Kat, and *P. syringae* CatF. The periplasm is also a common locale for superoxide dismutase and a combination of the two enzymes in the periplasm, preventing access of active oxygen species to sensitive inner membrane components and the cytoplasm, may be an effective alternative cellular repair process.

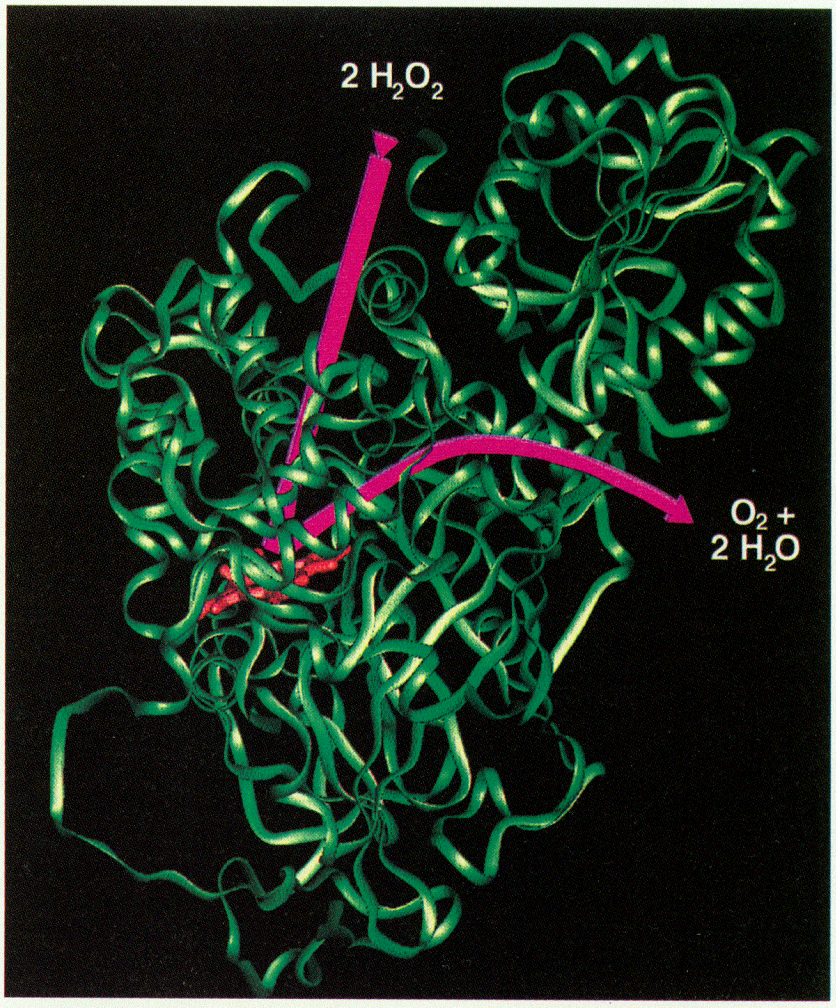
The crystal structures of six monofunctional catalases have been solved, including representatives from the animal (bovine liver catalase or BLC), small-subunit fungal (*Saccharomyces cerevisiae* SCC-A), large-subunit fungal (*Penicillium vitale*), small-subunit Group III bacterial (*Proteus mirabilis* and *Micrococcus lysodeikticus*), and

rge-subunit Group II bacterial (*E. coli* HPII). All six enzymes share a common β-barrel core structure containing the heme. Access to the deeply buried heme is via narrow channels that restrict access to small molecules explaining, at least in part, the weak peroxidatic activity among catalases.

The large-subunit enzymes differ from their small-subunit counterparts in having extensions at both the amino and carboxyl ends (Fig. 1). The N-terminal extension in HPII participates in an extensive series of interactions that are important in stabilizing the quaternary structure. The C-terminal extension is folded into a flavodoxin-like structure with no apparent function, although its removal interferes with folding and quaternary structure formation.

All of these catalases exhibit a unique pseudoknot or interweaving such that the N-terminal end of each subunit is overlapped or extends through a loop formed by the C-terminus of the adjacent subunit (Fig. 1). In the case of the small-subunit enzymes, about 25 residues are trapped, but in large-subunit enzymes over 80 residues are trapped. This feature contributes to the stability of the quaternary structure to an extent that HPII dimers do not dissociate at temperatures up to 95°C. This structural feature also imposes some very strict requirements on the folding pathway in which the core of the subunit fully folds only after dimers have associated.

FIGURE 2



A cartoon showing the approximate locations of the two channels that provide access to the active site heme (shown in red). The channel perpendicular to the heme is shown as providing an inlet for the substrate, and the channel that exits laterally from the heme is shown as the exhaust channel for the products.

In terms of their catalytic activities, catalases have very fast turnover rates (>10^5/s generally and 3.6 x 10^5/s for BLC at 300 mM H_2O_2), suggesting the presence of both inlet and outlet routes to prevent interference between incoming H_2O_2 and exhausting O_2. Indeed, two channels have been identified (Fig. 2). One channel approaches the active site perpendicular to the plane of the heme. It is 35 Å in length in small-subunit enzymes and 50 Å long in large-subunit enzymes. A second channel extends laterally at 90° to the first channel and is 35 Å long in both rge- and small-subunit enzymes. Recent evidence indicates that the lateral channel can provide access for inhibitors in HPII, raising questions about additional functions of the respective channels.

The catalase-peroxidases have so far resisted crystallization, and X-ray crystallographic information about their three-dimensional structure is not yet available. However, the sequence similarity of the catalase-peroxidases with plant and fungal peroxidases has provided some clues to the identity and location of key active site residues. Although the crystal structure of one Mn-containing or non-heme catalase was determined, its protein sequence has not been analyzed, meaning the overall picture remains vague.

Regulation of Catalase Biosynthesis

Cells synthesize catalase in response to a number of different stresses, with the precise pattern of synthesis being species dependent. Hassan and Fridovich investigated the regulation of catalase synthesis in *E. coli* more than 20 years ago, and the two decades since then have seen similar investigations applied to many other bacteria. Cells typically make more catalase as a response to oxidative stress. For example, an increase in the intracellular steady-state level of H_2O_2 in aerobically grown *E. coli* of ~0.2 μM induces synthesis of the catalase-peroxidase HPI, a component of the OxyR regulon.

Cells also may make more catalase as part of a generalized stress response, including to starvation, acid shock, and hypertonic shifts. For example, *E. coli* responds to starvation and the transition to stationary phase with the synthesis of HPII, as part of the RpoS regulon, which is regulated by a complex interplay of transcriptional, translational, and posttranslational processes. Variations on these two themes, response to oxidative stress and response to other stresses, are found throughout the prokaryotes with each organism presenting variations on this pattern presumably modified by environmental demands.

Catalase synthesis in eukaryotes ordinarily is regulated as part of very general responses to physiological and environmental circumstances. For example in yeast, the peroxisomal catalase SCC-A is synthesized along with other components found in this organelle, with promoter elements responding to glucose repression and activation by fatty acids. The second yeast catalase, SCC-T, is induced by a range of stress factors such as starvation, heat, high osmolarity, and H_2O_2. In plants, there are multiple catalases–for example, three in maize—and developmental expression often follows complex and individually unique patterns. In addition, environmental stresses such as pathogenesis, radiation, hormones, temperature extremes, oxygen extremes, and H_2O_2, also influence catalase expression.

The early stages in the assembly of catalases in bacteria are part of a complex process involving condensation of iron into protoporphyrin IX. Ferrochelatase catalyzes that condensation, forming heme, which is incorporated into the β-barrel core of a catalase subunit. This process requires the coordination of iron acquisition/storage, heme synthesis, and catalase gene transcription/translation.

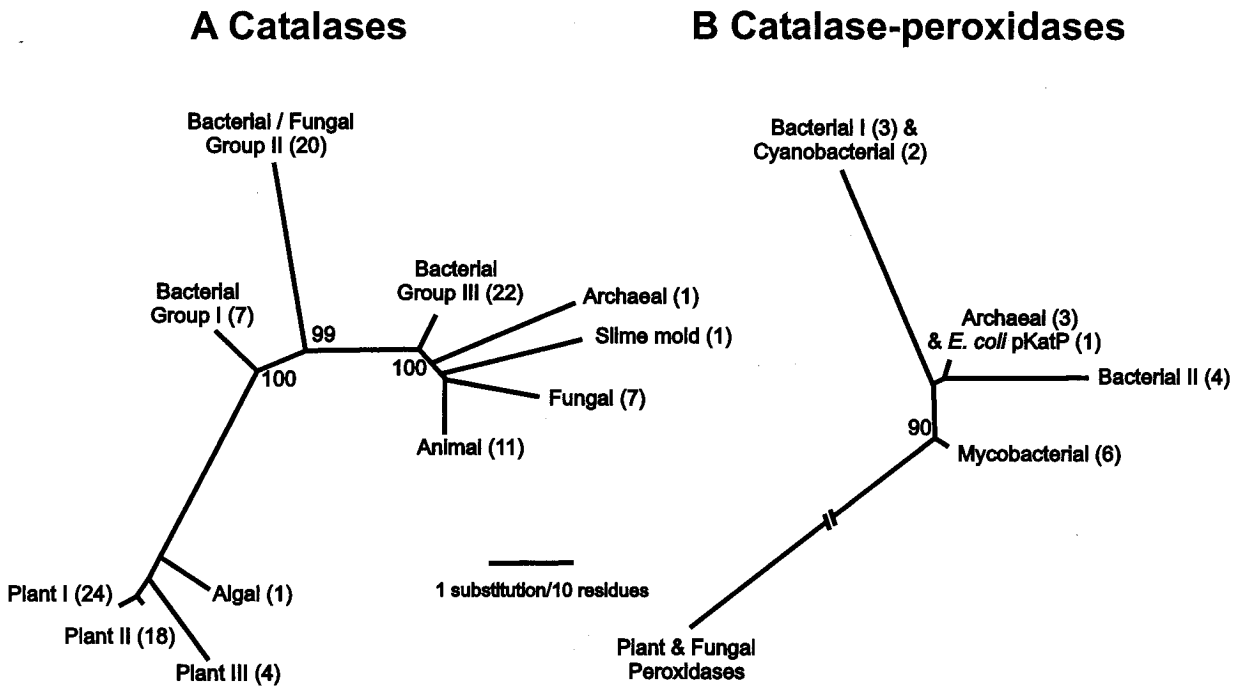
In some microorganisms, the iron storage proteins ferritin and bacterioferritin apparently play a role in this complex assembly process. For instance, *E. coli* and *Campylobacter jejuni* mutants lacking ferritin and bacterioferritin show increased sensitivity to H_2O_2 (only *E. coli*) and the redox-cycling antibiotic paraquat. The link between ferritin and catalase is further suggested by the proximity of *bfrA*, which encodes bacterioferritin A in *P. aeruginosa*, to *katA*, the major catalase locus, and by the 50% lower KatA activity, despite normal *katA* translational activity, in *bfrA* mutants. Because a similar *katA-bfrA* genetic arrangement exists in *P. putida* and a *Wolbachia* species (Fig. 1), we speculate that this proximity provides a "feeding" mechanism by which much of the iron from these bacterioferritins is earmarked for the heme group of catalase.

Posttranslational modifications provide catalases with a treasure trove of unusual features, not all of whose functions are understood

Posttranslational modifications provide catalases with a treasure trove of unusual features, not all of whose functions are understood. As early as 1984, investigators learned that NADPH is incorporated into bovine liver catalase, and speculated that this cofactor prevents the buildup of an inactive species called Compound II. The large-subunit catalases do not contain this nucleotide cofactor but also do not form significant amounts of Compound II.

Bovine liver catalase and all small subunit enzymes contain heme *b* or protoheme IX. In the two large-subunit catalases, HPII and *P. vitale* catalase, the heme is oxidized to a cis-spirolactone heme *d* derivative after assembly of the complete enzyme in a reaction requiring H_2O_2. In addition to being oxidized, the heme in the large-subunit enzymes is flipped 180° relative to its orientation in small-subunit enzymes. Curiously, the heme *d* structure is not essential for catalytic activity in HPII, and mutant variants with heme *b* exhibit near-wild-type specific activity. Adjacent to the essential active

FIGURE 3



Phylogenetic trees based on the amino acid sequences of 116 bacterial monofunctional catalases (A Catalases) and 19 catalase-peroxidases (B Catalase-peroxidases) constructed by parsimony and distance methods. The lengths of the branches are a reflection of the evolutionary distances as the average number of amino acid changes over a given length. The numbers at the nodes (only values greater than 80% are shown) represent the proportion of bootstrap samplings that support the topology shown.

site histidine, the small-subunit catalase from *P. mirabilis* contains a methionine residue that is oxidized to a sulfone. Like so many of the modifications in the catalases, this change is not essential to activity and not even present in most catalases. Another sulfur modification found in HPII involves one of the two cysteines being blocked by an alkali labile group with an uncharacterized mass of approximately 42 Da that is nonessential to enzyme function.

Perhaps the most unusual modification, not reported in any other protein, is the covalent linkage between the N^{δ} of His-392 and C^{β} of the essential Tyr-415 (fifth ligand of the heme) on the proximal side of the heme. The formation of this novel bond can be linked mechanistically to the heme oxidation, but, once again, the bond is not essential for enzyme activity in some mutant variants of HPII or the *P. vitale* catalase.

Yet another unusual linkage between a cysteine and the imidazole ring of the active site histidine has been characterized in a mutant variant of HPII. The modification presumably inactivates the enzyme by interfering with the ability of the imidazole to participate in the catalytic mechanism.

Molecular Diversity and Phylogenetic Relationships

Phylogenetic analysis of the core sequence from 116 monofunctional catalases produces a striking tree with separate unique groups of plant and animal catalases, two groups of fungal catalases, three groups of bacterial catalases, and a single archaeal catalase. The central core of the tree is supported at extremely high confidence limits (Fig. 3A). The only overlap among kingdoms within a single catalase group in this tree involves the large-subunit enzymes that are found in fungi and bacteria (group II catalases). Each of the three groups of bacterial catalases contain enzymes from diverse taxa, inconsistent with rRNA-based species phylogeny.

The tree can be interpreted in terms of a minimum of two gene duplication events that

produced a minimum of three catalase gene family members. Subsequent divergence of the catalases occurred during species evolution, and the loss of loci particularly in eukaryotes was the result of environmental pressures and genome economization. Two species of bacteria, *P. aeruginosa* and *Bacillus subtilis*, contain representatives from each of the three bacterial catalase groups (I, II, and III in Figure 3A), lending further credence to this proposal. The fact that these two species are found in many aerobic niches provides a likely explanation for the maintenance of the three enzymes in one organism.

The presence of a fourth catalase gene in *B. subtilis*, *ydbD*, encoding a Mn- or non-heme catalase illustrates the apparent importance of catalase for survival in these environments. *Methanosarcina barkeri* is, so far, unique among the archaebacteria in containing a catalase gene, and additional examples are needed to determine whether they form a distinct group. Little evidence for horizontal gene transfer is evident from this analysis, although one plasmid-encoded catalase diverges significantly from the AT/GC ratio of its host's genome.

The 19 catalase-peroxidases, all from bacteria or archaebacteria, are unrelated in sequence to the monofunctional catalases and are also separated from the distantly related ascorbate and cytochrome *c* peroxidases of plant and fungal origins (Fig. 3B). There are obvious groupings of archaeal and mycobacterial enzymes but not a clear definition of any other branches, and the confidence levels for the various groupings remain low. Although the plasmid-encoded KatP isolated in *E. coli* is found in the archaeal clade, the AT/GC content of the gene does not suggest donation from any of the three archaeal species in the tree.

SUGGESTED READING

Bravo, J., I. Fita, P. Gouet, H. M. Jouve, W. Melick-Adamyan, and G. N. Murshudov. 1997. Structure of catalases. *In* J. G. Scandalios (ed.), Oxidative stress and molecular biology of antioxidant defenses. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Deisseroth, A., and A. L. Dounce. 1970. Catalase: physical and chemical properties, mechanism of catalysis and physiological role. Physiol. Rev. 50:319–375.

Herbert, D., and J. Pinsent. 1948. Crystalline bacterial catalase. Biochem. J. 43:193–202.

Klotz, M. G., G. R. Klassen, and P. C. Loewen. 1997. Phylogenetic relationships among prokaryotic and eukaryotic catalases. Mol. Biol. Evol. 14:951–958.

Loewen, P. C. 1997. Bacterial catalases, p. 273–308. *In* J. G. Scandalios (ed.), Oxidative stress and molecular biology of antioxidant defenses. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Murthy, M. R. N., T. J. Reid III, A. Sicignano, N. Tanaka, M. G. Rossmann. 1981. Structure of beef liver catalase. J. Mol. Biol. 152:465–499.

Ruis, H., and F. Koller. 1997. Biochemistry, molecular biology, cell biology of yeast and fungal catalases, p. 309–342. *In* J. G. Scandalios (ed.), Oxidative stress and molecular biology of antioxidant defenses. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Scandalios, J. G., L. Guan, and A. N. Polidoros. 1997. Catalase in plants: gene structure, properties, regulation, and expression, p. 343–406. *In* J. G. Scandalios (ed.), Oxidative stress and molecular biology of antioxidant defenses. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Zhang, Y., B. Heym, B. Allen, D. Young, and S. Cole. 1992. The catalase-peroxidase gene and isoniazid resistance of *Mycobacterium tuberculosis*. Nature 358:591–593.