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Regulation of hydroperoxidase (catalase) expression in *Escherichia coli*

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Abstract

As part of its adaptive response to oxidative stress, *Escherichia coli* produces two inducible hydroperoxidases called HPI and HPII. Upon exposure to sublethal levels of hydrogen peroxide, HPI expression is induced at the transcriptional level by OxyR, a member of the LysR family of autoregulators. OxyR, functioning as both a sensor and transducer, contains a critical redox-sensitive Cys residue that is oxidized by hydrogen peroxide. This is thought to induce a conformational change in the tertiary structure of the OxyR tetramer altering its DNA-binding specificity and resulting in an increase in the transcription of *katG* and several other OxyR-dependent genes. In contrast, synthesis of the HPII enzyme is not induced by hydrogen peroxide. Expression of both HPI and HPII is growth phase-dependent levels of HPI and HPII are 10-fold higher in stationary phase than exponential phase cultures. These growth phase-dependent increases are largely dependent on RpoS, a stationary phase specific sigma factor that is itself subject to complex transcriptional and post-transcriptional controls. Several metabolic signals have been proposed to activate the RpoS regulon including hyperosmolarity, weak acids, homoserine lactone and UDP-glucose. Since both HPI and HPII are members of the RpoS regulon, elucidation of the mechanism of regulation of RpoS should contribute to our general understanding of hydroperoxidase regulation.

Keywords: *Escherichia coli*; Catalase; Hydroperoxidase; Stationary phase; Regulation; Oxidative stress; Hydrogen peroxide

1. Introduction

Hydroperoxidases, or catalases, are an integral component of the bacterial cell's response to oxidative stress. Together with superoxide dismutase and alkyl hydroperoxidase, hydroperoxidases are thought to limit the accumulation of reactive oxygen species. Hydroperoxidases reduce the intracellular concentration of hydrogen peroxide by catalysing the conversion of hydrogen peroxide to water and oxygen (Reaction I) or by oxidizing an intracellular reductant using hydrogen peroxide (Reaction II). Perhaps more importantly, elimination of hydrogen peroxide may also reduce the potential for transition metal-mediated OH · radical formation mediated by a Fenton-type reaction (Reaction III).

$$\text{Reaction I } 2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2$$

$$\text{Reaction II } AH_2 + H_2O_2 \xrightarrow{\text{Peroxidase}} 2H_2O + A$$

$$\text{Reaction III } Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH\cdot$$

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Biological sources of hydrogen peroxide are varied: hydrogen peroxide itself may be produced endogenously through the action of superoxide dismutase, the spontaneous dismutation of the superoxide anion or from the partial reduction of oxygen by the electron transport chain of actively respiring cells. Sources of exogenous hydrogen peroxide may include other competing bacteria in the intestinal tract, particularly lactobacilli, or the NADPH oxidase-catalysed respiratory burst of neutrophils. The relative importance of these various sources as mediators of oxidative stress has not been established. Possession of hydroperoxidase probably affords protection against all of these sources.

2. Structure and evolution

Escherichia coli produces two hydroperoxidases, called HPI and HPII, which differ in structure and kinetic properties (Table 1). HPI is expressed under both aerobic and anaerobic conditions and its synthesis is induced when cells are exposed to sublethal levels of hydrogen peroxide [1,2]. In contrast, HPII is produced at low levels when cells are grown anaerobically or are in exponential phase and is the principal hydroperoxidase in aerobically grown, stationary phase cultures [2]. HPI and HPII are distinct proteins. While HPI is composed of four identical 80-kDa subunits [3], HPII is a hexameric protein composed of 93-kDa subunits [4]. HPI is structurally similar to other peroxidases while HPII, despite its larger than normal subunit size, shares homology with the catalases of other organisms [5]. Despite DNA sequence similarities, HPII is in a phylogenetically unique group, separate from the catalases of other bacteria and of higher organisms [5].

3. Regulation of hydroperoxidase expression

3.1. Properties of catalase mutants

The availability of hydroperoxidase-deficient mutants has greatly aided studies of hydroperoxidase function and regulation and has facilitated the isolation of structural and regulatory genes that control catalase expression in *E. coli*. Genetic screens to isolate catalase deficient mutants are based on either hydrogen peroxide sensitivity (to isolate HPI-deficient mutants) or using a plate assay for catalase activity (to isolate HPII-deficient mutants). Wild-type *E. coli* colonies evolve gas bubbles when flooded with hydrogen peroxide while hydroperoxidase mutants, especially those deficient in HPII synthesis, exhibit reduced gas evolution. Several studies [5–9] have exploited this phenotypic difference to isolate point and insertion mutations in genes encoding structural and regulatory genes involved in *E. coli* hydroperoxidase biosynthesis. Other than a marked sensitivity to reactive oxygen species such as hydrogen peroxide, there is no distinguishing phenotype associated with a deficiency in hydroperoxidase synthesis in *E. coli*. However, mutations in regulatory genes controlling hydroperoxidase expression cause pleiotropic effects.

3.2. Regulation of HPI expression

HPI is a minor hydroperoxidase of *E. coli* in terms of total catalase activity but it is the most important cellular determinant for hydrogen peroxide resistance. Unlike HPII, HPI possesses both catalase and peroxidase activity [7]. An intracellular reductant that can be peroxidatively oxidized by HPI has not yet, however, been identified. The nucleotide se-

Table 1
Properties of hydroperoxidases of *Escherichia coli*

Hydroeroxidase	Enzymic activity	Subunit size (M_r)	Structure	Heme/subunit	K_m (μM)	V_{max} (μmol min^{-1} mg^{-1})	Structural gene
HPI	Catalase Peroxidase	80049	Tetramer	1/2 mol Protoheme IX	3.9	1496	*katG*
HPII	Catalase	93000	Hexamer	1 mol heme *d*		8924	*katE*

quence of the structural gene for HPI, *katG*, which maps to 89.2 min on the *E. coli* chromosome has been determined [3]. HPI hydroperoxidase is a member of a group of approximately 30 proteins that are induced when cells are exposed to sublethal levels of hydrogen peroxide [1]. A subset of this regulon, including HPI, is controlled at the transcriptional level by OxyR [1], a member of the LysR family of autoregulatory transcriptional activators [10]. The OxyR protein, under normal conditions, is inactive with respect to *katG* transcription. However, under conditions of oxidative stress (e.g. in the presence of hydrogen peroxide), a specific cysteine residue (Cys-199) in the OxyR protein is oxidized causing a conformational change in OxyR [11]. It is this modified form that functions as a transcriptional activator of the OxyR regulon. In the case of *katG*, transcription is more than 100-fold higher when OxyR is in the oxidized form [12]. Interestingly, OxyR also regulates its own expression by repressing transcription of the *oxyR* gene—both the reduced and oxidized forms of OxyR are equally effective as repressors [12]. As a consequence of this autoregulation, the amount of the regulator produced in the cell is relatively constant and activation of the OxyR regulon is caused almost solely by oxidation of OxyR to the active form ([12]; Fig. 1). Hydrogen peroxide-mediated induction of *katG* transcription by OxyR is probably transient in nature since the redox-sensitive regulator protein is thought to be readily reduced to the inactive form in the cell [12], lasting only until hydrogen peroxide has been consumed or decomposed by catalase. This may explain why oxidized OxyR, which can stimulate transcription of *katG* up to 100-fold in vitro [12], has a much more modest effect on HPI expression in in vivo experiments [1].

OxyR is thought to function as a tetramer under both reducing and oxidizing conditions with each monomer binding to an ATAGnt motif in the promoter region of OxyR regulated genes [13]. In the oxidized form, OxyR binds four adjacent major groove contacts while in the reduced form the tetramer binds two pairs of contacts separated by one helical turn [13]. Consistent with the activator role of OxyR in regulation of the HPI hydroperoxidase, 14 of 20 bases of the *katG* promoter match the consensus sequence recognized by the oxidized form of OxyR [13].

3.3. Regulation of HPII expression

The HPII hydroperoxidase in the major catalase produced in aerobically growing cultures. It has long been known that the levels of HPII are 10–20 higher in stationary phase cells than in exponentially growing cultures. Two genes, *katE* and *katF* (now generally referred to as *rpoS*), are involved in the synthesis of this hydroperoxidase [5,6]. The *katE* gene

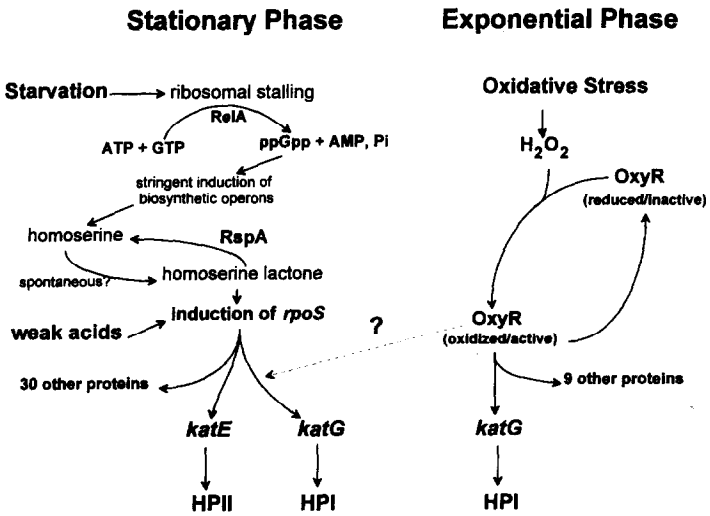

Fig. 1. Current model of HPI and HPII hydroperoxidase regulation in *Escherichia coli*. See text for an explanation of details.

maps to 37.2 min [5] while *rpoS* maps to 59 min [6] on the *E. coli* chromosome. HPII is not induced by hydrogen peroxide and the levels of HPII expressed in an *oxyR* deletion strain are similar to those found in a wild-type strain indicating that *katE* is not a member of the OxyR regulon [8]. The effect of *rpoS* mutations on the expression of *katE* [8,14] indicates that *rpoS* is a regulator of *katE*, the structural gene for HPII [4]. The *rpoS* gene is homologous to *rpoD*, the *E. coli* vegetative sigma factor [15], suggesting that *rpoS* is an alternative sigma factor that directs the transcription of a specific set of stationary phase genes. Consistent with this, purified RpoS can direct the transcription of *rpoS*-dependent genes in vitro [16].

Many other cellular proteins, in addition to hydroperoxidase, are found in greater amounts in stationary than in exponential phase cells. As a group, these have been called pex (*p*ost *ex*ponential) phase proteins [17]. Does RpoS control the expression of these other post-exponential proteins? Comparisons of the two-dimensional protein profiles of *rpoS* mutants to wild-type cells [17,18] indicate that up to thirty two proteins are controlled by RpoS (KatF). While the function of most of these proteins have not, as yet, been identified, the few which have been characterized have very different functions. These include hydroperoxidase, exonuclease III, glycogen synthetase, trehalose synthesis, acid phosphatase and factors important in bacterial virulence (for a review of *rpoS* regulon members see [19]). The diversity of these proteins reflects a need for many housekeeping functions required to maintain cellular viability when the cells are growing slowly or not at all.

What activates this stationary phase regulon? While the answer to this is not completely understood, several possibilities are being considered. The signal may be intrinsic (e.g. an alteration in DNA supercoiling or accumulation of an intracellular metabolic product) or it may be extrinsic (e.g. a fermentation product). Supporting the latter possibility, culture fluids taken from early stationary phase cultures can effectively induce *katE* expression in exponential phase cultures which normally exhibit low levels of expression [8]. Of the fermentation products produced by *E. coli*, only acetate is a good inducer of both *katG* [20] and *katE* [9] transcription. Other non-metabolic weak acids have also been found to be effective inducers [9,14], suggesting that it is not metabolism of weak acids per se but rather some other related effect such as a reduction of internal pH. Though it is not immediately clear why the cell would couple HPII regulation to the accumulation of weak acids, it is thought that the respiratory metabolism that accompanies catabolism of fermentation products may lead to an increase in reactive oxygen species and, concomitantly, an increase in the need for protective enzymes such as hydroperoxidase [9].

RpoS itself is subject to complex control involving transcriptional, translational and post-translational elements (for review see [19]) and thus expression of RpoS depends on other *E. coli* functions. For example, mutants that are deficient in the RelA-mediated synthesis of guanosine tetraphosphate (ppGpp) and are thus incapable of inducing the stringent response, produce low levels of RpoS protein and are unable to induce RpoS-dependent functions [21].

To identify other genes that affect RpoS expression, Huisman and Kolter [22] transformed a strain carrying a *lacZ* fusion to an RpoS-dependent promoter with a plasmid-borne *E. coli* genomic library and identified transformants that exhibited substantially reduced levels of β-galactosidase. Such transformants were presumed to be either unable to produce the signal that causes *rpoS* induction or, alternatively, able to efficiently remove the inducing signal. One of the clones obtained encoded a gene, *rspA*, that is homologous to a lactonizing enzyme of *Pseudomonas putida* [22]. From the inferred function of the gene product, Huisman and Kolter [22] deduced that the accumulating metabolite that induces *rpoS* expression might be homoserine lactone, a product derived from homoserine (or homoserine phosphate) which is an intermediate in the biosynthesis of threonine. Since acylated homoserine lactone derivatives, or autoinducers, have been implicated in a variety of colony density-dependent phenomena in Gram-negative bacteria and none has yet been described in *E. coli*, this represented an intriguing proposal. As expected, mutants deficient in biosynthetic steps prior to homoserine in the threonine pathway were unable to induce RpoS-dependent functions and this deficiency could be suppressed by the addition of exogenous homoserine lactone [22].

To reconcile the observed effects of homoserine lactone and ppGpp [21] on *rpoS* expression, Huisman and Kolter [22] proposed that starvation results in ribosomal stalling due to a net reduction in general translation (Fig. 1). This causes a RelA-mediated increase in ppGpp concentration which, in turn, leads to an increase in the expression of threonine biosynthetic genes. The accumulation of threonine precursors causes an increase of homoserine which can then be converted to homoserine lactone (Fig. 1). While this proposal accounts for a starvation sensing mechanism that is probably requisite in any model of RpoS regulation, it is not easily reconciled with the observation that non-starved cells can be induced to express RpoS in the absence of homoserine lactone [9,14]. Further complicating models of *rpoS* regulation, on the basis of observed effects of mutations in glucose metabolism on expression of an RpoS-dependent gene, Boehringer et al. [23] have recently shown that UDP-glucose levels in the cell also regulate RpoS expression.

There are several indications that other factors, independent of RpoS, may be involved in the regulation of *katE*. Levels of *katE* expression are higher under conditions of high osmolarity (H.E. Schellhorn and V.L. Stones, unpublished), a property shared by some, but not all RpoS-regulated genes [19]. Exponential phase cultures carrying a plasmid borne copy of *rpoS* exhibit only one-third of the level of *katE* expression found in stationary phase cultures [14], suggesting that RpoS is a necessary, but not sufficient condition for full HPII expression. Finally, promoters of RpoS-dependent genes appear to be very similar in sequence motifs to RpoD-directed promoters [15] which probably indicates that another factor or condition, in addition to RpoS itself, is required for the stationary phase-specific induction of HPII and other members of the RpoS regulon.

While the two *E. coli* hydroperoxidases appear to be independently regulated [2], there is recent evidence that expression of HPI is also RpoS-dependent. Basal levels of HPI, measured as peroxidase or catalase or using a *katG–lacZ* promoter fusion, are substantially reduced in a *rpoS*$^-$ strain [19,24]. Since RpoS plays a central regulatory role in stationary phase gene expression, these results raise the possibility that the cell's peroxide-inducible response may be operant in post-exponential as well as the exponential phase of growth. The potential for OxyR induction of protective proteins in stationary phase cultures has not yet, however, been examined.

4. Biological role of hydroperoxidases

Bacterial cells may encounter hydrogen peroxide from a variety of sources. Endogenous hydrogen peroxide may be produced through spontaneous or enzyme-catalysed (superoxide dismutase) dismutation of the superoxide anions or it can be released as a divalent reduction product from the respiratory chain in cells using oxygen as a terminal electron acceptor. As an opportunistic pathogen, *E. coli* may also encounter significant exposure to host-generated peroxides.

In view of the ready diffusibility of hydrogen peroxide across the cell envelope and the relatively small size of the bacterial cell, it is surprising that even hydroperoxidase producing bacteria can significantly alter the transmembrane hydrogen peroxide gradient and thus render the individual bacterial cell resistant to exogenous hydrogen peroxide. However, it has been well established that hydroperoxidase mutants, particularly those affected in HPI synthesis, are generally much more sensitive to millimolar concentrations of hydrogen peroxide than are wild-type strains. This apparent contradiction has been recently resolved [25]. Most studies that have tested the sensitivity of bacterial strains to exogenous hydrogen peroxide have been performed using relatively high density cultures. To test the idea that hydroperoxidase-competent bacteria, under such conditions, are more resistant to hydrogen peroxide simply because they eliminate hydrogen peroxide from culture supernate much faster than hydroperoxidase mutant strains, Ma and Eaton [25] examined the sensitivity of *E. coli* wild-type and hydroperoxidase mutants to hydrogen peroxide at various culture densities. Consistent with earlier reports, at relatively high culture densities wild-type cells were less sensitive to hydrogen peroxide than hydroperoxidase mutants because, through a mass action effect, high density cultures efficiently removed hydrogen peroxide. However, at low culture densities, where even wild-type strains cannot appreciably alter the concentration of extracellular hydrogen peroxide, wild-type

and mutant strains were found to be equally sensitive [25], indicating hydrogen peroxide sensitivity is cell density-dependent. This suggests while hydroperoxidase activity may be important for survival of colonial *E. coli* cultures, this enzymatic activity is of little benefit to the individual cell. In terms of the organisms normal habitat, the human intestine, where *E. coli* is found at high densities, the cell density-dependent response may protect the cell from competing hydrogen peroxide-producing lactobacilli. Wild-type *E. coli* survives co-culturing with lactobacillus much better than catalase-negative mutants [25].

5. Conclusions

Recent studies indicate that regulation of the two hydroperoxidases in *E. coli* is a complex phenomenon involving both common and independent factors. While models have been advanced to explain how the cell regulates hydroperoxidases, several questions remain. For example, is the cell's peroxide-inducible, OxyR-dependent regulon functional in stationary phase cultures? To date, most in vivo studies that have examined OxyR-mediated regulation have been performed using exponential phase cultures under conditions that probably do not adequately model culture growth in the mammalian intestinal environment where *E. coli* has relatively long generation times and experiences substantial variations in nutrient availability. More work must be done examining gene expression in culture growing under suboptimal conditions (e.g. in stationary phase or in nutrient limited chemostats) to address this point.

Though the induction of the HPI hydroperoxidase by hydrogen peroxide can be readily rationalized in terms of the detoxification function of this enzyme, it is not immediately obvious why the induction of HPII and HPI is coupled to changes in growth phase and/or starvation. There is now, however, considerable direct and circumstantial evidence that stationary phase genes, including *katE* and *katG*, require factors, other than RpoS, for full expression. Since RpoS-dependent genes lack a distinguishing structural feature such as a consensus promoter, information regarding these ancillary regulators may provide insight into the regulation of other genes that are specifically expressed in stationary phase.

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