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Intracellular location of catalase-peroxidase hydroperoxidase I of *Escherichia coli*

Alex Hillar [a], Larry Van Caeseele [b], Peter C. Loewen [a] *

[a] *Department of Microbiology, University of Manitoba, Winnipeg, Man. R3T 2N2, Canada*
[b] *Department of Botany, University of Manitoba, Winnipeg, Man. R3T 2N2, Canada*

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Abstract

The catalase-peroxidase hydroperoxidase I of *Escherichia coli* has been confirmed to be located in the cytoplasm using two independent methods. Catalase activity was found predominantly (>95%) in the cytoplasmic fraction following spheroplast formation. The cytoplasmic enzyme glucose-6-phosphate dehydrogenase and the periplasmic enzyme alkaline phosphatase were used as controls. The second method of immunogold staining for the enzyme in situ revealed an even distribution of the enzyme across the cell.

Keywords: Catalase-peroxidase; Cytoplasm; Immunogold staining

1. Introduction

The role of catalases and peroxidases is to scavenge H_2O_2 and moderate or prevent oxidative damage in the cell. Catalases are heme-containing proteins that convert H_2O_2 to water and oxygen in a two-step reaction cycle. In the first step, one molecule of H_2O_2 is converted to water while the heme moiety is oxidized to an intermediate referred to as compound I. A second H_2O_2 then donates electrons and an oxygen to compound I resulting in the formation of molecular oxygen. Heme-containing peroxidases follow a similar reaction pathway except that the reductant of compound I is usually an organic electron donor.

Escherichia coli, *Mycobacterium tuberculosis*, and a number of other bacterial [1] and fungal [2,3] species express a bifunctional catalase-peroxidase, hydroperoxidase I (also HPI and KatG). In *E. coli*, HPI is one of two enzymes with catalase activity, the second being the monofunctional catalase HPII. In *M. tuberculosis*, the catalase-peroxidase (MtHPI) is the sole catalase and considerable evidence has implicated it in the activation of the anti-tubercular drug isonicotinic acid hydrazide (isoniazid or INH). Its oxidation to an electrophilic species allows it to bind to, and inactivate, InhA, an acyl carrier protein reductase [4], or other potential targets [5] thereby inhibiting mycolic acid and cell wall synthesis. MtHPI supports higher levels of INH oxidation in vitro than HPI from *E. coli* [6] providing at least a partial explanation for the greater natural resistance of *E. coli* to INH, as compared to *M. tuberculosis*.

* Corresponding author. Tel.: +1 (204) 474-8334; Fax: +1 (204) 474-7603; E-mail: peter_loewen@umanitoba.ca

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The relative efficiencies of INH activation could also be influenced by the intracellular localization or concentration of the enzymes. Originally, HPI was localized to the periplasm of *E. coli* [7], but more recent work has suggested that HPI may be largely cytoplasmic [8]. In this report, we resolve the question of the cellular location of HPI using both enzymatic assays of spheroplast fractions and immunogold labeling in situ.

2. Materials and methods

2.1. Reagents and bacterial strains

E. coli strains used were MP180 (*thi-1*), HfrH [9], UM120 (asMP180 but *katE12::Tn10*) [10], UM202 (as MP180 but *katG17::Tn10*) [11], and *UM262* (*recA katG::Tn10 pro leu rpsL hsdM hsdR endI lacY*) [12] transformed with plasmid pBT22 a pBR322 derivative containing the *katG* gene of *E. coli* [13].

2.2. Cell culture and fractionation

Thirty-milliliter cultures were grown at 37°C with aeration for 5 h to late logarithmic phase. Ampicillin (100 μg ml^{-1}) was added as required. Cells were harvested by centrifugation and washed twice with 5% NaCl. The spheroplasting and fractionation procedure [14] was modified to omit the final centrifugation step that separates cytoplasmic and membrane associated proteins resulting in a combined cytoplasmic and inner membrane fraction.

2.3. Enzyme activity assays

Catalase activity was determined by the method of Rørth and Jensen [15], using a Gilson oxygraph equipped with a Clark electrode. One unit of catalase is defined as the amount that decomposes 1 μmol of H_2O_2 in 1 min at 37°C. Glucose-6-phosphate dehydrogenase activity was determined according to Merchante et al. [14] by monitoring NADP reduction at 340 nm in a Pharmacia Ultrospec 3000 UV-Visible. One unit of glucose-6-phosphate dehydrogenase activity corresponds to an increase of the A_{340} by 0.001 per min. All assays were performed in triplicate. Alkaline phosphatase activity was determined according to Torriani [16] by monitoring the appearance of free *p*-nitrophenol at 410 nm. One unit of alkaline phosphatase activity corresponds to an increase of the A_{410} by 1.0 per min at pH 8.2 and 37°C.

2.4. In situ immunogold staining

Cell suspensions were fixed in 4% formaldehyde prepared from paraformaldehyde in phosphate-buffered saline (PBS) pH 7.2 for 2 h at room temperature. The suspension was centrifuged and the pellet resuspended in 3% agarose, cooled, cut into cubes of approximately 1 mm^3 and washed in PBS pH 7.2. The cubes were dehydrated through a graded ethanol series to 100% ethanol and infiltrated with LR White using LR White plus 100% ethanol solutions of 1:1 for 16 h, 3:1 for 6.5 h, and 100% LR White for 16 h and then 6.5 h. The last suspension was polymerized at 50°C for 24 h in the absence of oxygen. Sections were cut on a Reichert Ultracut ultratome and collected on formvar-coated nickel grids. Sections were floated on 1% bovine serum albumin in PBS for 30 min and washed for 10 min in PBS. The grids were then floated on a solution of primary antibody raised against *E. coli* HPI diluted 1:25 in PBS containing 0.05% Tween 20 and 0.1% BSA for 3.5 h. After washing in PBS containing 1% BSA, the grids were floated for 1 h on a drop of goat anti-rabbit IgG conjugated to 10-nm gold spheres diluted 1:15 with PBS containing 0.1% BSA and 0.05% Tween 20. After washing, sections were stained in 2% aqueous uranylacetate for 10 s and Reynolds lead citrate [17] for 10 s, washed with water and examined with a Hitachi 7000 electron microscope. A series of control grids were prepared identically, except that the primary antibody was replaced with normal rabbit serum.

3. Results

3.1. Catalase levels in spheroplast fractions

Spheroplasts are generated by lysozyme solubilization of the cell wall and membrane under isotonic conditions. Periplasmic material is released into the medium and cytoplasmic material is retained within

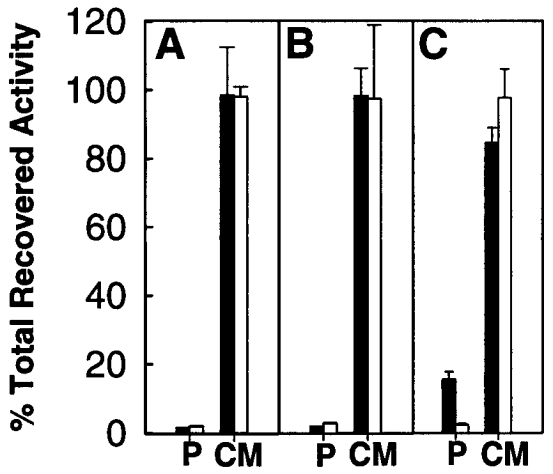

Fig. 1. Recovery of catalase and glucose-6-phosphate dehydrogenase activities from spheroplast fractions. Solid bars represent catalase activity, open bars represent glucose-6-phosphate dehydrogenase activity. P indicates periplasmic and CM indicates cytoplasmic and inner membrane fractions. Error bars indicate standard error of three individual assays. (A) Results for *E. coli* strain MP180 (wild-type). (B) Results for *E. coli* strain UM120 (*katE* or HPII deficient). (C) Results for *E. coli* strain UM202 (*katG* or HPI deficient).

the spheroplast creating a simple procedure for determining whether an enzyme is periplasmic or cytoplasmic. Spheroplasts are very fragile and care must be taken to prevent lysis which will lead to contamination of the periplasmic fraction with cytoplasmic components. It is therefore necessary to have a control that monitors the amount of spheroplast lysis and the cytoplasmic enzyme, glucose-6-phosphate dehydrogenase (GDH), serves this role. Essentially, any GDH activity found outside of the spheroplast is indicative of and can be used to quantitate spheroplast lysis. Under the conditions employed in this work, GDH was found predominantly (>95%) in the spheroplast fraction (cytoplasmic plus inner membrane) in all strains assayed (Fig. 1A–C) confirming that the isotonic protocol caused only minor lysis. Similarly, it was necessary to have a control confirming that a significant proportion of the cells had been converted to protoplasts. Alkaline phosphatase is found largely in the periplasm [18] and 67% of its activity was released during the protoplasting procedure confirming that at least that percentage of the cells had been converted to protoplasts.

Catalase activity in *E. coli* was found to have a distribution pattern between the periplasm and cytoplasm similar to that of GDH. The wild-type *E. coli* strain MP180 expressing both catalases, HPI and HPII, contained most of the catalase activity (>95%) in the cytoplasmic fraction (Fig. 1A). This suggested that both catalases were located predominantly, if not exclusively, in the cytoplasm and this was confirmed using strains lacking one or the other of the enzymes. When assayed individually, HPI in the *katE*-containing strain UM120 and HPII in the *katG*-containing strain UM202 were found predominantly (85–95%) in the cytoplasm (Fig. 1B,C). Even the overexpression of HPI in a strain transformed with a *katG*-encoding plasmid did not result in a significant increase in the periplasmic content of catalase activity (Fig. 2).

In the work that first concluded that HPI was periplasmic, the protocol used to liberate periplasmic contents did not involve rigorous control of the osmotic conditions to minimize spheroplast lysis [7].

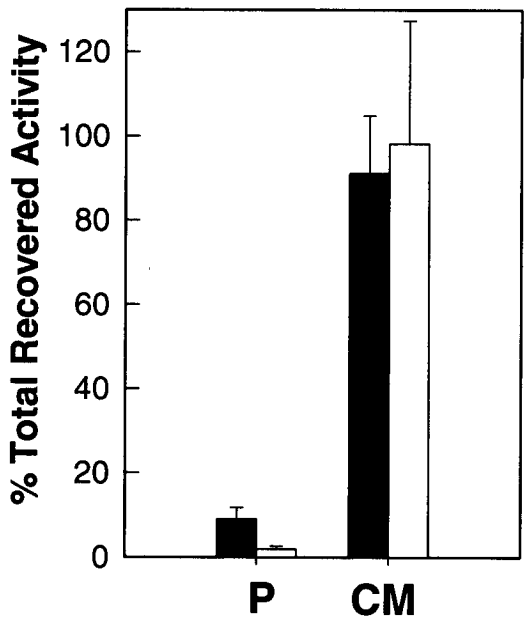

Fig. 2. Recovery of catalase and glucose-6-phosphate dehydrogenase activities from spheroplast fractions of *E. coli* strain UM262, harboring plasmid pBT22. Solid bars represent catalase activity, open bars represent glucose-6-phosphate dehydrogenase activity. P indicates periplasmic and CM indicates cytoplasmic and inner membrane fractions. Error bars indicate standard error of three individual assays.

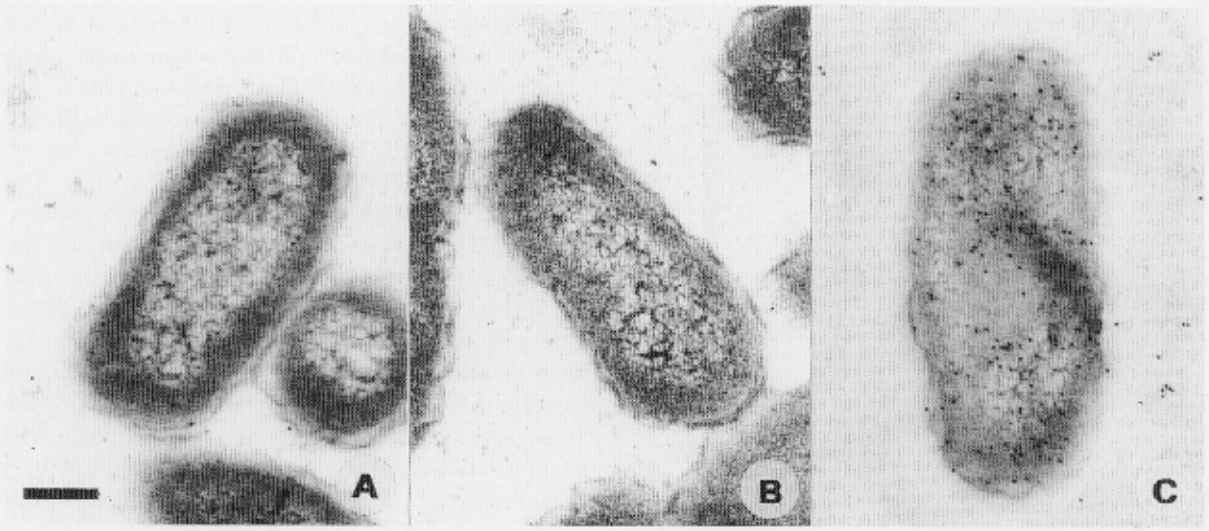

Fig. 3. Immunogold staining to visualize the intracellular location of catalase-peroxidase HPI in *E. coli* strains: (A) MP180, a wild-type strain; (B) UM202, a *katG*-containing strain; (C) UM262 [pBT22] which over-produces HPI. The bar in A represents 0.25 μm. The ratio of particles in the cell to particles in the background (averaged from 6 areas equivalent in size to the cell) was 10:2.7 in A, 0:1.8 in B and 156:8.2 in C.

To compare results for isotonic and non-isotonic procedures, strains MP180, UM120, and UM202 were converted to spheroplasts using the non-isotonic osmotic shock procedure [7]. Up to 35% of catalase and GDH activities were found in the 'periplasmic' fraction using this procedure, indicative of significant spheroplast lysis and cytoplasmic contamination of the periplasmic fractions (data not shown).

3.2. Immunogold staining for in situ location of HPI

In order to provide an independent confirmation of the localization of catalases, in situ immunogold labeling of HPI was carried out. Wild-type strain MP180 revealed a sparse, but even, distribution of the gold label across the cell section (Fig. 3A). For comparison, the *katG*-containing mutant strain UM202 exhibited no gold staining (Fig. 3B). Strain UM262 harboring plasmid pBT22, encoding *katG*, and producing high levels of HPI, exhibited a pattern of very dense gold label that was evenly distributed across the cell (Fig. 3C). In both Fig. 3A,C, there were significantly more gold particles within the cell than in an equivalent background area. The gold labeling pattern is indicative of HPI being distributed throughout the cytoplasm with no bias towards the periplasm.

4. Discussion

The conflicting reports about whether HPI is periplasmic or not [7,8] have been resolved by the application of two independent methods that confirm that the enzyme is distributed throughout the cytoplasm and is not localized in the periplasm. Using known cytoplasmic and periplasmic markers, glucose-6-phosphate dehydrogenase and alkaline phosphatase, respectively, for comparison, both *E. coli* catalases were found to have distribution patterns typical of a cytoplasmic enzyme. Secondly, the use of immunogold labeling in situ confirmed an even distribution of the enzyme throughout the cytoplasm. While the two normal catalases of *E. coli* are not periplasmic, the enterohemorrhagic *E. coli* strain O157:H7 produces the plasmid-encoded KatP as a periplasmic enzyme [8]. Other bacteria that also produce periplasmic and extracellular catalases include phytopathogenic *Pseudomonas syringae* [19,20], *Vibrio fischeri* [21], *Brucella abortus* [22], *Bacillus subtilis* [23] and *Caulobacter crescentus* [24].

A major criticism of the enzyme assay data is that spheroplast lysis will give rise to a false indication of periplasmic location. However, the protocol used here maintained isotonic conditions, and caused less than 5% lysis as determined by GDH levels in the periplasmic fraction. These levels of spheroplast lysis in isotonic solutions are consistent with the reports of $<10\%$ lysis by other workers [14,18,20]. This does not fully explain the 100% periplasmic localization described by Heimberger and Eisenstark [7], but may provide a partial explanation.

The greater resistance of *E. coli* to INH as compared to *M. tuberculosis* may, at least in part, arise from the *E. coli* HPI having a lower efficiency for INH oxidation than the *M. tuberculosis* enzyme [6]. The cytoplasmic location of HPI in *E. coli* may provide a second reason for its lower sensitivity to INH. The drug must pass through both the cell wall and two membranes before coming in contact with a relatively low concentration of the enzyme. A periplasmic or membrane associated localization would provide the enzyme with a greater chance of coming in contact with the drug. The intracellular locale of HPI in *M. tuberculosis* must now be determined for comparison.

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