Structure-function analyses of the catalase-peroxidase HPI from

Escherichia coli

by

Ajith Balakrishnan

A thesis

submitted to the Faculty of Graduate Studies in

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STRUCTURE-FUNCTION ANALYSES OF THE CATALASE-PEROXIDASE HPI
FROM *ESCHERICHIA COLI*

BY

AJITH BALAKRISHNAN

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University

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ABSTRACT

Catalase-peroxidases (CP's) are bifunctional hemoproteins that are important components of the oxidative defense system of bacterial and fungal cells. Despite the sequence similarity between yeast cytochrome *c* peroxidase and CP's, they differ dramatically in their reactivities to hydrogen peroxide and electron-donating peroxidatic substrates. Smaller differences also exist among different bacterial CP's such as EcHPI (*katG*-encoded CP from *E. coli*) and MtKatG (CP from *M. tuberculosis*), which has been implicated in the peroxidatic activation of isoniazid. Previous site-directed mutagenesis studies on the putative active site residues in EcHPI have suggested the importance of Trp105 in the catalase activity of the enzyme. Trp residues in and around the putative active site of EcHPI are highly conserved and show a specific pattern of distribution among CP's. The primary objective of this thesis is to investigate the catalytic and structural role of Trp88, Trp89 and Trp197 in EcHPI. Using site-directed mutagenesis on plasmid-encoded *katG,* these residues were replaced with either Phe or Leu and the variant enzymes were purified for characterization. Both the wild type and variant enzymes contained more heme and exhibited higher activities when prepared from cells grown at 28°C compared to 37°C. Physicochemical, spectral, biochemical, kinetic and heme inhibition studies together indicate the absence of any significant differences between wild type EcHPI and its Trp-replacement variants. It can thus be implied that Trp88, Trp89 and Trp197 may be dispensable for the normal functioning of EcHPI. The only notable effect the mutations may be actually causing are the shifts in the proportions of the different heme-containing tetrameric enzyme species, as reflected in the reduced heme/protein ratios of most variants in relation to the wild-type.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

A	Absorbance
ABTS	2,2-*azinobis*(3-ethylbenzothiazolinesulfonicacid)
Amp^R	Ampicillin resistant
AP	Apurinic
APX	Ascorbate peroxidase
ATP	Adenosine triphosphate
BER	Base excision repair
bp	Base pairs(s)
CCP	Cytochrome *c* peroxidase
CP	Catalase-peroxidase
Ci	Curie
CIP	Calf intestinal alkaline phosphatase
CpeB	Catalase-peroxidase B
Da	Dalton
DEAE	Diethylaminoethyl
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphate
EcHPI	*Escherichia coli* Hydroperoxidase I
EDTA	Ethylenediaminetetraacetic acid
EHEC	Enterohaemorrhagic *Escherichia coli*
EXAFS	Extensive X-ray absorption fine structure analysis
GPX	Glutathione peroxidase
HPI	Hydroperoxidase I
HPII	Hydroperoxidase II
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
INH	Isonicotinic acid hydrazide (isoniazid)

k_{cat}	Turnover number
kDa	kiloDalton
K_m	Michealis-Menten constant
KPB	Potassium phosphate buffer pH 7.0.
LB	Luria-Bertani
MES buffer	2-[N-Morpholino]-ethane sulfonic acid buffer
NADH	Nicotinamide adenine dinucleotide (reduced)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
OD	Optical density
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PMSF	Phenylmethylsulfonyl fluoride
RNA	Ribonucleic acid
RNAse	Ribonuclease
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate
SOD	Superoxide dismutase
TE buffer	Tris-EDTA buffer
Tris	Tris(hydroxymethyl)aminomethane
V	Volts
V_{max}	Maximum velocity
w/v	Weight per unit volume
v/v	Volume per unit volume

1. GENERAL INTRODUCTION

1.1. Oxygen toxicity and oxidative stress

Aerobic organisms use molecular oxygen (O_2) for respiration or oxidation of nutrients to obtain energy. The role of oxygen as a terminal electron acceptor in such organisms is absolutely essential for their survival. Although, the complete four-electron reduction of oxygen to water is thermodynamically highly favorable, the accomplishment of this process without the formation of free partially reduced species is mechanistically difficult (Jones and Wilson, 1978). The electronic structure of O_2 predisposes it to reduction by the univalent pathway. The paramagnetism of O_2 in the ground state indicates that it contains two unpaired electrons with parallel spin states. In contrast, all the electrons in stable organic molecules are arranged in pairs with antiparallel spin states. Moving a pair of electrons from any stable organic compound into O_2 will require the inversion of an electronic spin state, since two electrons cannot occupy the same orbital unless their spins are antiparallel (Fridovich, 1999). This may be illustrated as follows:

Inversion of electronic spin states, by interaction with nuclear spins, does occur, but on a time scale several orders of magnitude longer than the lifetime of collisional complexes. It is thus unlikely to occur while the O_2 and the reductant are in contact. But it can occur during the intervals between collisions; so the electrons are preferentially transferred to O_2 one at a time (Fridovich, 1999). Four electrons (and

four protons) are needed to reduce O_2 to two molecules of water. Hence the univalent

pathway necessitates intermediates, as follows:

$$O_2 \xrightarrow{e^-} O_2^{\cdot -} \xrightarrow{e^-} H_2O_2 \xrightarrow{e^-} OH^{\cdot} \xrightarrow{e^-} H_2O$$

| Oxygen | Superoxide Anion | Hydrogen Peroxide | Hydroxyl Radical | Water |
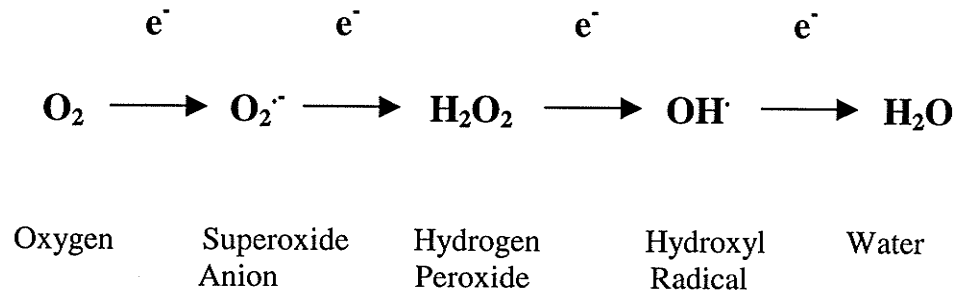
Oxygen-derived free radicals such superoxide anion ($O_2^{\cdot -}$), hydroperoxyl ($HOO^{\cdot}$),

and hydroxyl ($OH^{\cdot}$) radicals are generally termed as Reactive Oxygen Species (ROS).

Other ROS which contain even number of electrons include hydrogen peroxide (H_2O_2)

and hypohalous acids (HOX) (Grisham, 1992). ROS may originate from both within

and without the cell. They may be formed as a consequence of several enzymatic

reactions within the cell, such as catalysis by different oxidases, dehydrogenases, and

redox enzymes such as peroxidases and P-450 oxygenases and superoxide-dismutases

(SOD). Experimental data indicate that, in *Escherichia coli*, the respiratory chain can

account for as much as 87% of total H_2O_2 production (González-Flecha and Demple,

1995). Within mammalian cells, mitochondria have been shown to produce $O_2^{\cdot -}$ and

H_2O_2 (Dionisi *et al.*, 1975) as a consequence of "leaks" in the electron flow into O_2

during respiration. Non-enzymatic sources of ROS originating intracellularly include

auto oxidation of catecholamines, reduced flavins, hydroquinone, and reaction with

transition metals in reduced form (Farr and Kogoma, 1991). There are numerous

extraneous sources of ROS, most of which exert their toxic effects following

metabolic breakdown into free radicals. Various toxic chemicals including certain

herbicides, azo dyes, anthracycline anticancer drugs and nitroheterocyclic antibiotics such as metronizadole, have all been shown to form free radical metabolites that can react with oxygen to generate ROS (Mason, 1992). Environmental sources of oxidants include such diverse factors as air pollution, natural radiation (ultraviolet rays), and macrophage response to bacterial infections (Ames, 1983; Cerutti, 1991; Babior, 1981).

The presence of pro-oxidants in the cell is the operational definition of oxidative stress, and oxygen toxicity occurs when the degree of oxidative stress exceeds the capacity of the cell's antioxidant defense systems (Farr and Kogoma, 1991). Unless intercepted, ROS can have deleterious effects on the cell. The primary biological targets of ROS are cellular macromolecules such as DNA, RNA, proteins and lipids. All reactive oxygen molecules can cause varying degrees of damage to cells although, most of the damage is caused by hydroxyl radicals, which are generated from H_2O_2 via the Fenton reaction, which requires iron (or another divalent metal ion, such as copper) and a source of reducing equivalents to regenerate the metal. ROS may react with DNA to produce base modifications or strand breaks (Ames and Shigenaga, 1992; Farr and Kogoma, 1991; Sies and Menck, 1992), lesions that would in turn block DNA replication. ROS also have been shown to be capable of oxidizing proteins and enzymes (Weser, 1984; Gardner and Fridovich, 1991). At a cellular level, when proteins are exposed to ROS, modifications of amino acid side chains occur, and consequently, the protein structure is altered. These modifications lead to functional changes that disturb cell metabolism. ROS can directly attack poly-unsaturated membrane fatty acids and initiate peroxidation (Chance *et al.*, 1979; Cadenas and Sies, 1984; Koster *et al.*, 1984). The primary effect of lipid peroxidation

is a decrease in membrane fluidity, which alters membrane properties and disrupts membrane-bound proteins significantly. Thus, the end result of oxidant-induced modifications or damage to cellular macromolecules is cell death or tissue damage.

1.2. Anti-oxidant defense mechanisms

1.2.1. Avoidance of formation of Reactive Oxygen Species

"Prevention is better than cure" is an age-old and time-tested adage. Many biological oxidases utilize mechanisms that accomplish divalent and even tetravalent reduction of O_2, without releasing intermediates. Cytochrome c oxidase is such an enzyme; by virtue of two heme and two Cu(II) prosthetic groups it can accumulate four reducing equivalents, which are then transferred to bound O_2, via a bound peroxo intermediate, to produce two molecules of water (Malatesta $et\ al.$, 1995). So the first line of defense is avoidance, which is very effective since in most cells under normal conditions only 0.1-1.0% of the electrons transferred to O_2 produce O_2^- (Fridovich, 1999). However, the rate of O_2 consumption is such that even a 0.1% yield of O_2^- would impose an intolerable oxidative burden were there not additional defenses.

1.2.2. Pre-damage defense mechanisms

The appearance of oxygen in the atmosphere led to the development of defense mechanisms that kept the concentration of O_2-derived free radicals at acceptable levels. Organisms have evolved various enzymatic and non-enzymatic antioxidants that serve to prevent or limit oxidative damage caused by toxic oxygen species. These include enzymes such as catalases, peroxidases, and superoxide dismutases. Non-

enzymatic antioxidants include various low molecular weight scavengers, reductants and some iron binding proteins.

Superoxide dismutases (SODs) catalyze the conversion of O_2^- into H_2O_2 and O_2 and do so with unmatched efficiency, being limited only by the rate of diffusion (Fridovich, 1999). Since the creation of an oxygen-rich atmosphere by photosynthetic organisms must have imposed a common selection pressure on a varied anaerobic biota, it is not surprising that several families of SODs currently exist. In eukaryotes there is a MnSOD in the mitochondria, a CuZnSOD in the cytosol and in the other organelles, and a different CuZnSOD in the extracellular spaces (Fridovich, 1999). Plants have a MnSOD, a CuZnSOD, and also an FeSOD (Fridovich, 1999). The bacterium *Escherichia coli* is known to produce four SOD isoenzymes: a MnSOD (Keele *et al.*, 1970), an FeSOD (Yost and Fridovich, 1973), an FeMnSOD (Clare *et al.*, 1984), and a CuZnSOD (Battistoni and Rotilio, 1995). Recently a NiSOD has been found in *Streptomyces* species (Choudhury *et al.*, 1999). In all cases, the active site metal of the SOD is reduced by one O_2^- and then reoxidized by the next. In this way it transfers an electron from one O_2^- to the next while avoiding the electrostatic repulsion that would hinder direct electron transfer between one O_2^- and another (Fridovich, 1999).

The H_2O_2 produced from O_2^-, and directly by specific oxidases, must be eliminated because it can be converted into the highly reactive cytotoxic OH^- radical (Fridovich, 1999). H_2O_2 is metabolized and eliminated by catalases and peroxidases. Catalases catalyze the mutual oxidation-reduction of two molecules of H_2O_2 to yield O_2 and H_2O. Most catalases are heme-containing enzymes but *Lactobacilli* and related bacteria, which cannot synthesize heme, can produce a very effective catalase,

the prosthetic group of which is a binuclear cluster of Mn atoms (Beyer and Fridovich, 1985).

Peroxidases use a variety of reductants to reduce H_2O_2 to 2 H_2O, although the initial oxidation step utilizes a molecule of H_2O_2 similar to the first step of the catalase reaction. This property has lead to the conception that peroxidases are functionally similar to catalases and that they have a protective effect while using hydrogen donors other than H_2O_2. Thus there are peroxidases that utilize glutathione, ascorbate, ferrocytochrome c, reduced thioredoxin, NADH, and even iodide and chloride. Plant peroxidases are notoriously broad in their utilization of reductants. Most peroxidases are heme-based enzymes; but the prosthetic group of the Glutathione peroxidases (GPX), so important in higher animals, have a selenocysteine residue. GPX use reduced glutathione as the hydrogen donor (Ren *et al.*, 1997), the reaction products being oxidized glutathione and water. Oxidized glutathione is then reduced via the enzyme glutathione reductase, which utilizes the cofactor NADPH. Levels of reduced intracellular glutathione are normally maintained high relative to the oxidized form (Grisham, 1992), allowing efficient interception of ROS. The GPX deserve special mention for another reason and that is their ability to catalyze the reduction of alkylhydroperoxides, such as those generated by the oxidation of polyunsaturated lipids to alcohols. The alkyl hydroperoxidase AhpC of *Escherichia coli* and *Salmonella typhimurium* (Storz and Tartaglia, 1992; Sherman *et al.*, 1996; Jacobson *et al.*, 1989) is capable of reducing organic peroxides such as cumene hydroperoxide, and the di-heme cytochrome c peroxidase from *Pseudomonas aeruginosa*, a periplasmic enzyme, is believed to provide protection against toxic peroxides (Fülöp *et al.*, 1995).

In addition to the enzymatic antioxidant mechanisms described above, there are a number of non enzymatic antioxidants including small molecule substances as well as antioxidant proteins that contribute to removal of ROS species both intracellularly and extracellularly. Examples of such molecules found in human blood plasma include ascorbic acid, glutathione, α-tocopherol, β-carotene, albumin (Halliwell and Gutteridge, 1986), lactoferrin, transferrin and metallothioneines (Frei *et al.*, 1992). Bacterioferritin and glutathione are examples of two such molecules that have been described to play an important role in antioxidant defense in *E. coli* cells (Andrews *et al.*, 1989; Smith and Schrift, 1979).

1.2.3. Post-damage defense mechanisms

In addition to the primary defense mechanisms mentioned above, there are secondary mechanisms that serve to repair the damage caused by toxic oxygen species. These include DNA repair systems and proteolytic and lipolytic enzymes. All damage produced by free-radical attack on DNA molecules are repaired by the ubiquitous DNA repair process called base excision repair (BER) (Demple and Harrison 1994). The enzymatic components of BER include DNA N-glycosylases that recognize damaged purines and pyramidines, 5'-apurinic (AP) endonucleases that process strand breaks, sites of base loss, and the products of DNA glycosylase/AP lyase action, and DNA polymerases and DNA ligases. BER also processes certain alkylated bases as well as uracil-derived from deamination of cytosine. BER enzymes have been found throughout all phylogenetic kingdoms and, where examined, retain the fundamental characteristics of the prototypic activities that have been identified in *E. coli* (Wallace, 1997).

1.3. Catalases

1.3.1. Introduction and Properties

Catalases have been the subject of observation and study for well over one

hundred years, with the first report of a biochemical characterization and naming of

the enzyme appearing in the year 1900 (Loew, 1900). All catalases whether

eukaryotic, prokaryotic, or archae, are predominantly heme containing enzymes with

a striking ability to evolve molecular oxygen via dismutation of hydrogen peroxide.

The overall reaction for the classical enzyme is very simple on paper, $2H_2O_2 \rightarrow 2H_2O$

$+ O_2$. Because of its prevalence in many mammalian tissues, particularly blood, the

catalase enzyme proved easy to isolate, facilitating its purification to provide

sufficient protein for detailed biochemical studies. Bacterial catalases have been

studied extensively since the first report of the purification of the catalase from

Micrococcus luteus was published in 1948 (Herbert and Pinsent, 1948). Interest in

bacterial catalases also derived from the potential role of the enzyme in the

pathogenesis wherein the infecting bacterium frequently has to survive a burst of

active oxygen species, including H_2O_2 generated by the infected tissue.

Generally, most catalases are homotetramers having subunit sizes ranging

between 55-84 kDa (Nicholls *et al.*, 2001). Each subunit contains a non-covalently

bound heme prosthetic group characterized as protoferriheme (protoporphyrin IX)

(Stern, 1936) in the high spin Fe(III) state (Maeda *et al.*, 1973). High resolution

crystal structures of various catalases (Reid *et al.*, 1981; Murthy *et al.*, 1981; Fita and

Rossmann, 1985; Vainshtein *et al.*, 1986; Murshudov *et al.*, 1992; Bravo *et al.*, 1995)

have shown that the axial ligand at the fifth coordination position of the heme iron is

a tyrosine residue, while the sixth coordination position of iron is probably occupied by a water molecule (Oakes, 1986; Schonbaum and Chance, 1976).

Catalases show pseudo-first order saturation kinetics (Michealis-Menten kinetics) with a kinetic constant for the overall reaction being independent of pH in the range of 4.7-10.5. The kinetic behavior of classical catalases remains widely misunderstood, as evidenced by the frequent quoting of K_m and k_{cat}/K_m values for catalases without any rider explaining that these parameters do not have the meaning they possess for standard Michelis-Menten enzymes. Mammalian catalases have been reported to have specific kinetic constants in the range of 0.01 to 6 x 10^7 M^{-1} s^{-1} and classically low K_m values of about 1.0 M (Sevinc et al., 1999; Jones and Wilson, 1978). The reliability of the kinetic parameters are questionable, as it has been demonstrated that H_2O_2 causes inactivation of the enzyme at concentrations of less than 1M (Sevinc et al., 1999; DeLuca et al., 1995), probably making it impossible to achieve true saturation conditions for the enzyme with H_2O_2.

There are several inhibitors of catalase activity that include the classical heme inhibitors such as cyanide, azide and hydroxylamine (Nicholls and Schonbaum, 1963; Maj et al., 1996), which bind reversibly to the heme iron. 3-amino 1,2,4-triazole irreversibly inhibits catalases by forming a covalent bond with the histidine residue on the oxidized catalytic intermediate of catalase (compound I) (Chang and Schroeder, 1972). Other catalase inhibitors include t-butylhydroperoxide, which causes suicide inhibition through formation of an inactive catalytic intermediate (Pichorner et al., 1993); and thiol reagents, superoxide, and hydrogen peroxide all of which may directly or indirectly lead to oxidation of amino acid residues on the enzyme (Takeda et al., 1980; Kono and Fridovich, 1982; DeLuca et al., 1995).

1.3.2. The catalase reaction

The heme chromophore of catalases provided a convenient tool for workers intent on studying the catalase reaction mechanism, eventually leading to the definition of the two distinct stages in the reaction pathway. The first stage of the catalase reaction involves oxidation of the heme iron using hydrogen peroxide as substrate to form compound I, an oxyferryl species with one oxidation equivalent located on the iron and a second oxidation equivalent delocalized in a heme cation radical (reaction 1). Compound I is a short-lived catalytic intermediate with a distinct absorption spectrum characterized by reduction in absorption intensity of the Sorêt band (at approximately 400nm; a band which is diagnostic of hemoproteins). The formation of compound one is believed to be initiated by a histidine side-chain in the active site above (distal to) the plane of the heme. The histidine acts as a general base donating electrons to the H_2O_2 molecule coordinated to the heme iron, forming a transition complex which is stabilized by additional charge interactions provided by other residues in the active site. This allows scission of the peroxide O-O bond, to form compound I and one molecule of water (Fita and Rossman, 1985).

Enz (Por-Fe^{III}) + $H_2O_2 \rightarrow$ compound I (Por$^{+\cdot}$-Fe^{IV}=O) + H_2O (1)
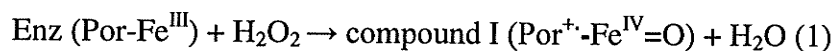
The second stage, or reduction of compound I, employs a second molecule of peroxide as electron donor providing two oxidation equivalents (reaction 2). The second H_2O_2 molecule enters the active site and is preoriented for reaction by hydrogen bonding and charge interactions with the side chains of the active site histidine and asparagines residues. As the charge density on the H_2O_2 molecule shifts, due to interaction with the amino acid side chains, it is believed that the O-O bond

takes on double bond character while the oxygen-iron bond is broken. The active site histidine side-chain then acquires greater nucleophilic character, allowing a rearrangement in the relative positions of the hydroxyl group closest to the active side chains, while a hydroxyl is simultaneously formed on the heme iron. Water is then thought to be formed through transfer of a hydrogen and electron to the OH group still coordinated to the iron, as the O_2 molecule is released from the coordination with the histidine residue (Fita and Rossmann, 1985).

compound I (Por$^{+\cdot}$-Fe^{IV}=O) + H_2O_2 → Enz (Por-Fe^{III}) + H_2O + O_2 (2)
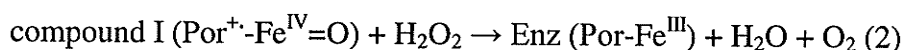
Compound I can also undergo a one-electron reduction with or without a proton, resulting in the formation of an inactive compound II (Reaction 3 or 3a) with one oxidation equivalent above that of the ferric heme (Nicholls and Schonbaum, 1963). Reduction of compound I to the spectrally distinct compound II may either occur in the presence of H_2O_2 or hydrogen donors such as ferrocyanide and ascorbate. Addition of large excesses of H_2O_2 to catalases leads to formation of inactive catalase compound III or the oxycatalase form of the enzyme.

Compound I (Por$^{+\cdot}$-Fe^{IV}=O) + e$^-$ → compound II (Por-Fe^{IV}=O) (3)

Compound I (Por$^{+\cdot}$-Fe^{IV}=O) + e$^-$ + H$^+$ → compound II (Por-Fe^{IV}-OH$^+$) (3a)
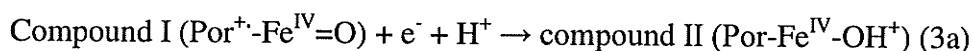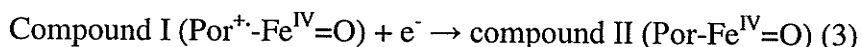
Catalases from various prokaryotes and eukaryotes have been shown to contain one tightly bound NADPH molecule per enzyme subunit (Kirkman and Gaetani, 1984; Gouet *et al.*, Hillar *et al.*, 1994; Yusifov *et al.*, 1989). The bound NADPH has been postulated to prevent the enzyme from accumulating compound II, (Kirkman *et al.*,

1987) or a compound II-like species in which a tyrosinate based radical is present (Hillar and Nicholls, 1992; Ivancich *et al.*, 1997; Kirkman *et al.*, 1999).

1.3.3. Catalase: Diversity and Categorization

Structural diversity among catalases, evident in the variety of subunit sizes the number of quaternary structures, the different heme prosthetic groups, and the variety of sequence groups allows them to be organized into four main groups: the "classical" monofunctional catalases (type A), the catalase-peroxidases (type B), the nonheme catalases (type C), and miscellaneous proteins with minor catalatic activities (type D) (Nicholls *et al.*, 2001).

Monofunctional catalases (type A):

Monofunctional catalases are the largest and most extensively studied group of catalases. The dismutation of H_2O_2 is their predominant activity; any peroxidatic activity is minor and restricted to small substrates. The group can be further subcatergorized based on subunit size with an accompanying attention to heme content (Nicholls *et al.*, 2001). Bovine liver catalase, a small subunit enzyme containing heme *b* and hydroperoxidase II, a large subunit catalase from *Escherichia coli* containing heme *d* are among the well-studied enzymes in this group.

Catalase-peroxidases (type B):

Catalase-peroxidases constitute the second largest group of catalases. They are bifunctional hemoprotein enzymes that exhibit a significant organic peroxidase activity in addition to the catalatic activity (Nicholls *et al.*, 2001). They have been

characterized in both fungi and bacteria. This group will be discussed in detail in section 1.5.

Non-heme catalases (type C):

Currently the smallest group, there are only three nonheme catalases so far characterized, (*Lactobacillus plantarum, Thermoleophilum album,* and *Thermus thermophilus*) but several more have been sequenced, all of bacterial origin. The active site of each of the three enzymes contains a manganese-rich reaction center rather than a heme group (Kono and Fridovich, 1983; Allgood and Perry, 1986; Waldo *et al.*, 1991).

Minor catalases (type D):

This group includes heme-containing enzymes including most peroxidases (Arnao *et al.*, 1990) that have been observed to exhibit a low level of catalatic activity. The chloroperoxidase from *Caldariomyces fumago* exhibits the greatest reactivity as a catalase (Sun *et al.*, 1994; Sundaramoorthy et al., 1995). Despite the fact that there is yet only one such example to consider, it provides an alternate mechanism for the catalatic reaction (Nicholls *et al.*, 2001)

1.4. Peroxidases

1.4.1. Introduction

Peroxidases played a key role in the development of modern enzymology. Much of the early definitive work on peroxidases was done by Keilin and Theorell in the 1930's. Horseradish peroxidase was for a long time, the best known, most worked

upon and the simplest example of a heme-containing enzyme. The explosion in

discoveries about enzymes in the last half of the twentieth century dwarfs all previous

knowledge. Research on peroxidases and heme enzymes has both contributed to and

benefited from this explosion. Most peroxidases like catalases are hemoprotein

enzymes, which catalyze the overall reaction $H_2O_2 + 2RH \rightarrow H_2O + 2R\cdot$, where RH

represents reduced substrate hydrogen donor and R represents the oxidized hydrogen

donor, which may be a free radical species. Unlike catalases, however, there is a great

deal of variability in peroxidases with regard to their structural properties. This is

probably a consequence of many different roles that individual peroxidases play in

organisms from which they are derived. While a majority of peroxidases are

hemoproteins, such as horseradish peroxidase (HRP), cytochrome c peroxidase (CCP),

and other mammalian, plant and fungal peroxidases, there are non-heme peroxidases,

such as the vanadium-containing chloroperoxidase enzyme from *Curvularia*

inaequalis (Messerschmidt and Wever, 1996), the FAD cysteine redox center NADH

peroxidase from *Streptococcus faecalis* (Stehle *et al.*, 1991), the human peroxiredoxin

(peroxidase) (Choi *et al.*, 1998), and the selenium-containing glutathione peroxidases

(GPXs) (Arthur, 2000).

1.4.2. The peroxidase reaction

The peroxidase reaction proceeds in three steps. Reaction (1) or compound I

formation is the first step and is common to catalases and peroxidases. However they

differ in the second and third steps. While catalases proceed with reactions (2) or (3)

for compound I reduction, peroxidases use single electron donor substrates (organic

or inorganic) to reduce compound I, as shown in reactions (4) and (5).

compound I (Por^{+}·-Fe^{IV}=O) + RH → compound II (Por-Fe^{IV}-OH) + R· (4)

compound II (Por-Fe^{IV}-OH) + RH → Enz (Por-Fe^{III}) + R· (5)
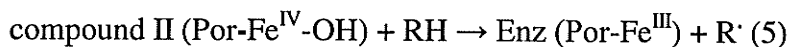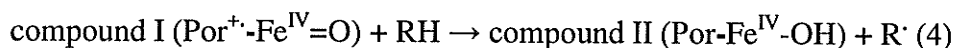
1.4.3. Peroxidase: Categorization and Properties

On the basis of sequence homologies, Welinder has proposed three families of peroxidases, which belong to the plant peroxidase superfamily (Welinder, 1992). The class I family constitutes intracellular peroxidases of prokaryotic origin. These include CCP, chloroplast and cytosolic ascorbate peroxidases (APXs), and bacterial catalase-peroxidases such as HPI of *Escherichia coli*. Class II family comprises of secreted fungal peroxidases such as the lignin and manganese peroxidases of *Phanerocheate chrysosporium* and the ink cap mushroom peroxidase from *Coprinus cinereus*. Class III family is made up of classical secretory plant peroxidases. The most extensively studied example is HRP isoenzyme C (Dunford, 1999a). Although, the three classes only show about 20% sequence similarity, they have conserved residues in the heme cavity, namely the distal Arg and His, and the proximal His hydrogen-bonded with a buried Asp side-chain, which are functionally and structurally important (Welinder, 1992; Welinder and Gajhede, 1993). The Welinder classification systematizes existing knowledge of peroxidases, leads to predictions about unknown properties, and provides a framework for future work on plant peroxidases. Heme-containing animal peroxidases such as thyroid, eosinophil, lacto- and myeloperoxidases constitute a separate superfamily (Dunford, 1999b).

Historically, many important discoveries and achievements in peroxidase research were first made by investigations of CCP and HRP and comparisons to them. Between the two of them, they provide an excellent starting point to discuss all of the

known peroxidases. The CCP enzyme from *Saccharomyces cerevisiae* is a single polypeptide chain of 294 amino acids with a single heme (protoporphyrin IX group) prosthetic group bound non-covalently and has a molecular weight of 35.4 kDa (Bosshard *et al.*, 1991). It is present in the mitochondria and is induced under aerobic conditions (Welinder, 1992). The HRP enzyme found in horseradish roots exists in the form of several isoenzymes, of which isoenzyme C (neutral), is the most predominant and also the best characterized form. HRP is a monomeric protein with 308 amino acid residues having a non-covalently bound protoporphyrin IX group. In addition, the enzyme possesses two calcium ions, an N-terminal signal peptide for excretion, four conserved disulfide bridges and eight neutral carbohydrate side chains attached through Asn residues. The molecular weight of HRP is 34.5 kDa excluding the carbohydrate content and 42.1 kDa if the carbohydrate side chains are taken into account (Dunford, 1991).

Both CCP and HRP have been successfully cloned and expressed in *E. coli* as apoenzymes, and then reconstituted with heme (Fishel *et al.*, 1987 and Smith *et al.*, 1990). The crystal structure of CCP was solved in the mid-eighties (Poulos *et al.*, 1980; Finzel *et al.*, 1984) while that of HRP was solved only in the late nineties (Gajhede *et al.*, 1997). Critical amino acid residues on both the proximal and distal sides of the heme are conserved between CCP and HRP. The conserved residues in the substrate accessible distal heme cavity include distal histidine (His52 in CCP, His42 in HRP) and arginine (Arg48 in CCP, Arg38 in HRP). In addition, Trp51 of CCP and the corresponding Phe41 of HRP are both key distal cavity residues involved in enzyme catalysis. Both enzymes have histidine side chains as proximal

axial ligands to the heme (His175 in CCP and His170 in HRP) (Welinder *et al.*, 1992; Dunford, 1999b).

The original Poulos and Kraut (Poulos and Kraut, 1980) mechanism of compound I formation that was based on the 1980 crystal structure of CCP laid the foundation for understanding compound I formation in plant peroxidases. Two features of their mechanism for the transfer of an oxygen atom from hydrogen peroxide to iron (III) have become accepted for all other plant peroxidases including HRP. Poulos and Kraut suggested that the distal histidine was involved as a proton acceptor from hydrogen peroxide and the distal arginine acted as a charge stabilizer during compound I formation (Poulos, 1987).

Both CCP and HRP react rapidly with H_2O_2 to form a very stable compound I species that has two oxidizing equivalents above the ferric resting enzyme. The ferric iron of both enzymes is oxidized in compound I to an oxyferryl ($Fe^{IV}=O$) species, but the site that contributes the second electron differs. In HRP an electron is removed from the porphyrin to give a porphyrin radical π-cation (Dolphin *et al.*, 1971), but in CCP the electron is removed from the proximal Trp191 to give a protein radical (Sivaraja *et al.*, 1989). The optical spectrum of HRP compound I consists of a Sorêt band reduced in intensity by a factor of ~2 compared to the band of native HRP, and a rather featureless visible spectrum. Because the free radical electron in CCP, is undergoing little interaction with the $Fe^{IV}=O$ group, the optical spectrum of CCP is very distinct from that of HRP and in fact resembles that of compound II of HRP (Dunford, 1999c). One-electron substrate oxidation reduces the porphyrin or protein radical and gives the one-electron oxidized ferryl species known as compound II, which in turn is reduced via a one-electron substrate oxidation to the resting ferric

state enzyme. Thus the conventional wisdom has been that the reaction cycle of CCP and HRP are very similar except for the atypical structure of compound I of CCP and its atypically large reducing substrate, cytochrome c (Ortiz de Montellano $et\ al.$, 1994; Dunford, 1999c).

In addition to effecting the conversion of H_2O_2 to H_2O plant peroxidases have also been suggested to have wider roles in reactions of biological significance such as in the peroxidation of plant hormones and lignin precursors. The links between peroxidases and such reactions are apparent from the requirement for different reducing substrates in the catalytic process (Smith and Veitch, 1998). In CCP the compound I reductants are two molecules of ferrocytochrome c, while in HRP; the possible reductants comprise a wide range of compounds such as aromatic phenols, anilines, amines, and even NADH/NADPH (Smith $et\ al.$, 1995; Dunford, 1991; Haliwell, 1978). Reduction of CCP compound I is extremely rapid, with a maximum steady state turnover reported to be $1.4 \times 10^4\ s^{-1}$ (Yonetani and Ray, 1966). The kinetics of this reduction are very complex, as each reduction step requires the formation of CCP-cytochrome c complex followed by intramolecular electron transfer reaction donation to the CCP active site. A recently proposed model for the mechanism proposes a two binding site model, where the affinity of the two sites for cytochrome c differs by 1000-fold and rapid electron transfer occurs only at the high affinity site (Miller, 1996). Reduction of HRP has been described as following a type of ping-pong (ordered two-substrate, two-product) reaction (Dunford, 1991), which proceeds through sequential one-electron reductions of the enzyme, and yields an optically observable second HRP reaction intermediate known as compound II, which is one oxidizing equivalent above that resting (ferric) enzyme. Reduction of HRP

compounds I and II usually generates radical products. This feature, in combination with the involvement of mediating molecules such as scopoletin and oxygen, allows HRP to carry out peroxidatic as well as oxidatic reactions (Saikumar *et al.*, 1994; DeSandro *et al.*, 1991), and can often lead to inactivation of the enzyme by formation of substrate radical adducts (Gilfoyle *et al.*, 1996) or other types of substrate inhibition (Bayton *et al.*, 1994). The most parsimonious mechanisms for HRP compound I and compound II degradation propose that the hydrogen donor donates an electron to reduce the porphyrin radical and simultaneously donates its hydrogen to the distal basic histidine chain in reducing compound I to compound II, and then a second donor molecule donates both its hydrogen and one electron to the oxoferryl group which is hydrogen bonded to the protonated distal histidine, resulting in the reduction of the iron and formation of the water leaving group (Dunford, 1991).

In addition to peroxidatic and oxidatic reactions, HRP can also catalyze other reactions *in vitro*, such as superoxide anion mediated production of yet another spectral intermediate, an inactive form of the enzyme termed compound III or oxyperoxidase (Nakajima and Yamazaki, 1987), and formation of compound IV, a similar but irreversibly inactivated species (Baynton *et al.*, 1994). However, these types of reactions, like those for catalases, may not be physiologically relevant.

1.5. Catalase-Peroxidases

A new class of bacterial enzymes, the catalase-peroxidases emerged in the late seventies with the first detections of the catalase-peroxidase hydroperoxidase I (HPI) from *Escherichia coli* (Claiborne and Fridovich, 1979). Subsequently, many different catalase-peroxidases were isolated and characterized from bacteria (Loewen, 1997)

and fungi (Fraaije *et al.*, 1996; Levy *et al.*, 1992). The characteristic feature of these enzymes as their name suggests, is their bifunctional catalytic behaviour. These enzymes were later recognized as a part of the class I family of plant peroxidases due to their high sequence homology with class I members such as CCP and ascorbate peroxidase. Interestingly, catalase- peroxidases show very little sequence homology with eukaryotic-type monofunctional catalases (Welinder, 1991; Loewen, 1997).

Catalase peroxidases range in size from one to four subunits (Fukumori *et al.*, 1985; Dunford, 1999d). They have a protoporphyrin IX (heme *b* or protoheme) prosthetic group with subunit sizes of about 80 kDa (Loewen, 1997). They show a sharp maximum in their catalatic activity at pH 6.0-6.5, and unlike classical catalases are not inhibited by 3-amino-1,2,4-triazole. Catalase-peroxidases are inactivated by an ethanol-chloroform mixture and also lose half of their activity when dialysed against 2 mM hydrogen peroxide for about 20-30 minutes (Dunford, 1999d). In contrast, classical catalases have a broad pH range for activity (5.5-10), are unaffected by the ethanol-chloroform mixture and do not lose activity following dialysis against hydrogen peroxide for periods up to one hour. Catalase-peroxidases are not activated by temperature increase, whereas classic catalases are (Dunford, 1999d).

The catalase-peroxidase from *Mycobacterium tuberculosis* MtKatG has been implicated in the activation of the antitubercular drug isoniazid (INH). 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 (Zhang *et al.*,

1992; Loewen *et al.*, 2000).

The importance of isoniazid as an antitubercular drug and the prevalence of *katG*-induced drug resistance generated a lot of interest worldwide, in determining the crystal structure of KatG. Unfortunately, attempts to crystallize a number of different catalase-peroxidases including MtKatG were unsuccessful until very recently when the crystallization of the enzymes from *Holoarcula marismortui* (Yamada *et al.*, 2001), *Synechococcus* (Wada *et al.*, 2002), *Burkholderia pseudomallei* (Carpena *et al.*, 2002) and of the C-terminal domain of *Escherichia coli* HPI were reported (Carpena *et al.*, 2002). Hence most of the structure-function studies of catalase-peroxidases were originally modeled on the high-resolution crystal structure of CCP (Finzel *et al.*, 1984).

Based on homology and modeling with yeast CCP, important predictions have been made about catalase-peroxidases (Welinder, 1991; Welinder, 1992). Whereas the peroxidases of plants, fungi and yeast are monomeric and contain 290-350 amino acid residues, the subunit of bacterial catalase-peroxidases contains approximately 730 residues. The approximate doubling of the molecular weight has been suggested to be caused by gene duplication and fusion during evolution, leading to the formation of two domains (Welinder, 1991). The N-terminal domain has a high degree of sequence homology with CCP and is considered the active half of catalase-peroxidases. A heme binding consensus sequence has been detected in the N-terminal half of every subunit. The C-terminal half evolved with a greater sequence deviation without bound heme and is considered as the inactive half of these enzymes (Dunford, 1999d). It was recently reported that the C-terminal half may be involved in the manganese peroxidase activity of the catalase-peroxidase CpeB from *Streptomyces*

reticuli (Zou and Schrempf, 2000). The key residues in the active site of CCP, which play a part in catalysis, folding and structural stability, are either invariant or highly conserved in all bacterial catalase-peroxidases (Welinder, 1991).

Figure 1.1 shows the orientation of catalytic residues in the putative active site of catalase-peroxidases based on the structure of yeast CCP. Ferriprotoporphyrin IX or heme *b* that is present in the active site of yeast CCP is also believed to be present in the putative active site of catalase-peroxidases. The residue numbers shown in Figure 1.1 are for the catalase-peroxidase HPI from *Escherichia coli* and the numbers in parentheses are for corresponding residues in yeast CCP. The distal amino acid residues shown in the figure (Trp51, His52 and Arg48) have been shown to be essential for catalysis in CCP (Dunford, 1999b). In CCP, the fifth coordination position of the iron molecule in the heme facilitates the attachment of the heme to the apoprotein by covalent bonding with the imidazole side-chain of the proximal histidine (His175), which is also known as the proximal fifth ligand (Dunford, 1999b). The sphere shown on the distal side of the heme iron in Figure 1.1 represents a water molecule (Hillar *et al.*, 2000).

1.6. Catalase enzymes of *Escherichia coli*

Three different enzymes having catalase activity have been identified in *Escherichia coli*. Hydroperoxidase I [HPI] (Claiborne and Fridovich, 1979) and Hydroperoxidase II [HPII] (Claiborne *et al.*, 1979) are the chromosomally encoded catalase enzymes, both of which have been well studied. HPI is a catalase-peroxidase and HPII is a monofunctional catalase. A third catalase enzyme from *E. coli* termed KatP has been described more recently (Brunder *et al.*, 1996). It is a novel catalase-
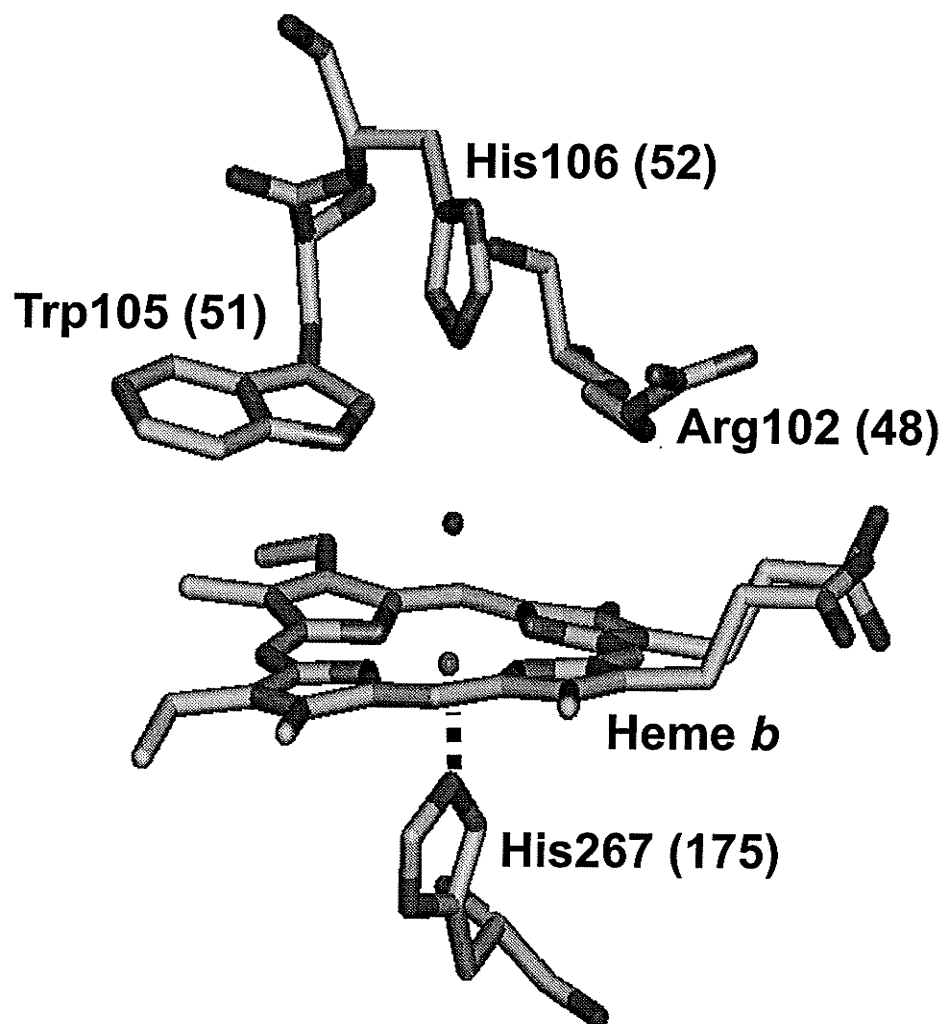
Figure 1.1: Orientation of residues in the active site of catalase-peroxidase EcHPI based on the structure of yeast CCP.

peroxidase encoded by the large plasmid of the enterohaemorrhagic *E. coli* (EHEC) strain O157:H7. Nucleotide sequencing revealed an ORF of 2208 bp and predicted a molecular mass of 81.8 kDa. In addition, an N-terminal signal sequence was found, suggesting that KatP is exported to, and functions in the periplasm. PCR analysis of representative strains of all enteric *E. coli* pathogroups (i.e. enterohaemorrhagic, enterotoxigenic, enteropathogenic, enteroaggregative and enteroinvasive *E. coli*) revealed a close association between the occurrence of EHEC-haemolysin and the *katP* gene in Shiga-like-toxin-producing *E. coli* O157 strains (Brunder *et al.*, 1996).

1.6.1. HPII of *Escherichia coli*

HPII, the monofunctional catalase is encoded by the *katE* gene which is located at 37.8 min on the *E. coli* chromosome (Loewen, 1984). HPII with 753 residues per sub-unit is the largest catalase so far characterized, existing as a homotetramer of 84.2 kDa subunits (von Ossowski *et al.*, 1991). Each subunit has a non-covalently associated heme *d* cis prosthetic group, and lacks the NADPH binding site found in other catalases. The enzyme has been extensively studied and its high-resolution crystal structure has been solved (Bravo *et al.*, 1995; Bravo *et al.*, 1999). HPII has a high catalase specific activity of ≈15,000 U/mg (Loewen and Switala, 1986) and is found in the cytoplasm. Like typical catalases, HPII is sensitive to 3-amino-1,2,4-triazole (Loewen and Switala, 1986) and is stable in 0.1% SDS or 7M urea, at 70°C (Meir and Yagil, 1985; Loewen and Switala, 1986). HPII maintains full catalatic activity over a pH range of 5-11 (Loewen and Switala, 1986) and is extremely thermotolerant, with a temperature inactivation midpoint of 83°C (Switala *et al.*, 1999). The enzyme is only expressed at low levels during the exponential phase. It is

produced primarily in the stationary phase when there is a ten- to twenty-fold increase in its levels (Loewen *et al.*, 1985a). Synthesis of HPII is regulated predominantly by the alternate sigma factor σ^S (RpoS), which is the central control element for a generalized stress response, including starvation, acid shock and hypertonic shift (Loewen and Hennge-Aronis, 1994).

1.6.2. HPI of *Escherichia coli*

HPI (EcKatG or EcHPI) is a catalase-peroxidase enzyme that is encoded by the *katG* gene located at 89.2 min on the *E. coli* chromosome (Loewen *et al.*, 1985b). It was first purified and described by Claiborne and Fridovich in 1979. It was originally described as a tetrameric enzyme made up of identical 80 kDa subunits containing two heme *b* (protoporphyrin IX) prosthetic groups per tetramer (Claiborne and Fridovich, 1979). However, recent studies have shown that the heme occupation of HPI like some other catalase-peroxidases is partial, at an average of 0.5 heme per subunit in a heterogenous mixture of dimers and tetramers with 0, 1 and 2 or 1, 2 and 3 hemes respectively (Hillar *et al.*, 2000). The amino acid sequence of the enzyme subunits has been deduced from the DNA sequence of the cloned *katG* to consist of 726 amino acids (Loewen *et al.*, 1983; Triggs-Raine *et al.*, 1988). The primary sequence (Triggs-Raine *et al.*, 1988), physical and enzymatic properties of HPI are very similar to those of *katG* gene products from other bacteria, such as *Salmonella typhimurium* (Loewen and Stauffer, 1990), *Mycobacterium tuberculosis* (Zhang *et al.*, 1992) and *Bacillus stearothermophilus* (Loprasert *et al.*, 1989). The cloned plasmid-encoded HPI enzyme has a catalase-specific activity of about 2000 U/mg (Loewen *et al.*, 1990; Hillar *et al.*; 2000) which is only about one-seventh of that reported for the

classical catalase HPII from *E. coli* (Loewen and Switala, 1986). Kinetic studies have shown that HPI has apparent catalatic K_m and. k_{cat} values of about 5.9 mM and 5300 sec^{-1} respectively (Hillar *et al.*, 2000). In addition to the catalase activity, the enzyme has also been shown to elicit a significant peroxidatic activity ($\approx$3.2 U/mg with o-dianisidine and $\approx$660 U/mg with 2,2-*azinobis* [ABTS]; Hillar *et al.*, 2000) towards various aromatic hydrogen donors such as 2,2-*azinobis* [ABTS], o-dianisidine, guaiacol, pyrogallol, p-phenyldiamine (Claiborne and Fridovich, 1979; Hillar *et al*; 2000) and also towards 3-diaminobenzidine which is used for *in situ* localization of peroxidase activity in polyacrylamide gels (Loewen and Switala, 1986).

HPI is not as robust as HPII, showing a relatively sharp pH optimum (6.5-7) for catalase activity (Loewen *et al.*, 1990) and a much lower temperature inactivation mid-point of 53°C (Switala *et al.*, 1999). HPI is inhibited by the classical heme inhibitors such as cyanide and azide but is resistant to inhibition by 3-amino-1,2,4-triazole. Recent studies have confirmed that HPI is distributed throughout the cytoplasm (Hillar *et al.*, 1999) and is not localized in the periplasm as previously reported (Heimberger and Eisenstark, 1988). The enzyme is expressed constitutively in the exponential phase under the control of the alternate sigma factor σ^S (RpoS) and its levels increase about two-fold as cells enter stationary phase, presumably because of elevated σ^S levels (Ivanova *et al.*, 1994; Visick and Clark, 1997). Addition of hydrogen peroxide directly to the medium of exponential phase cells causes a 10- to 20-fold increase in HPI levels (Loewen *et al.*, 1985a). This is a result of activation of OxyR regulatory protein which in response to oxidant levels in the cell induces the expression of nine different genes encoding antioxidant proteins such as HPI, alkylhydroperoxidase and gluthathione peroxidase (Christman *et al.*, 1989).

1.6.3. Importance of studying structure-function in EcHPI

HPI has a high degree of amino acid homology with other catalase-peroxidases, which indicates a common evolutionary origin for members of this group (Zamocky *et al.*, 2000). In addition, HPI has significant (~70%) sequence similarity with MtKatG, the catalase-peroxidase enzyme of *Mycobacterium tuberculosis*, that has been implicated in the peroxidatic activation of the antitubercular drug isoniazid (INH). MtKatG and HPI have similar physical and chemical properties including comparable kinetic parameters, subunit sizes, and susceptibility to classical heme inhibitors such as cyanide. Despite these similarities, HPI is not as proficient as MtKatG in the peroxidation of INH (Hillar and Loewen, 1995), nor are *E. coli* cells susceptible to INH, even at high concentrations of the drug, although over expression of MtKatG in *E. coli* results in the increased susceptibility of cells to INH (Zhang *et al.*, 1992). Recent spectral and EXAFS (Extensive X-ray absorption fine structure analysis) studies point to only minor differences in the heme iron environment between EcKatG and MtKatG, suggesting that greater differences in the protein structure beyond the immediate redox centre of the enzymes are responsible for differences in how they interact with INH (Powers *et al.*, 2001).

Previous site-directed mutagenesis studies on HPI (Hillar *et al.*, 2000) and SynKatG, the catalase-peroxidase from the *Synechocystis* PCC 6803 (Regelsberger *et al.*, 2000) have suggested a role for the three active site residues Arg, Trp and His (Arg102, Trp105 His106 in HPI) in the catalatic mechanism, which can be modeled on the original peroxidatic mechanism (Poulos and Kraut, 1980; English and

Tsaprailis, 1996). Interestingly, yeast CCP which also has the same Trp, Arg, His triad in its active site in corresponding positions does not show any catalatic activity and in fact uses a large molecule, cytochrome c, as a reducing substrate. The properties of the HPI variants that were generated at the three amino acid positions (Arg102, Trp105 and His106) were generally consistent with the properties of variants of CCP with changes in equivalent residues. For example, changing either of the distal side His (52 in CCP) or Arg (48 in CCP) caused significant reductions in peroxidatic activity of the enzyme (Vitello *et al.*, 1993; Erman *et al.*, 1993). Minor structural differences between HPI and CCP may thus be responsible for letting HPI behave predominantly as a catalase and yeast CCP as a peroxidase.

Tryptophan residues in and around the putative active site of HPI are highly conserved among catalase-peroxidases (Figure 1.2). Because of their aromaticity, hydrophobicity, large size and specific pattern of distribution (Figure 1.2), tryptophan residues may be important constituents involved in the reactivity, substrate access and stability of the crystal structure of the protein. Studies on HPI (Hillar *et al.*, 2000) and SynKatG, the catalase-peroxidase from the *Synechocystis* PCC 6803 (Regelsberger *et al.*, 2000) have suggested that the indole ring of the distal Trp (Trp 105 in *E. coli*) is essential for the two-electron reduction of compound I of both enzymes. The reasoning for this was based on the observations that in the Trp-replacement mutants the catalase activity was significantly reduced (Hillar *et al.*, 2000) or even lost (Regelsberger *et al.*, 2000), whereas the peroxidatic-to-catalatic ratio was increased dramatically (Hillar *et al.*, 2000), indicating that compound I formation was not influenced by this mutation.

1.7. Objectives of thesis

The objective of this thesis is to investigate the catalytic and structural role of certain conserved tryptophan (Trp) residues in and around the putative active site of catalase-peroxidase HPI from *Escherichia coli*, using site-directed mutagenesis methodology. The objective would be achieved by creation and construction of active mutant variants of phagemid-borne *katG*-encoded HPI. Trp88, Trp89 and Trp197 were chosen as the target residues for the study. They will be substituted with either phenylalanine (Phe) or Leucine (Leu). The Trp-replacement variants would then be characterized using various parameters such as catalase activity, peroxidase activity, heme binding, kinetic studies and sensitivity to common heme inhibitors, in order to ascertain the role of target residues in enzyme catalysis.

Enzymatic activity of HPI and other catalase-peroxidases has been associated with the amino-terminal domain and currently no function has been assigned to the carboxy-terminal domain, although it may play role in substrate binding. Studies in MtKatG have shown that deletion of the carboxy-terminal domain results in inactive enzyme (Heym *et al.*, 1993). A limited study of the carboxy-terminal domain of HPI would also be initiated in the present study to ascertain its role in catalysis or structure of the enzyme. This study would be performed by progressive truncation of the carboxy-terminus of HPI by insertion of stop codons into phagemid-borne *katG* in place of lysine (Lys). Lys705 and Lys654 were randomly chosen as the target residues, which will be mutagenized into termination codons in order to achieve truncation at those positions.

1 MSTSDDIHNTTATGKCPFHQGGHDQSAGAGTTTRDWWPNQ

41 LRVDLLNQHSNRSNPLGEDFDYRKEFSKLDYYGLKKDLKA

81 LLTESQPWWPADWGSYAGLFIRMAWHGAGTYRSIDGRGGA

121 GRGQQRFAPLNSWPDNVSLDKARRLLWPIKQKYGQKISWA

161 DLFILAGNVALENSGFRTFGFGAGREDVWEPDLDVNWGDE

201 KAWLTHRHPEALAKAPLGATEMGLIYVNPEGPDHSGEPLS

241 AAAAIRATFGNMGMNDEETVALIAGGHTLGKTHGAGPTSN

281 VGPDPEAAPIEEQGLGWASTYGSGVGADAITSGLEVVWTQ

321 TPTQWSNYFFENLFKYEWVQTRSPAGAIQFEAVDAPEIIP

361 DPFDPSKKRKPTMLVTDLTLRFDPEFEKISRRFLNDPQAF

401 NEAFARAWFKLTHRDMGPKSRYIGPEVPKEDLIWQDPLPQ

441 PIYNPTEQDIIDLKFAIADSGLSVSELVSVAWASASTFRG

481 GDKRGGANGARLALMPQRDWDVNAAAVRALPVLEKIQKES

521 GKASLADIIVLAGVVGVEKAASAAGLSIHVPFAPGRVDAR

561 QDQTDIEMFELLEPIADGFRNYRARLDVSTTESLLIDKAQ

601 QLTLTAPEMTALVGGMRVLGANFDGSKNGVFTDRVGVLSN

641 DFFVNLLDMRYEWKATDESKELFEGRDRETGEVKFTASRA

681 DLVFGSNSVLRAVAEVYASSDAHEKFVKDFVAAWVKVMNL

721 DRFDLL

Figure 1.2. Amino acid sequence of catalase-peroxidase EcHPI.

2. MATERIALS AND METHODS

2.1. *Escherichia coli* strains, plasmids and bacteriophage

The *E. coli* strains, plasmids and bacteriophage used in this study are listed in Table 2.1. Phagemids pSK+ and pKS- from Stratagene Cloning Systems were generally used as vectors for mutagenesis, sequencing and cloning. The *E. coli katG* gene encoding HPI was originally cloned into pAT153, to generate pBT22 (Triggs-Raine and Loewen, 1987) and then was later cloned into pKS- (Stratagene Cloning Systems) as pM*katG* or pAH7 for site-directed mutagenesis and subsequent variant protein expression. Plasmid pAB42 was created by modification of pKS- via removal of the *Eco*RI and *Bam*HI restriction sites located within its multiple cloning site. This procedure was performed using Klenow polymerase according to the procedure of Ausubel *et al.*, 1989. The plasmid KS- was first cut with *Eco*RI and *Bam*HI and then treated with 5 U of Klenow (Large fragment of DNA polymerase I) in the presence of 0.5 mM of each dNTP at 30°C for about 30 minutes. The reaction was stopped by heating the mixture to 75°C for 10 minutes or by adding 1μl of 0.5 EDTA. The plasmid pAB45 was created by inserting *katG* gene into pAB42.

Strain CJ236, harbouring plasmids pAH2, pAH10 or pAB30, containing subcloned fragments I or II or III of the *E. coli katG* gene (Figure 2.1.) respectively were used for generation of single-stranded, uracil containing DNA templates employed for site-directed mutagenesis. Helper phage R408 was used for infection of the strain CJ236 to generate single-stranded DNA. Strain NM522 was used for cloning and plasmid propagation. Strain JM109 was used for production of plasmid DNA for double-stranded DNA sequencing. Subclone fragments from pAH10 or pAH2 or pAB30, with the desired

mutations were used to reconstruct the complete *katG* gene using fragments from other plasmids namely pAH9, pAH7, pAB45 or pAB42 (Section2.4.2b). Strain UM262, which is catalase negative, was used for routine expression of catalase proteins from various plasmids.

2.2. Biochemicals and common reagents

All biochemicals and reagents, including antibiotics used during the course of these studies, were usually purchased from either Sigma Chemical Co. (St. Louis, Missouri), or from Fisher Scientific Ltd. (Mississauga, Ontario). Restriction nucleases, polynucleotide kinase, T4 DNA ligase and most other enzymes used in the study were purchased from Invitrogen Canada Inc. (Burlington, Ontario). The dideoxy sequencing kit was obtained from USB Corporation (Cleveland, Ohio). Components for the media used for growth of bacterial cell cultures were usually obtained from Invitrogen Canada Inc. (Burlington, Ontario). Most solutions were normally prepared using reverse osmosis distilled water.

2.3. Media, growth conditions and storage of stock cultures

E. coli cultures were routinely grown in LB (Luria-Bertani) medium containing 10 g/l tryptone, 5 g/l yeast extract, and 5 g/l NaCl. Solid LB media contained between 14 and 20 g/l agar. Ampicillin was added to a concentration of between 100 and 250 µg/ml for maintenance of selection pressure on plasmid-harboring strains grown in liquid media, and to a concentration of 100 µg/ml for growth on solid media. In addition, chloramphenicol was added to 40 µg/ml in order to maintain the presence of F′ episome for the growth of the strain CJ236.

Table 2.1. List of *Escherichia coli* strains, plasmids and bacteriophage used in this study.

	Genotype or characteristics	Source
Strains		
CJ236	*dut*1 *ung*1 *thi*-1 *rec*A1/pCJ105/·cam^{r}F′	Kunkel *et al.*, 1987
NM522	supE Δ(*lac-pro*AB) *hsd*-5 [F′*pro*AB *lac*I^q *lac*ZΔ15]	Mead *et al.*, 1985
JM109	*rec*A1 *sup*E44 *end*A1 *hsd*A1 *hsd*R17 *gyr*A96 *rel*A1 *thi* Δ(*lac-pro*AB)	Yanisch-Perron *et al.*, 1985
UM262	*rec*A *kat*G::Tn*10 pro leu rps*L *hsd*M *hsd*R *end*I *lac*Y	Loewen *et al.*, 1990
Plasmids		
pSK+, pKS-	Amp^R	Stratagene Cloning Systems
pAB42 (pKS- minus *Eco*RI and *Bam*HI sites	Amp^R	This study
pBT22 (pAT153, *E. coli katG* clone)	Amp^R	Triggs-Raine and Loewen, 1987
pM*katG* (pKS- + *E. coli katG* clone)	Amp^R	This study
pAH2 (pSK+, subclone I)	Amp^R	Hillar, 1999; *PhD. Thesis*
pAH10 (pSK+, subclone II)	Amp^R	Hillar, 1999; *PhD. Thesis*
pAB30 (pKS-, subclone III)	Amp^R	This study
pAH9	Amp^R	This study
pAH7 (pKS- + *E. coli katG* clone)	Amp^R	This study
pAB45 (pAB42 + *E. coli katG* clone)	Amp^R	This study
Bacteriophage		Stratagene Cloning Systems
R408 (helper phage)		
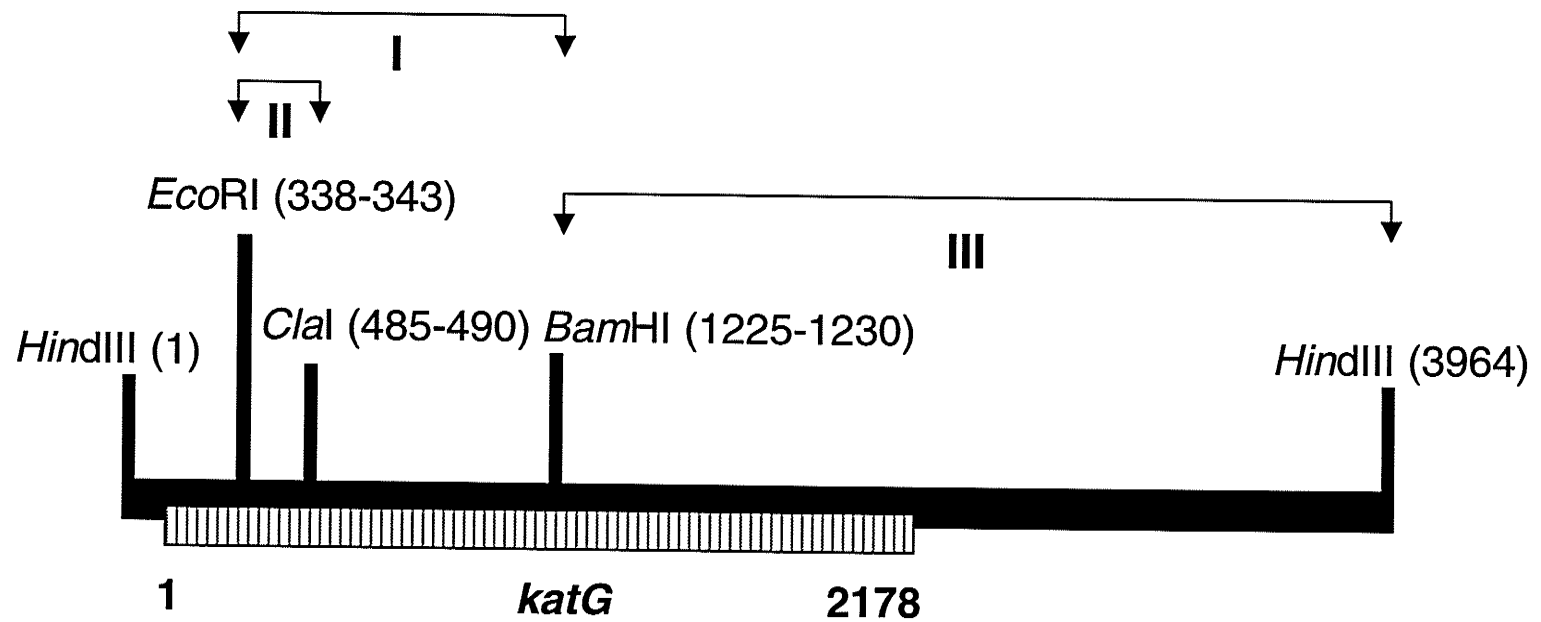
Figure 2.1. Simplified restriction maps of the chromosomal inserts containing the *E. coli* gene in plasmids pBT22, pAH7, pM*katG* and pAB45. The 2178 bp long *katG* open reading frame ▦ is indicated as part of the chromosomal insert ▬ as are the three insert fragments **I**, **II** and **III** used to construct the subclone vectors employed in site-directed mutagenesis of *E. coli katG*.

E. coli strains in general were grown in solid or liquid media at 37° C. However, cell cultures in liquid media for expression of wild type and variant proteins were either grown at 28°C or at 37°C in shake flasks with good aeration. Long term storage of stock cultures was done in 8% dimethylsulfoxide (DMSO) at -60°C. Bacteriophage R408 was stored at 4°C in LB culture supernatant.

2.4. DNA manipulation

2.4.1. Preparation of synthetic oligonucleotides

Oligonucleotides used for site-directed mutagenesis were synthesized using a PCR-Mate DNA synthesizer (Applied Biosystems Inc.). Extraction of oligonucleotide DNA from synthesis columns was accomplished by gently and repetitively washing the columns with 1 ml volumes of concentrated NH_4OH. The NH_4OH wash was then incubated at 55° C overnight, evaporated to dryness under vacuum, and resuspended in HPLC grade distilled water and stored at -20°C until further use. Concentrations of oligonucleotide DNA were determined spectrophotometrically at 260 nm, where one unit of absorbance ≈ 20 μg / ml single-stranded oligonucleotide DNA (Sambrook *et al.*, 1989).

Oligonucleotides used for site-directed mutagenesis were phosphorylated at the 5′-end using T4 kinase (Invitrogen Canada Inc.) according to the procedure of Ausubel *et al.*, (1989). Approximately 100 ng of oligonucleotide DNA in a volume of 25 μl, containing 1 mM ATP and 10 units of kinase were incubated in appropriately diluted buffer supplied by the manufacturer at 37°C for 30 minutes. The reaction was terminated by heat inactivation at 65°C for 5 minutes.

2.4.2a. Site-directed mutagenesis strategy

Targeted base changes on phagemid-borne *E. coli katG* were generated according

to the *in vitro* mutagenesis protocol described by Kunkel *et al.*, (1987). Subclones were

constructed from parts of the chromosomal insert containing the *katG* gene on pKS or

pSK. A simplified restriction map of *E. coli katG* indicating the locations of individual

subclones is shown in figure 2.1. The subclones rather than the whole gene were

mutagenized in order to limit the amount of subsequent sequencing required to confirm

mutations in the subclones both after mutagenesis and after reinsertion into the *E. coli*

katG gene for protein expression. Target codons for mutagenesis were selected from the

DNA sequence of *E. coli katG* shown in figure 2.3. DNA sequences for oligonucleotides

used in the mutagenesis procedure are listed in Table 2.2. Mutagenesis was performed by

annealing the phosphorylated oligonucleotides encoding the desired base modifications to

uracil-containing single-stranded DNA templates obtained from the appropriate

bluescript phagemid subclone. The complementary DNA strand was then synthesized *in*

vitro by unmodified T7 DNA polymerase (New England Biolabs) using the annealed

oligonucleotide as a primer. The 3′ and 5′ ends of the completed complementary strand

were ligated by including T4 DNA ligase (Invitrogen Canada Inc.) in the reaction mixture.

The DNA was then transformed into NM522 cells where the uracil-containing templates

were degraded. Plasmid DNA recovered from the transformants was further transformed

into JM109, recovered from this strain, used to screen for the desired mutation in the

plasmid subclone by DNA sequencing. Once the correct mutant was identified, the

complete subclone sequence was determined in order to ensure that no base changes apart

from those desired had been introduced. The mutated subclones were then used to

reconstruct the complete *E. coli katG* gene, which was then sequenced over the region containing the mutation for final confirmation. Reconstructed, mutant *E. coli katG* clones were then transformed into UM262 for determination of enzyme activities and visualization of protein from crude extracts or whole cells by SDS-PAGE. Clones expressing significant levels of variant HPI enzyme were then grown on a larger scale (4-8 liters) for purification and characterization of the enzyme.

2.4.2b. Reconstruction of *katG* subclones with desired mutation

An outline of the reconstruction protocol is illustrated in Figure 2.2. The plasmids have been drawn in a linearized form for reasons of simplicity. Reconstruction of mutant *katG*'s for W88F and W88L involved three stages. Plasmid pAH10$^+$ with the desired mutation and pAH9 were first cut with *Cla*I; the *Cla*I-*Cla*I fragment of pA10$^+$ was ligated into pAH9 to form the 4 kb plasmid pABx1. In the second stage, the *Eco*RI-*Bam*HI fragment from pABx1 was ligated into pAH7 that was also cut with *Eco*RI and *Bam*HI, to give the 4.2 kb plasmid called pABx2. Finally, the complete form of the variant *katG* called pABx3 ($\approx$2.1 kb) was generated by inserting the *Bam*HI-*Bam*HI fragment of pAH7 into pABx2 cut with *Bam*HI. The final plasmid with variant *katG* had a total size of $\approx$6.9 kb. W197F was reconstructed in a very similar manner but since the mutation was generated on plasmid pAH2, the construction directly proceeded to the second stage; the 900 bp *Eco*RI-*Bam*HI fragment from pAH2 was ligated to the 3300 bp *Eco*RI-*Bam*HI fragment from pAH7 to give pABx2, which was then used to construct pABx3 as done in the previous case.

Since the reconstruction processes (the steps involving single enzyme digestions) were a bit cumbersome, an alternate protocol was designed and implemented during the

later stages of the study, for the reconstruction of W89F, W89L and W197L variants. Plasmid pAB45 was substituted in the reconstruction process of W89F, W89L and W197L with some final modifications in the construction steps, which primarily involved cutting pABx2 and pAB45 with *Eco*RI and *Bam*HI (instead of the single *Bam*HI digestion) in the third stage and subsequent ligation to generate pABx3. Plasmid pAB45 is very similar to pAB7 but lacks the *Eco*RI and *Bam*HI sites that are indicated by * in Figure 2.2. The K705 variant was constructed by co-ligating the 2.7 kb *Bam*HI-*Hind*III fragment from pAB30 along with the 1.2 kb *Hind*III-*Bam*HI fragment from pAB45 into *Hind*III-cut pAB42. All the variant constructs were verified for correct positioning and orientation using various restriction digestion studies at each step in the construction process.

2.4.3. DNA isolation and purification

Plasmid DNA was isolated according to Sambrook *et al.*, (1989). All procedures were carried out at 4°C unless mentioned otherwise. Plasmids containing cells from 5 ml LB cultures grown to stationary phase were pelleted by centrifugation and resuspended in 0.2 ml volumes of Tris-glucose-EDTA buffer (25 mM Tris-HCl, pH 8.0, 1% glucose, 10mM Na-EDTA). The cells were then lysed by addition of 0.4 ml 1% SDS (w/v), 0.2 M NaOH solution and gently mixed. This was then neutralized by addition of 0.3 ml of 6.2 M ammonium acetate, pH 5.9. After 10-15 minutes incubation, the mixture was centrifuged twice to remove all precipitates. Plasmid DNA was then precipitated by addition of 0.55 ml propanol to the remaining supernatant followed by a 15-minute incubation at room temperature. It was then pelleted by centrifugation, washed twice with

Figure 2.2: General reconstruction protocol used for generation of variant *katG's* from

mutagenized subclones I or II, for protein expression.

Cutting with
EcoRI and BamHI

900 bp

pABx1
4.0 kb

Cutting with
EcoRI and BamHI

3300 bp

pAB x2
4.2 kb

pAH 7
6.9 kb

katg (2.1kb)
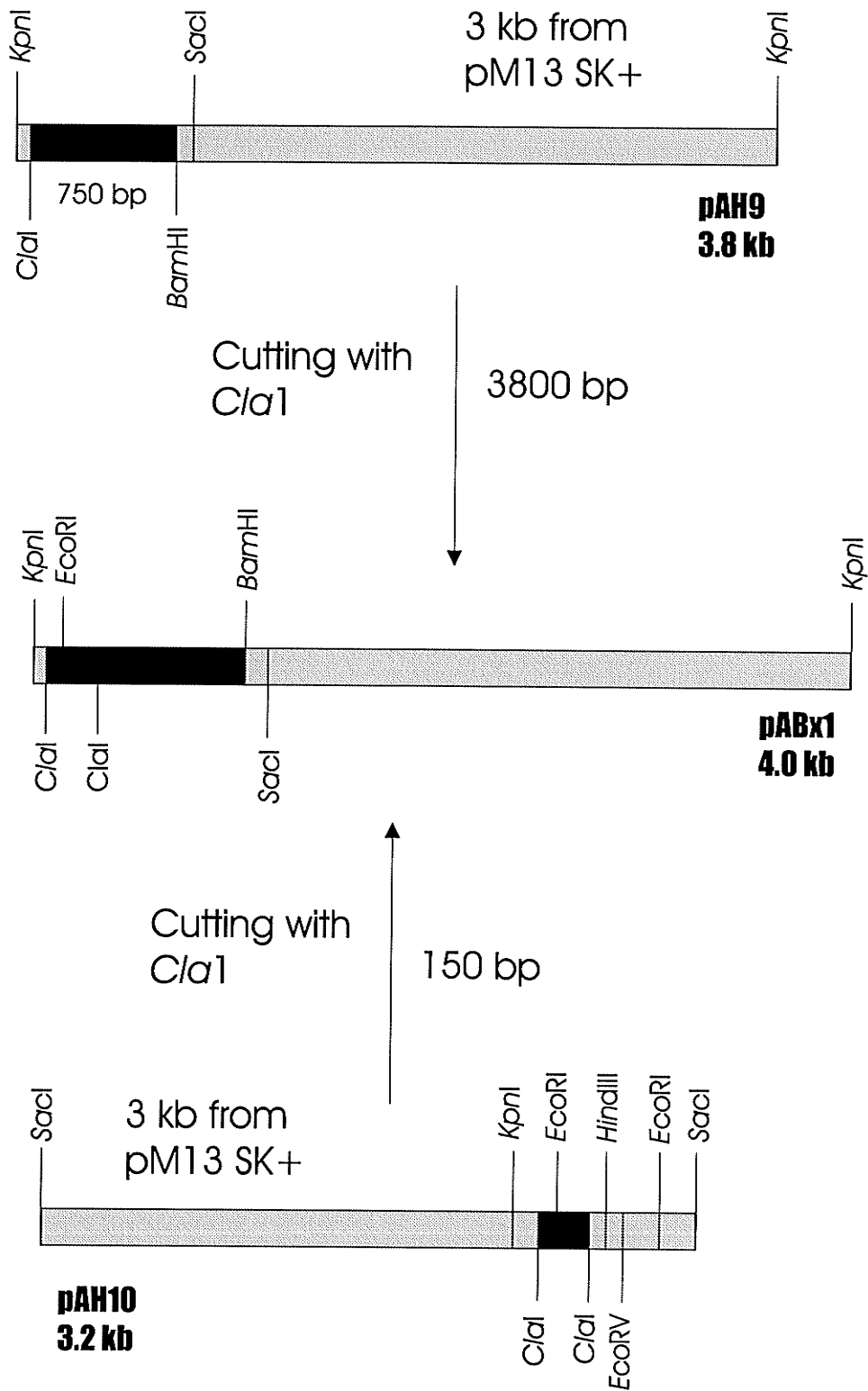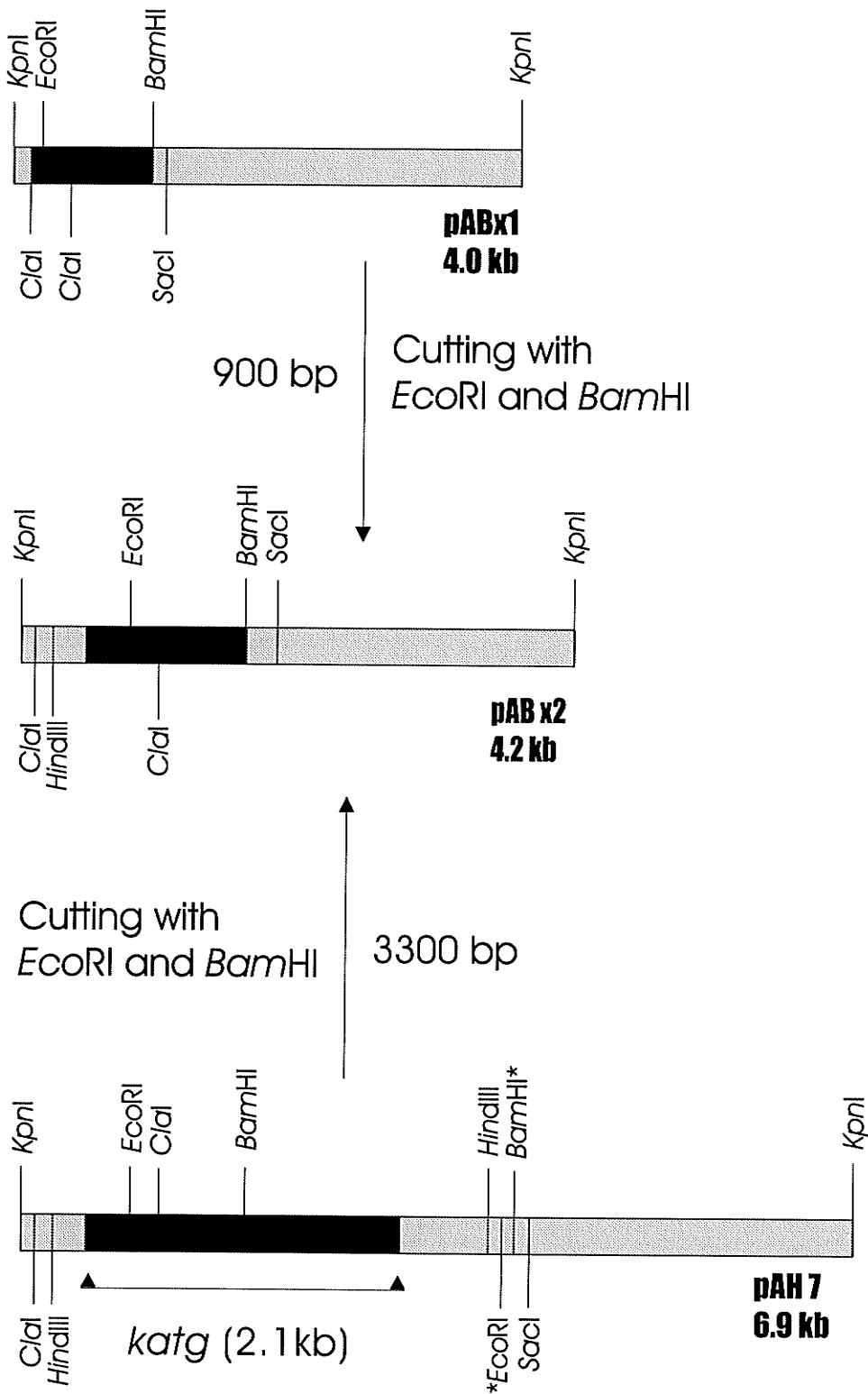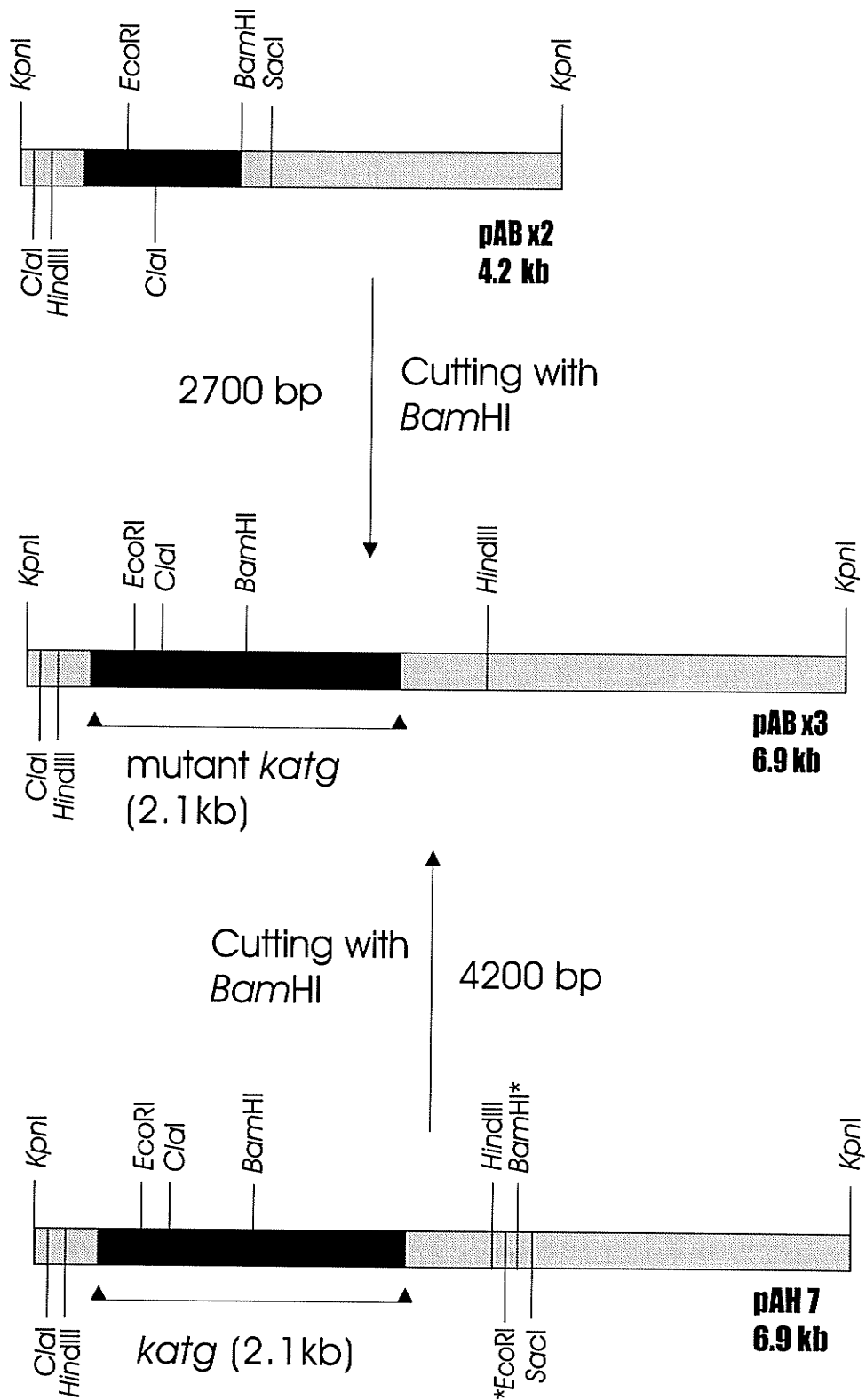
Figure 2.3: Nucleotide sequence of *E. coli katG* in the chromosomal insert of pBT22, pAH7, pM*katG* and pAB45 plasmids. Sequencing primers and mutagenic oligonucleotides showing base changes are indicated, as are restriction endonuclease recognition sequences, used for subcloning and excision of an internal gene fragment. The promoter sequences (−**35** and −**10**), the Shine-Dalgarno sequence (**SD**), and the putative transcription terminator sequences are also indicated in the figure (revised and modified from Triggs-Raine *et al.*, 1988, with appended sequence data from Blattner *et al.*, 1997, indicating an open reading frame {**ORF**} in the *katG-gldA* intergenic region).

```
AAGCTTAATTAAGATCAATTTGATCTACATCTCTTTAACCAACAATATGTAAGATCTCAA        60
TTCGAATTAATTCTAGTTAAACTAGATGTAGAGAAATTGGTTGTTATACATTCTAGAGTT
HindIII


CTATCGCATCCGTGGATTAATTCAATTATAACTTCTCTCTAACGCTGTGTATCGTAACGG       120
GATAGCGTAGGCACCTAATTAAGTTAATATTGAAGAGAGATTGCGACACATAGCATTGCC
                      -35                              -10


                        M  S  T  S  D  D  I  H  N  T  T  A       (12)
TAACACTGTAGAGGGGAGCACATTGATGAGCACGTCAGACGATATCCATAACACCACAGC      180
ATTGTGACATCTCCCCTCGTGTAACTACTCGTGCAGTCTGCTATAGGTATTGTGGTGTCG
          SD                                    EcoRV


   T  G  K  C  P  F  H  Q  G  G  H  D  Q  S  A  G  A  G  T  T    (32)
CACTGGCAAATGCCCGTTCCATCAGGGCGGTCACGACCAGAGTGCGGGGGCGGGCACAAC      240
GTGACCGTTTACGGGCAAGGTAGTCCCGCCAGTGCTGGTCTCACGCCCCCGCCCGTGTTG
      ------AGC---------(C16S)G17→


   T  R  D  W  W  P  N  Q  L  R  V  D  L  L  N  Q  H  S  N  R    (52)
CACTCGCGACTGGTGGCCAAATCAACTTCGTGTTGACCTGTTAAACCAACATTCTAATCG      300
GTGAGCGCTGACCACCGGTTTAGTTGAAGCACAACTGGACAATTTGGTTGTAAGATTAGC
                                                G1→


   S  N  P  L  G  E  D  F  D  Y  R  K  E  F  S  K  L  D  Y  Y    (72)
TTCTAACCCACTGGGTGAGGACTTTGACTACCGCAAAGAATTCAGCAAATTAGATTACTA      360
AAGATTGGGTGACCCACTCCTGAAACTGATGGCGTTTCTTAAGTCGTTTAATCTAATGAT
                                   EcoRI


   G  L  K  K  D  L  K  A  L  L  T  E  S  Q  P  W  W  P  A  D    (92)
CGGCCTGAAAAAAGATCTGAAAGCCCTGTTGACAGAATCTCAACCGTGGTGGCCAGCCGA      420
GCCGGACTTTTTTCTAGACTTTCGGGACAACTGTCTTAGAGTTGGCACCACCGGTCGGCT
                          ←G11
                          --------TTC--------W88F→
                          --------CTG--------W88L→
                              --------TTC--------
                              --------CTG--------
```

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    W   G   S   Y   A   G   L   F   I   R   M   A   W   H   G   A   G   T   Y   R      (112)
CTGGGGCAGTTACGCCGGTCTGTTTATTCGTATGGCCTGGCACGGCGCGGGGACTTACCG             480
GACCCCGTCAATGCGGCCAGACAAATAAGCATACCGGACCGTGCCGCGCCCCTGAATGGC
-W89F→
-W89L→


    S   I   D   G   R   G   G   A   G   R   G   Q   Q   R   F   A   P   L   N   S      (132)
TTCAATCGATGGACGCGGTGGCGCGGGTCGTGGTCAGCAACGTTTTGCACCGCTGAACTC             540
AAGTTAGCTACCTGCGCCACCGCGCCCAGCACCAGTCGTTGCAAAACGTGGCGACTTGAG
    ClaI


    W   P   D   N   V   S   L   D   K   A   R   R   L   L   W   P   I   K   Q   K      (152)
CTGGCCGGATAACGTAAGCCTCGATAAAGCGCGTCGCCTGTTGTGGCCAATCAAACAGAA             600
GACCGGCCTATTGCATTCGGAGCTATTTCGCGCAGCGGACAACACCGGTTAGTTTGTCTT
                                G2→


    Y   G   Q   K   I   S   W   A   D   L   F   I   L   A   G   N   V   A   L   E      (172)
ATATGGTCAGAAAATCTCCTGGGCCGACCTGTTTATCCTCGCGGGTAACGTGGCGCTAGA             660
TATACCAGTCTTTTAGAGGACCCGGCTGGACAAATAGGAGCGCCCATTGCACCGCGATCT


    N   S   G   F   R   T   F   G   F   G   A   G   R   E   D   V   W   E   P   D      (192)
AAACTCCGGCTTCCGTACCTTCGGTTTTGGTGCCGGTCGTGAAGACGTCTGGGAACCGGA             720
TTTGAGGCCGAAGGCATGGAAGCCAAAACCACGGCCAGCACTTCTGCAGACCCTTGGCCT


    L   D   V   N   W   G   D   E   K   A   W   L   T   H   R   H   P   E   A   L      (212)
TCTGGATGTTAACTGGGGTGATGAAAAAGCCTGGCTGACTCACCGTCATCCGGAAGCGCT             780
AGACCTACAATTGACCCCACTACTTTTTCGGACCGACTGAGTGGCAGTAGGCCTTCGCGA
    HpaI G8A→
    --------TTC---------W197F→
    --------CTG---------W197L→


    A   K   A   C   L   G   A   T   E   M   G   L   I   Y   V   N   P   E   G   P      (232)
GGCGAAAGCACCGCTGGGTGCAACCGAGATGGGTCTGATTTACGTTAACCCGGAAGGCCC             840
CCGCTTTCGTGGCGACCCACGTTGGCTCTACCCAGACTAAATGCAATTGGGCCTTCCGGG
                                              HpaI                  --


    D   H   S   G   E   P   L   S   A   A   A   A   I   R   A   T   F   G   N   M      (252)
GGATCACAGCGGCGAACCGCTTTCTGCGGCAGCAGCTATCCGCGCGACCTTCGGCAACAT             900
CCTAGTGTCGCCGCTTGGCGAAAGACGCCGTCGTCGATAGGCGCGCTGGAAGCCGTTGTA
----CTC--------(H234L)G16→    G3→


    G   M   N   D   E   E   T   V   A   L   I   A   G   G   H   T   L   G   K   T      (272)
GGGCATGAACGACGAAGAAACCGTGGCGCTGATTGCGGGTGGTCATACGCTGGGTAAAAC             960
CCCGTACTTGCTGCTTCTTTGGCACCGCGACTAACGCCCACCAGTATGCGACCCATTTTG


    H   G   A   G   P   T   S   N   V   G   P   D   P   E   A   A   P   I   E   E      (292)
CCACGGTGCCGGTCCGACATCAAATGTAGGTCCTGATCCAGAAGCTGCACCGATTGAAGA             1020
GGTGCCACGGCCAGGCTGTAGTTTACATCCAGGACTAGGTCTTCGACGTGGCTAACTTCT
```

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     Q   G   L   G   W   A   S   T   Y   G   S   G   V   G   A   D   A   I   T   S      (312)
ACAAGGTTTAGGTTGGGCGAGCACTTACGGCAGCGGCGTTGGCGCAGATGCCATTACCTC          1080
TGTTCCAAATCCAACCCGCTCGTGAATGCCGTCGCCGCAACCGCGTCTACGGTAATGGAG


     G   L   E   V   V   W   T   Q   T   P   T   Q   W   S   N   Y   F   F   E   N      (332)
TGGTCTGGAAGTAGTCTGGACCCAGACGCCGACCCAGTGGAGCAACTATTTCTTCGAGAA          1140
ACCAGACCTTCATCAGACCTGGGTCTGCGGCTGGGTCACCTCGTTGATAAAGAAGCTCTT


     L   F   K   Y   E   W   V   Q   T   R   S   P   A   G   A   I   Q   F   E   A      (352)
CCTGTTCAAGTATGAGTGGGTACAGACCCGCAGCCCGGCTGGCGCAATCCAGTTCGAAGC          1200
GGACAAGTTCATACTCACCCATGTCTGGGCGTCGGGCCGACCGCGTTAGGTCAAGCTTCG
     ------TTT-------(Y336F)G4→


     V   D   A   P   E   I   I   P   D   P   F   D   P   S   K   K   R   K   P   T      (372)
GGTAGACGCACCGGAAATTATCCCGGATCCGTTTGATCCGTCGAAGAAACGTAAACCGAC          1260
CCATCTGCGTGGCCTTTAATAGGGCCTAGGCAAACTAGGCAGCTTCTTTGCATTTGGCTG
                          BamHI


     M   L   V   T   D   L   T   L   R   F   D   P   E   F   E   K   I   S   R   R      (392)
AATGCTGGTGACCGACCTGACGCTGCGTTTTGATCCTGAGTTCGAGAAGATCTCTCGTCG          1320
TTACGACCACTGGCTGGACTGCGACGCAAAACTAGGACTCAAGCTCTTCTAGAGAGCAGC


     F   L   N   D   P   Q   A   F   N   E   A   F   A   R   A   W   F   K   L   T      (412)
TTTCCTCAACGATCCGCAGGCGTTCAACGAAGCCTTTGCCCGTGCCTGGTTCAAACTGAC          1380
AAAGGAGTTGCTAGGCGTCCGCAAGTTGCTTCGGAAACGGGCACGGACCAAGTTTGACTG


     H   R   D   M   G   P   K   S   R   Y   I   G   P   E   V   P   K   E   D   L      (432)
GCACAGGGATATGGGGCCGAAATCTCGCTACATCGGGCCGGAAGTGCCGAAAGAAGATCT          1440
CGTGTCCCTATACCCCGGCTTTAGAGCGATGTAGCCCGGCCTTCACGGCTTTCTTCTAGA


     I   W   Q   D   P   L   P   Q   P   I   Y   N   P   T   E   Q   D   I   I   D      (452)
GATCTGGCAAGATCCGCTGCCGCAGCCGATCTACAACCCGACCGAGCAGGACATTATCGA          1500
CTAGACCGTTCTAGGCGACGGCGTCGGCTAGATGTTGGGCTGGCTCGTCCTGTAATAGCT
     G5→                                                          ClaI


     L   K   F   A   I   A   D   S   G   L   S   V   S   E   L   V   S   V   A   W      (472)
TCTGAAATTCGCGATTGCGGATTCTGGTCTGTCTGTTAGTGAGCTGGTATCGGTGGCCTG          1560
AGACTTTAAGCGCTAACGCCTAAGACCAGACAGACAATCACTCGACCATAGCCACCGGAC


     A   S   A   S   T   F   R   G   G   D   K   R   G   G   A   N   G   A   R   L      (492)
GGCATCTGCTTCTACCTTCCGTGGTGGCGACAAACGCGGTGGTGCCAACGGTGCGCGTCT          1620
CCGTAGACGAAGATGGAAGGCACCACCGCTGTTTGCGCCACCACGGTTGCCACGCGCAGA


     A   L   M   P   Q   R   D   W   D   V   N   A   A   A   V   R   A   L   L   V      (512)
GGCATTAATGCCGCAGCGCGACTGGGATGTGAACGCCGCAGCCGTTCGTGCTCTGCCTGT          1680
CCGTAATTACGGCGTCGCGCTGACCCTACACTTGCGGCGTCGGCAAGCACGAGACGGACA


     L   E   K   I   Q   K   E   S   G   K   A   S   L   A   D   I   I   V   L   A      (532)
TCTGGAGAAAATCCAGAAAGAGTCTGGTAAAGCCTCGCTGGCGGATATCATAGTGCTGGC          1740
AGACCTCTTTTAGGTCTTTCTCAGACCATTTCGGAGCGACCGCCTATAGTATCACGACCG
                                      EcoRV          G6→
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       G  V  V  G  V  E  K  A  A  S  A  A  G  L  S  I  H  V  P  F        (552)
     TGGTGTGGTTGGTGTTGAGAAAGCCGCAAGCGCCGCAGGTTTGAGCATTCATGTACCGTT        1800
     ACCACACCAACCACAACTCTTTCGGCGTTCGCGGCGTCCAAACTCGTAAGTACATGGCAA


       A  P  G  R  V  D  A  R  Q  D  Q  T  D  I  E  M  F  E  L  L        (572)
     TGCGCCGGGTCGCGTTGATGCGCGTCAGGATCAGACTGACATTGAGATGTTTGAGCTGCT        1860
     ACGCGGCCCAGCGCAACTACGCGCAGTCCTAGTCTGACTGTAACTCTACAAACTCGACGA


       E  P  I  A  D  G  F  R  N  Y  R  A  R  L  D  V  S  T  T  E        (592)
     GGAGCCAATTGCTGACGGTTTCCGTAACTATCGCGCTCGTCTGGACGTTTCCACCACCGA        1920
     CCTCGGTTAACGACTGCCAAAGGCATTGATAGCGCGAGCAGACCTGCAAAGGTGGTGGCT
         MunI


       S  L  L  I  D  K  A  Q  Q  L  T  L  T  A  P  E  M  T  A  L        (612)
     GTCACTGCTGATCGACAAAGCACAGCAACTGACGCTGACCGCGCCGGAAATGACTGCGCT        1980
     CAGTGACGACTAGCTGTTTCGTGTCGTTGACTGCGACTGGCGCGGCCTTTACTGACGCGA


       V  G  G  M  R  V  L  G  A  N  F  D  G  S  K  N  G  V  F  T        (632)
     GGTGGGCGGCATGCGTGTACTGGGTGGCAACTTCGATGGCAGCAAAAACGGCGTCTTCAC        2040
     CCACCCGCCGTACGCACATGACCCACCGTTGAAGCTACCGTCGTTTTTGCCGCAGAAGTG
         SphI                                        G7→


       D  R  V  G  V  L  S  N  D  F  F  V  N  L  L  D  M  R  Y  E        (652)
     TGACCGCGTTGGCGTATTGAGCAATGACTTCTTCGTGAACTTGCTGGATATGCGTTACGA        2100
     ACTGGCGCAACCGCATAACTCGTTACTGAAGAAGCACTTGAACGACCTATACGCAATGCT
                                                                 - - - -


       W  K  A  T  D  E  S  K  E  L  F  E  G  R  D  R  E  T  G  E        (672)
     GTGGAAAGCGACCGACGAATCGAAAGAGCTGTTCGAAGGCCGTGACCGTGAAACCGGCGA        2160
     CACCTTTCGCTGGCTGCTTAGCTTTCTCGACAAGCTTCCGGCACTGGCACTTTGGCCGCT
     - - - -TAA- - - - - - - - - →K654*


       V  K  F  T  A  S  R  A  D  L  V  F  G  S  N  S  V  L  R  A        (692)
     AGTGAAATTTACGGCCAGCCGTGCGGATCTGGTGTTTGGTTCTAACTCCGTCCTGCGTGC        2220
     TCACTTTAAATGCCGGTCGGCACGCCTAGACCACAAACCAAGATTGAGGCAGGACGCACG


       V  A  E  V  Y  A  S  S  D  A  H  E  K  F  V  K  D  F  V  A        (712)
     GGTGGCGGAAGTTTACGCCAGTAGCGATGCCCACGAGAAGTTTGTTAAAGACTTCGTGGC        2280
     CCACCGCCTTCAAATGCGGTCATCGCTACGGGTGCTCTTCAAACAATTTCTGAAGCACCG
                                - - - - - - - -TAG- - - - - - - - →K705*


       A  W  V  K  V  M  N  L  D  R  F  D  L  L  *                       (726)
     GGCATGGGTGAAAGTGATGAACCTCGACCGTTTCGACCTGCTGTAATCTGACCCCGTTCA        2340
     CCGTACCCACTTTCACTACTTGGAGCTGGCAAAGCTGGACGACATTAGACTGGGGCAAGT


     GCGGCTGCTTGCTGGCAGTCGCTGAACGTTCTTTACCAGCGTATAGTGGGCGAACGAAAA        2400
     CGCCGACGAACGACCGTCAGCGACTTGCAAGAAATGGTCGCATATCACCCGCTTGCTTTT
     transcription terminator


                 ORF→
     CTACACACTGGATCTCTCATGTCTGCCGCAGGAAAGAGCAACCCACTGGCAATCAGTGGC        2460
     GATGTGTGACCTAGAGAGTACAGACGGCGTCCTTTCTCGTTGGGTGACCGTTAGTCACCG
```

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CTGGTTGTGCTCACACTTATCTGGAGTTATAGCTGGATTTCATGAAGCAAGTCACCAGTT          2520
GACCAACACGAGTGTGAATAGACCTCAATATCGACCTAAAGTACTTCGTTCAGTGGTCAA
  ←G14------TTC------------                           ←G12

ACATCGGTGCCTTCGACTTTACCGCCTTACGCTGCATTTTCGGCGCTCTCGTTTTATTCA          2580
TGTAGCCACGGAAGCTGAAATGGCGGAATGCGACGTAAAAGCCGCGAGAGCAAAATAAGT

TCGTCCTTTTATTACGTGGTCGCGGAATGCGCCCGACACCGTTTAAATACACCTTAGCCA          2640
AGCAGGAAAATAATGCACCAGCGCCTTACGCGGGCTGTGGCAAATTTATGTGGAATCGGT

TTGCCCTGTTACAAACCTGCGGGATGGTTGGTCTGGCGCAGTGGGCGTTGGTCAGCGGAG          2700
AACGGGACAATGTTTGGACGCCCTACCAACCAGACCGCGTCACCCGCAACCAGTCGCCTC

GTGCGGGGAAGGTGGCGATCCTGAGCTATACCATGCCGTTCTGGGTGGTGATTTTCGCCG          2760
CACGCCCCTTCCACCGCTAGGACTCGATATGGTACGGCAAGACCCACCACTAAAAGCGGC

CGTTGTTTCTCGGTGAACGCCTGCGACCTGGGCAATATTTCGCGATTCTGATTGCCGCTT          2820
GCAACAAAGAGCCAGTTGCGGACGCTGGACCCGTTATAAAGCGCTAAGACTAACGGCGAA

TCGGCTTATTTTTGGTGTTGCAGCCGTGGCAACTCGATTTCTCTTCGATGAAAAGTGCCA          2880
AGCCGAATAAAAACCACAACGTCGGCACCGTTGAGCTAAAGAGAAGCTACTTTTCACGGT

TGCTGGCAATCCTCTCCGGCGTCAGTTGGGGGGCGAGCGCGATTGTTGCTAAACGTCTGT          2940
ACGACCGTTAGGAGAGGCCGCAGTCAACCCCCCGCTCGCGCTAACAACGATTTGCAGACA

ATGCCCGTCATCCGCGCGTGGATTTATTGTCGTTAACATCCTGGCAGATGCTGTATGCGG          3000
TACGGGCAGTAGGCGCGCACCTAAATAACAGCAATTGTAGGACCGTCTACGACATACGCC

CGCTGGTGATGAGTGTGGTCGCTTTACTGGTGCCGCAACGTGAAATTGACTGGCAGCCCA          3060
GCGACCACTACTCACACCAGCGAAATGACCACGGCGTTGCACTTTAACTGACCGTCGGGT

CCGTGTTCTGGGCGCTGGCCTACAGTGCGATTCTGGCGACGGCACTGGCGTGGAGCTTAT          3120
GGCACAAGACCCGCGACCGGATGTCACGCTAAGACCGCTGCCGTGACCGCACCTCGAATA

GGTTGTTTGTATTGAAAAACTTGCCTGCCAGTATTGCCAGCTTAAGCACACTGGCCGTTC          3180
CCAACAAACATAACTTTTTGAACGGACGGTCATAACGGTCGAATTCGTGTGACCGGCAAG

CCGTTTCGCCGCTACTCTTTTCCTGGTGGCTGCTCGGCGAGAATCCGGGGGGCCGTTGAAG          3240
GGCAAAGCGGCGATGAGAAAAGGACCACCGACGAGCCGCTCTTAGGCCCCCGGCAACTTC

GTAGCGGTATTGTGCTGATTGTGCTGGCACTGGCGCTGGTGAGCCGTAAGAAAAAAGAAG          3300
CATCGCCATAACACGACTAACACGACCGTGACCGCGACCACTCGGCATTCTTTTTTCTTC
                   ←ORF
CCGTCAGTGTAAAAAGGATCTGAATTTTTTCTTCATGTGGGGCGATCTCTTATTTAACAA          3360
GGCAGTCACATTTTTCCTAGACTTAAAAAAGAAGTACACCCCGCTAGAGAATAAATTGAA

AATAACGATAATGCCCCACCATCCGCCAGTTAAACAGCACATCTTCTTCCTGCGCGCCTG          3420
TTATTGCTATTACGGGGTGGTAGGCGGTCAATTTGTCGTGTAGAAGAAGGACGCGCGGAC

CGCCAATGTTATGTATCACCAGCGGCGTACCGTCGCGGGCGAAGCCATCTGAAACCACCC          3480
GCGGTTACAATACATAGTGGTCGCCGCATGGCAGCGCCCGCTTCGGTAGACTTTGGTGGG
```

```
CAATATGTGCCAGCCCGTTATCCAGTCGCCAGGAGACAATATCGCCCGCTTGATAGTCAC          3540
GTTATACACGGTCGGGCAATAGGTCAGCGGTCCTCTGTTATAGCGGGCGAACTATCAGTG
```

```
TGGGGTTCTTGCTGGTGGGGCGTGTTTTATCATGGCGGCTAAACCAGGTTTCCAGATTAG          3600
ACCCCAAGAACGACCACCCCGCACAAAATAGTACCGCCGATTTGGTCCAAAGGTCTAATC
```

```
GCACCCGACGGTGATCGATGTTGCTGTCCGGGCGCTTTAACTTCCATTTTTGCGGGTACT          3660
CGTGGGCTGCCACTAGCTACAACGACAGGCCCGCGAAATTGAAGGTAAAAACGCCCATGA
```

```
CAGCAAAATTCTTCGCCATATCTTCGTGAACCAGTTTCTGCAAATCGACCTTCTGGCTGC          3720
GTCGTTTTAAGAAGCGGTATAGAAGCACTTGGTCAAAGACGTTTAGCTGGAAGACCGACG
```

```
GCAATGCGCGGATCACCACATCGGAACATACACCGCGTTCTTGCGGAACATCACCGCCAG          3780
CGTTACGCGCCTAGTGGTGTAGCCTTGTATGTGGCGCAAGAACGCCTTGTAGTGGCGGTC
```

```
GATAAGTAAGCTGCACATACGCCGGATCGTAAAATAGCGTGCTGCCAATTTGCTGTCTGG          3840
CTATTCATTCGACGTGTATGCGGCCTAGCATTTTATCGCACGACGGTTAAACGACAGACC
```

```
CACCGTCTGCGATGGCAAGGTTGGTATTGGCCTGGATTTGTACCACGGTTGGTGGAACGG          3900
GTGGCAGACGCTACCGTTCCAACCATAACCGGACCTAAACATGGTGCCAACCACCTTGCC
```

```
CGGGAGATTTTAAGGAGTGGCTGGTAAATGCCGTTAGCAGGCTGAGCAGCGCCAGTGAAGCTT     3963
GCCCTCTAAAATTCCTCACCGACCATTTACGGCAATCGTCCGACTCGTCGCGGTCACTTCGAA
                                                        HindIII
```

Table 2.2. Sequences of oligonucleotides used for site-directed mutagenesis of *E. coli katG*.

Primer name	Base changed	Oligonucleotide sequence
W88F	GG → TC	CTCAACCGTTCTGGCCAGCC
W88L	TG → CT	CTCAACCGCTGTGGCCAGCC
W89F	GG → TC	AACCGTGGTTCCCAGCCGAC
W89L	TG → CT	AACCGTGGCTGCCAGCCGAC
W197F	GG → TC	ATGTTAACTTCGGTGATGAA
W197L	TG → CT	ATGTTAACCTGGGTGATGAA
K705Stop	A → T	CCCACGAGTAGTTTGTTAA

70% (v/v) ice-cold ethanol, and then dried under vacuum. The DNA pellet was either stored in this condition at -20°C or was suspended in HPLC grade water or TE buffer (10mM Tris, pH 8, 1mM Na-EDTA) prior to storage at -20°C until further use.

Single-stranded template DNA for site-directed mutagenesis or DNA sequencing was isolated and purified according to the procedure of Vieira and Messing (1987). Plasmid-containing cells in a 5 ml LB culture in early exponential phase were infected with 50 µl of helper phage R408 (10^{11}-10^{12} PFU per ml) (in the presence of 50 µl 1M $MgSO_4$) and grown overnight. 1-2 ml of the overnight grown culture was centrifuged in order to remove the cells and debris. A solution of 300 µl of 1.5M NaCl, 20% PEG 6000 was added per ml of medium supernatant and mixed by inversion. This mixture was then incubated at room temperature for 15 minutes and then centrifuged to pellet the phage particles. The pellet was then resuspended in TE buffer on ice and extracted first with an equal volume of buffer-saturated phenol, followed by extraction with an equal volume of water-saturated chloroform. Single-stranded DNA was precipated by addition of an equal volume of 7.5 M ammonium acetate, pH 7.5 and 4 volumes of ice-cold 95% ethanol followed by incubation at -20°C for 30 minutes. Single stranded DNA was recovered by centrifugation and the pellet was washed three times with 70% (v/v) ethanol. The dried pellet was stored at -20°C until further use.

2.4.4. Restriction endonuclease digestion of DNA

Restriction nucleases and buffers used in this study were products of Invitrogen Canada Inc. Restriction digestions were performed at 37°C for 2-5 hours in total volumes of 10 µl (or 20µl), containing 1 µg RNase, 1µl of 10X appropriate buffer provided by the

supplier, ~1-5 µg DNA, and 0.5-1 µl (50-2,500 Units) of endonuclease. 5′ Phosphate groups of vector DNA were removed during the latter 0.5-1 hour digestion by addition of 12.5-25 units calf intestinal alkaline phosphatase [CIP] (Amersham Pharmacia Biotech Inc.). Alternatively, the restriction digested DNA was ethanol precipitated (Sambrook *et al.*, 1989), the dried DNA pellet was resuspended and then treated with CIP 0.5-1 hour at 37°C, after which the reaction was stopped by heating to about 75°C for 10 minutes or extracting with phenol, and then precipitating with ethanol (Ausbel *et al.*, 1989).

2.4.5. Agarose gel electrophoresis

Electrophoresis of the restriction endonuclease digested DNA was performed according to Sambrook *et al.*, (1989). Agarose gels containing 1-2% (w/v) agarose and 0.1 µg/ ml ethidium bromide were prepared in TAE buffer (40 mM Tris-Acetate and 1mM EDTA, pH 8.0) and cast in Bio-Rad Mini Sub Cell Plexiglass horizontal electrophoresis trays. Samples of 10 µl volumes were mixed with 1-3 µl Stop buffer (40% [v/v] glycerol, 10 mM EDTA pH 8.0, 0.25% [w/v] bromophenol blue). 1 kb plus DNA ladder (Invitrogen Canada Inc.) was used as a molecular weight size standard. Electrophoresis was carried out at 40-60 mA constant current in TAE buffer, usually until the bromophenol blue marker dye front had migrated approximately half the length of the gel. Following electrophoresis, the DNA bands were visualized with ultraviolet light and recorded in a digitized form using a Gel Doc 1000 image capture system (Bio-Rad).

2.4.6. Ligation

DNA fragments to be ligated were excised from agarose gels and purified using the Ultraclean ^{TM}15 DNA purification kit (Bio/Can Scientific Inc.) according to the

instructions supplied by the manufacturer. In cases where the fragments of DNA were less than 200 bp in length, the incubation step with Ultrabind was carried out 30 minutes longer than recommended, to allow for better DNA adherence and recovery. Ligation of insert DNA into vector was carried out according to the procedure of Sambrook *et al.* (1989). Purified DNA was mixed in a ratio of 2-3 of insert to vector in 10 µl volumes (and some modifications) containing 1 unit T4 DNA ligase (Invitrogen Canada Inc.), and the manufacturer's supplied buffer at the appropriate concentration. Ligation mixtures were incubated overnight at 15°C. A mixture without the insert DNA added was used as a control.

2.4.7. Transformation

Transformation of *E. coli* cells with the various plasmids was achieved according to Chung *et al.*, (1989). 5 ml LB cultures of cells grown to exponential phase (2-4 hours) were harvested by centrifugation and made competent by resuspension in ice-cold 0.1 M $CaCl_2$ for at least 30 minutes. 2-10 µg DNA was usually added to 100 µl of this cell suspension, followed by a further 30-45 minute incubation on ice, and a 90 second heat shock at 42°C. The cells were then added to 0.9 ml LB medium and incubated at 37°C for 45-60 minutes without aeration. The mixture was then either spread, or (in the case of ligation mixture transformations) mixed with 2.5-3 ml molten (50°C) R-Top agar (0.125 g yeast extract, 1.25 g tryptone, 1 g NaCl, 1 g agar per 125 ml volume with 0.25 ml 1M $CaCl_2$ and 0.42 ml 30% glucose sterile solutions added after autoclaving) and poured onto ampicillin-containing LB plates.

2.4.8. DNA sequencing

Sequencing of DNA was performed according to method described by Sanger *et al.*, (1977). Sequencing was carried out manually with either single or double stranded DNA templates using the primers shown in Table 2.2. For preparation of double stranded DNA template, 5 μg plasmid DNA was resuspended and denatured in a 40 μl volume of 0.4 M freshly prepared NaOH. This mixture was incubated for ten minutes at 37°C, and then reprecipitated by addition of 10 μl 3M sodium acetate, pH 4.8 and 140 μl ice-cold 95% ethanol. Following incubation at -20°C for 30 minutes, the DNA pellet was recovered by centrifugation, washed once with 1ml 95% ice-cold ethanol, and once with 0.1 ml 70% ice-cold ethanol, and then evaporated to dryness under vacuum in a dessicator. Annealing and sequencing reactions were carried out using a T7 Sequencing Kit (USB Corporation) according to the manufacturer's specifications and using 25 μCi or 1 mCi [α-^{35} S] dATP (Amersham Biosciences). Reaction mixtures were separated and resolved on 8% (w/v) polyacrylamide vertical slab gels containing 7 M urea, 0.13 M boric acid, and 10 mM EDTA. Electrophoresis was carried out using 18-24 mA constant current in TBE buffer (90 mM Tris, 89 mM borate, 2.2 mM EDTA) for 1.5-4 hours as required. Gels were mounted on 3 mm paper (Whatman), covered with clear plastic film, and dried at 80°C for about 1 hour on a slab gel drier under vacuum (Savant). Dried gels were exposed to X-ray film (Kodak X-OMAT AR) in order to visualize and record the DNA bands.

2.5. Purification of *E. coli* HPI and its variants

For small scale crude extracts used in determination of relative levels of protein expression, as well as catalase activity, plasmid containing cells were grown in either 5 ml LB medium in test tubes, or in 125 ml flasks of the same medium at 37°C or 28°C for

16-20 hours. The cells were pelleted and resuspended in 1-2 ml 50 mM potassium phosphate buffer, pH 7.0, sonically disrupted, and centrifuged to remove unbroken cells and debris; followed by assay for catalase activity and/or expression profile by SDS-PAGE. If only protein visualization was to be carried out (in case of mutations that had low or no catalase activity), 10-50 µl of aliquots of cell suspensions were pelleted by centrifugation, directly resuspended in SDS protein sample buffer, and analyzed via SDS-PAGE.

For large scale preparations of EcHPI and its variant proteins, UM262 cells overexpressing the desired protein from the plasmid-borne *katG* genes were grown in 500 ml volumes (in shake flasks) of LB media containing 100 µg/ml of ampicillin, for 16-20 hours, at either 37°C or 28°C with good aeration. Isolation of HPI and its proteins was done according to the procedure of Loewen and Switala (1986). All isolation and purification steps were carried out at 4°C. Cells were harvested from the growth media by centrifugation. The cell pellet was resuspended in about 150-250 ml of 50 mM potassium phosphate buffer, pH 7.0 (KPB). The protease inhibitor PMSF (100 mM stock solution) was added to a final concentration of 100 µM, and EDTA (0.5 M stock solution) was added to a final concentration of 5 mM. Cells were disrupted by a single pass through a French pressure cell at 20,000 psi. Unbroken cells and debris were removed by centrifugation, yielding the crude extract, to which was added streptomycin sulfate to a final concentration of 2.5 % (w/v). The resulting precipitates were removed by centrifugation and discarded. Solid $(NH_4)_2SO_4$ was then added in appropriate amounts with gentle stirring, to achieve the required concentration followed by centrifugation, to precipitate the desired protein. EcHPI and its variants were found to precipitate in

$(NH_4)_2SO_4$ at 20-25% saturation. Pellets from the $(NH_4)_2SO_4$ precipitations were resuspended in 20-40 ml of KPB. The presence of the desired protein in the pellets was confirmed by assays for catalase activity and visualization on SDS gels. Resuspensions were centrifuged to remove any remaining precipitates, and dialyzed overnight using a 12,000-14,000 molecular weight cutoff membrane, against 100 volumes of KPB.

The dialyzed resuspensions were centrifuged and loaded onto a 2.5 cm x 23 cm column of DEAE-cellulose A-500 (Cellufine, Amicon) equilibrated with KPB. The column was washed with KPB until the A_{280} of the column fractions was below 0.025. The protein of interest was then eluted with a 0-0.5 M NaCl linear gradient in KPB, usually in a total volume of 1 liter. Eighty-drop fractions of the eluate proteins from the column were collected throughout. Purity of the recovered column fractions was determined based on A_{280} and catalase activity elution profiles. Selected fractions were pooled and concentrated under nitrogen in a stirred pressure cell (Model 8050, Amicon) using a YM-30 (Amicon) membrane, to volumes of between 8-12 ml. The concentrated protein sample was then dialyzed against approximately 100-500 volumes of KPB overnight. The concentrated, dialyzed, clarified samples were checked for purity based on catalase activity, spectrophotometrically from $A_{407/280}$ (heme/protein) ratios and visualization using SDS-PAGE. No further purification was usually necessary for EcHPI and its variants. The purified protein samples were aliquoted into eppendorf tubes in 0.5 - 1 ml volumes and either dried and/or stored frozen at -60°C until use.

2.6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Denaturing SDS-PAGE was carried out according to Weber *et al.*, (1972). Discontinuous 4% stacking and 8% separating polyacrylamide gels were cast as vertical slabs of

dimensions 10 x 10 cm and 0.5 mm thickness (mini gels). Samples loaded onto the mini gels usually contained between 5-25 μg of protein. Protein samples were mixed with equal volumes of reducing denaturing sample buffer (3.4 mg/ml NaH_2PO_4, 10.2 mg/ml Na_2HPO_4, 10 mg/ml SDS, 0.13 mM 2-mercaptoethanol, 0.36 g/ml urea and 0.15% bromophenol blue) and boiled for three minutes before loading onto the gels. Samples were run with 125-175 V constant voltage in a vertical BIO-RAD Mini-Protean II electrophoresis system, using running buffer containing 14 g glycine, 3 g Tris base, and 1 g SDS per liter. Gels were stained for protein by incubation in staining solution containing 0.5 g/l Coomassie Brilliant Blue R-250, 30% ethanol and 10% acetic acid for about one hour. This was followed by destaining of gels in a solution containing 15% methanol and 7% acetic acid, until the background was judged to be clear. Finally the gels were soaked in a destaining solution containing 7% acetic acid and 1% glycerol for about one hour. Gels were then mounted on 3 mm (Whatman) paper, covered with a clear plastic film, and dried at 60-80°C for 1-2 hours on a slab gel drier under vacuum (Savant).

2.7. Enzymatic assays and protein quantitation

Catalase was determined by the method of Rørth and Jensen (1967) in a Gilson oxygraph equipped with a Clark electrode. One unit of catalase activity is defined as the amount of enzyme that decomposes 1 μmol of substrate in 1 minute in 60 mM H_2O_2 at 37°C, pH 7. Appropriately diluted samples of enzyme or cell culture aliquots were incubated in 1.8 ml of 50 mM potassium phosphate buffer, pH 7.0 for 0.5-1.0 minutes at 37°C followed by addition of H_2O_2 to a final concentration of 60 mM. Catalase activity in units/ml was determined from the slope of the plot representing oxygen evolution. Specific catalase activity was expressed as units $\bullet$ ml^{-1} $\bullet$ mg^{-1} purified protein. Specific

activity in whole cells (units/mg dry cell weight) was determined by converting the cell turbidity values at 600 nm to Klett values. Specific activity was always determined as the average of a minimum of three or more determinations. pH profile studies for catalase activity of wild type EcHPI and variants were performed using 50 mM phosphate buffer over a pH range of 2.5 to 10 (Loewen *et al.*, 1990).

Peroxidase activity was determined spectrophotometrically using two different substrates namely o-dianisidine and ABTS {3-ethylbenzothiazolinesulfonicacid} (2,2-*azinobis*). Since the pH optima for the peroxidation of both substrates by EcHPI has never been ascertained before, pH profile studies for o-dianisidine and ABTS peroxidation were set up. Three different buffers together covering a pH range of 2.5 to 7 were used for the study: MES (2-[N-Morpholino]-ethane sulfonic acid) buffer (pKa-6.15) was used between pH 5.5 and 7, sodium acetate buffer (pKa-4.75) between pH 3.5 and 5.0 and potassium phosphate buffer (pKa-2.12) was used for determinations between pH 2.5 and 3.0. Ideal pH and buffering conditions for peroxidatic activity of both EcHPI and its variants were ascertained and incorporated into the assay conditions. o-Dianisidine assay was done according to the method described in the Worthington Enzyme Catalogue with some modifications (Worthington Chemical Co., 1969). Assays were carried out at room temperature in 1 ml final assay volumes containing 1 mM H_2O_2, 0.34 mM o-dianisidine in 50 mM acetate buffer, pH 5. Aliquots (5-20 µl) of the appropriately diluted enzymes were added to initiate the reaction. Peroxidatic activity was determined by the ΔA_{460}/min average over periods of 2-3 minutes and expressed as units $\bullet$ ml^{-1} $\bullet$ mg^{-1} purified protein calculated as: (ΔA_{460}/min average)/ (11.3 x mg enzyme/ml reaction mixture). ABTS peroxidation by EcHPI and its variants was assayed spectro-

photometrically using the method described by Smith *et al.*, (1990) with some

modifications. Assays were carried out in 1 ml final assay volumes containing 2.5 mM

H_2O_2, 0.5 mM ABTS in 50 mM sodium acetate buffer pH 4.3 or pH 4.5. Aliquots (5-20µl)

of the appropriately diluted enzymes were added to initiate the reaction. Peroxidase

activity was determined by the ΔA_{405}/min. average over periods of 0.25-2 minutes.

Activities were expressed as $\mu moles \bullet min^{-1} \bullet ml^{-1}$ by using a molar absorption coefficient

of the ABTS product of 36.8 $mM^{-1} cm^{-1}$. Protein concentration (mg/ml) was estimated

spectrophotometrically based on the A_{280}/A_{260} ratios (Layne, 1957). Specific activities

were always determined as the average of a minimum of three or more individual

determinations.

2.8. Absorption spectrometry

Absorption spectra, time courses, and peroxidatic assays were performed using a

Pharmacia Ultrospec 4000 Spectrometer or a Milton Roy MR3000 Spectrometer.

All experiments were performed at ambient temperature in 1 ml quartz, semimicro

cuvettes. Proteins were normally diluted in 50 mM potassium phosphate buffer, pH 7,

unless otherwise stated, and the same buffer was used as a reference. For the ABTS and

o-dianisidine peroxidation assays, proteins were diluted in 50 mM sodium acetate buffer,

and the same buffer was used as a reference. For preparation of spectral and time course

plots, data collected were transferred to Sigma Plot software.

2.9. Effects of inhibitors

The effects of classical heme inhibitors, KCN and NaN_3 on catalase activities of wild

type EcHPI and its variants were studied. For assays of catalase activity in presence of

different concentrations of KCN and NaN$_3$, the enzyme was incubated for 1 minute in the

reaction mixture containing one of the above compounds prior to initiation of the reaction

by addition of H$_2$O$_2$.

3. RESULTS

3.1. Construction of EcHPI variants

The high degree of aminoacid homology of EcHPI with other catalase-peroxidases, indicates a common evolutionary origin for members of the catalase-peroxidase group (Zamocky *et al.*, 2000). Alignment of the EcHPI protein sequence to that of CCP, allowed identification of residues in EcHPI that corresponded to catalytic residues identified in CCP. Not surprisingly, many of these residues are conserved among peroxidases and catalase-peroxidases (Loewen, 1997). While it was confirmed by previous studies on EcHPI that Arg102, Trp105 and His106 were indeed active site residues and that they were involved in the catalatic mechanism, there was also a very interesting finding regarding the Trp105 residue (Hillar *et al.*, 2000). In all catalase-peroxidases and CCP the distal Trp (Trp105 in EcHPI) is present in corresponding positions, but in many other peroxidases there is a Phe residue in that position. Replacement of Trp105 with either Phe or Leu reversed the relative levels of catalase and peroxidase activities of EcHPI, producing a predominant peroxidase from a catalase, thus giving a clue as to where to draw the line between behaving as a peroxidase or a catalase (Hillar *et al.*, 2000).

One of the major challenges in investigations on catalase-peroxidases like EcHPI is to explain their bifunctional capability in molecular terms. Due to the absence of high-resolution crystal data for EcHPI, structure-function investigations have to rely strongly on information from amino acid sequence alignment studies, in order to identify target residues for site-directed mutagenic analysis. Amino acid sequence alignment studies between EcHPI and other catalase-peroxidases in our laboratory have shown that tryptophan residues in and around the putative active site of HPI are highly conserved and

show a specific pattern of distribution among catalase-peroxidases. Thus the goal of this study was primarily to investigate the possible involvement of some of these conserved Trp residues in the catalysis of HPI. Trp88, Trp89 and Trp197 were chosen as the target residues for this analysis. Trp88 and Trp89 were chosen primarily because of their sequential proximity to the putative active site of EcHPI. All target residues were replaced with either Phe or Leu by site-directed mutagenesis of the phagemid-borne *katG* gene.

The role of the carboxy-terminal domain of EcHPI in catalysis or structure is unclear. Recent studies on MtKatG have detected intra-molecular domain interactions between the amino and carboxy-terminal domains, which may have a role in the mechanism of the enzyme (Wilming and Johnsson, 2001). In order to investigate the role of the carboxy-terminal domain, a mutagenesis-based approach involving progressive truncation of the carboxy-terminal domain region of the phagemid-borne *katG* gene was initiated. Unfortunately, the studies were unsuccessful primarily because the very first variant created using that method (Lys 705) produced insufficient protein to allow isolation and characterization.

3.2. Purification of EcHPI and its variants

Plasmid-borne wild type or variant EcKatG's were expressed in the catalase-deficient *E. coli* strain UM262 and grown on a large scale in batch cultures either at 28°C or 37°C for 16-20 hours. The enzyme was purified using the same basic protocol (section 2.5), which involves breaking the cells using a French press, streptomycin sulfate treatment, ammonium sulfate precipitation, and ion-exchange chromatography (DEAE-cellulose). A course of typical purification of EcHPI enzyme from UM262 expressed via plasmid pBT22/pMKatG/pAH7 is shown in Table 3.1. W88F, W88L, W89F, W89L, W197F and

W197L variants, were also purified using the protocol in Table 3.1. HPI and its variants precipitated between 20-25% concentrations of ammonium sulfate.

3.3. Comparison of the effect of culture growth temperature on the heme/subunit ratios and specific activities of purified wild type EcHPI and its variants

Wild type EcHPI binds more heme and correspondingly shows higher specific activity when prepared from cells grown at 28°C compared to 37°C (Table 3.2). Heme/subunit ratios increased by about 1.7 times and specific activity increased almost 2-fold when EcHPI protein was expressed in *E. coli* cultures grown at 28°C when compared to 37° C. There was also a remarkable improvement in the heme/subunit ratios (1.5 to 3-fold increases) and specific activities (1.5 to 4-fold increases) of the Trp-replacement variants when cultures were grown at 28°C (Table 3.2). All the variants whether grown at 37°C or at 28°C had reduced specific activities when compared to the wild type. The results indicate that the lower temperature (28°C) facilitates better heme binding in EcHPI and its Trp-replacement variants when compared to 37°C.

3.4. Physicochemical characterization of EcHPI variants

Purified EcHPI and its variants that were isolated from both 28°C and 37°C cultures were analyzed by SDS-PAGE, as shown in Figure 3.1a and 3.1b respectively. EcHPI and its variants show very similar electrophoretic mobilities and express a predominant band with an apparent molecular mass of about 80 kDa. The presence of larger bands at ≈ 160 kDa probably represent the dimeric forms of the enzymes, which may be the result of some covalent cross-links which are not labile to reduction by either β-mercaptoethanol or dithiothreitol added to the sample buffer. All further studies and characterizations on

Table 3.1. Purification of wild type HPI from the *Escherichia coli* strain UM262 harboring plasmid-borne *katG*, grown at 28°C.

Purification Step	Total protein (mg)	Total catalase activity (units x 10^3)	Specific catalase activity (units/mg)	Recovery (%)	Purification (fold)	$A_{407}/_{280}$ (Heme/ Subunit)
Crude extract	1450	750	517	100	1	0.110
(NH$_4$)$_2$SO$_4$ precipitation	135	338	2507	45	4.8	0.570
Anion exchange (DEAE-A-500)	59	189	3200	25	6.2	0.624

W88F, W88L, W89F, W89L, W197F, W197L variants were also purified using the above protocol.

Table 3.2. Comparison of the effect of culture growth temperature on the heme/subunit ratios and specific activities of purified wild-type EcHPI and its variants.

Variant	$A_{407}/_{280}$		Specific activity (units/mg)	
	28°C	37°C	28°C	37°C
Wt-HPI	0.624	0.359	3200	1500
W88F	0.522	0.321	2100	1300
W88L	0.469	0.164	2400	630
W89F	0.516	0.254	1900	860
W89L	0.346	0.159	1600	500
W197F	0.638	0.279	2300	620
W197L	0.534	0.296	1100	590
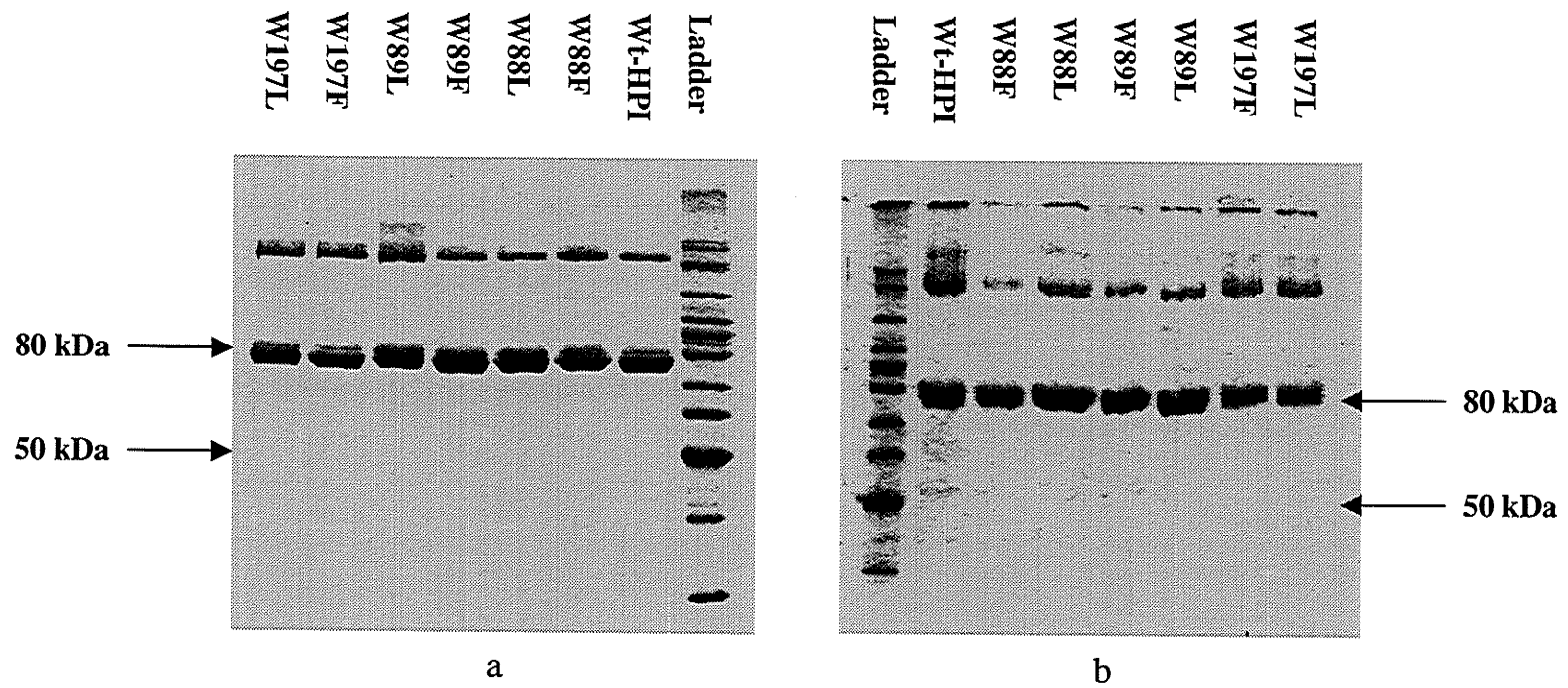
Figure 3.1: SDS-polyacrylamide gel electrophoretic analysis of purified EcHPI and its variants, isolated from the 28°C cultures (a) and the 37°C cultures (b).

EcHPI and its variants were performed using the purified proteins isolated from the 28°C cultures.

Figure 3.2 shows the optical absorption spectra of the variants compared to that of the wild type EcHPI. The Sorêt maxima of the wild type EcHPI and its Trp197 variants are at 407 nm while that of the Trp88 and 89 replacement variants differ by one nm or less from that of the wild type. No significant shifts are observed in the positions of the charge transfer bands of the variants in comparison to that of the wild type. Shifts in the charge transfer bands are generally indicators of changes in the hydrogen bond network in the vicinity of the heme. Table 3.2 lists the $A_{407/280}$ values (heme/protein ratios) of the wild type and the variants enzymes isolated from the 28°C cultures. All variants except W197F show lower heme/protein ratios when compared to that of the wild type. The percentage reductions in the heme content of such variants ranged between 14-45% in comparison to the wild type.

3.5. Biochemical characterization of EcHPI and its variants

3.5.1. pH profile for catalase and peroxidase activities

The pH profiles for catalase and peroxidase activities of wild type EcHPI are shown in Figure 3.3. The pH optima for peroxidase activity were measured using two substrates, o-dianisidine and ABTS. The pH optima for catalatic and peroxidatic activities differ significantly, with catalatic pH optima between 6.5-7 and peroxidatic pH optima depending on the substrate, between pH 4.3 (ABTS) and pH 5.0 (o-dianisidine). All the Trp- replacement variants exhibit catalatic pH optima between 6.5-7 and peroxidatic
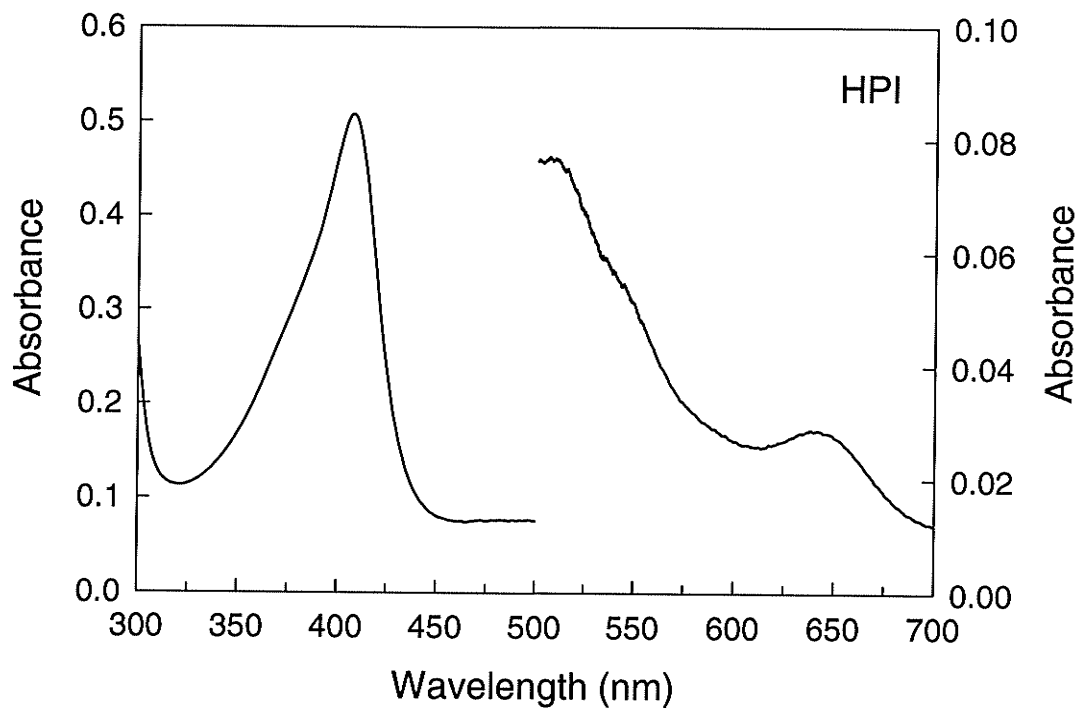
Figure 3.2: Absorption spectra of wild type EcHPI and its
Trp-replacement variants. Left axis scales apply to the
300-500 nm wavelength range. Right axis scales apply
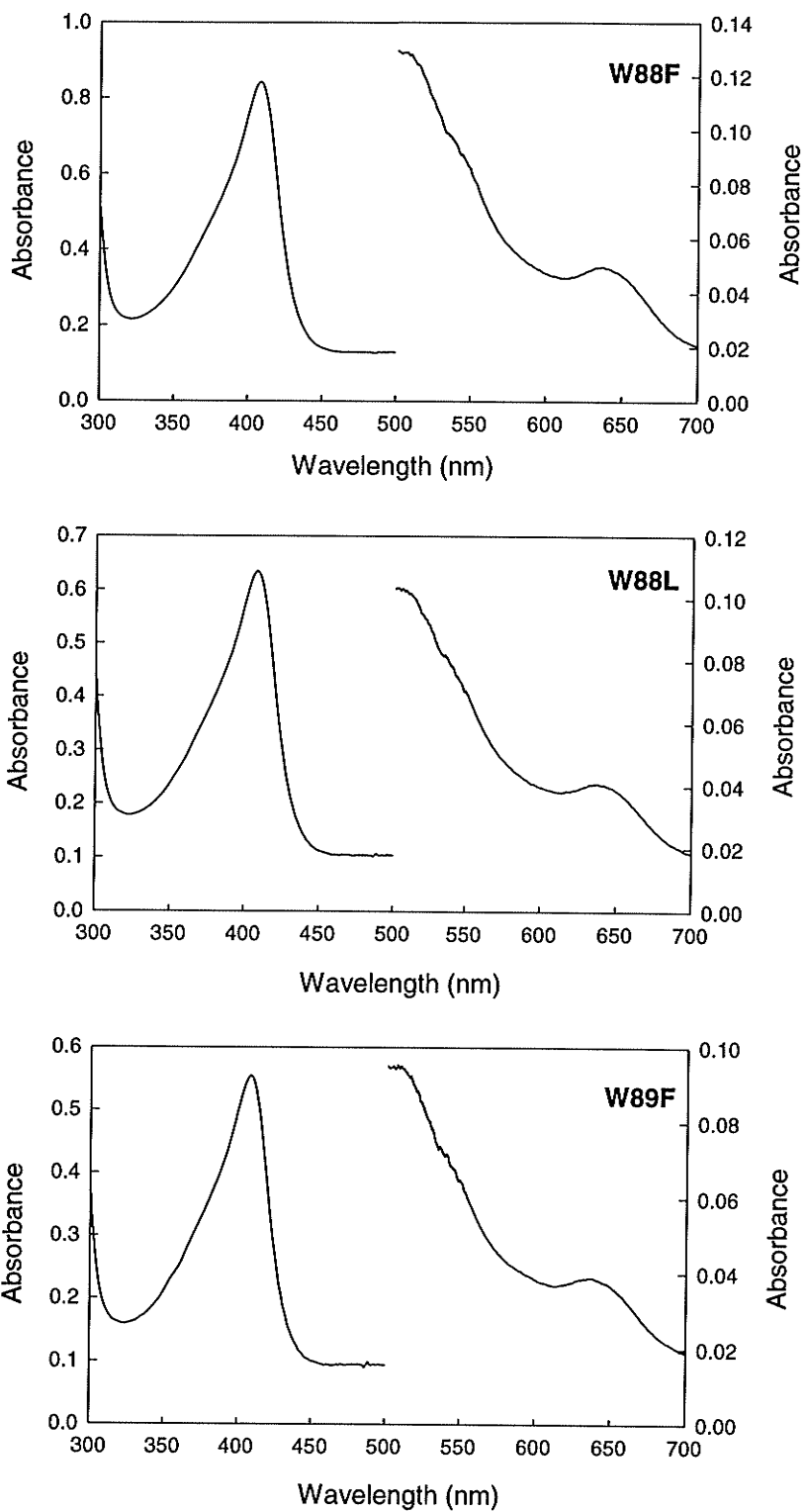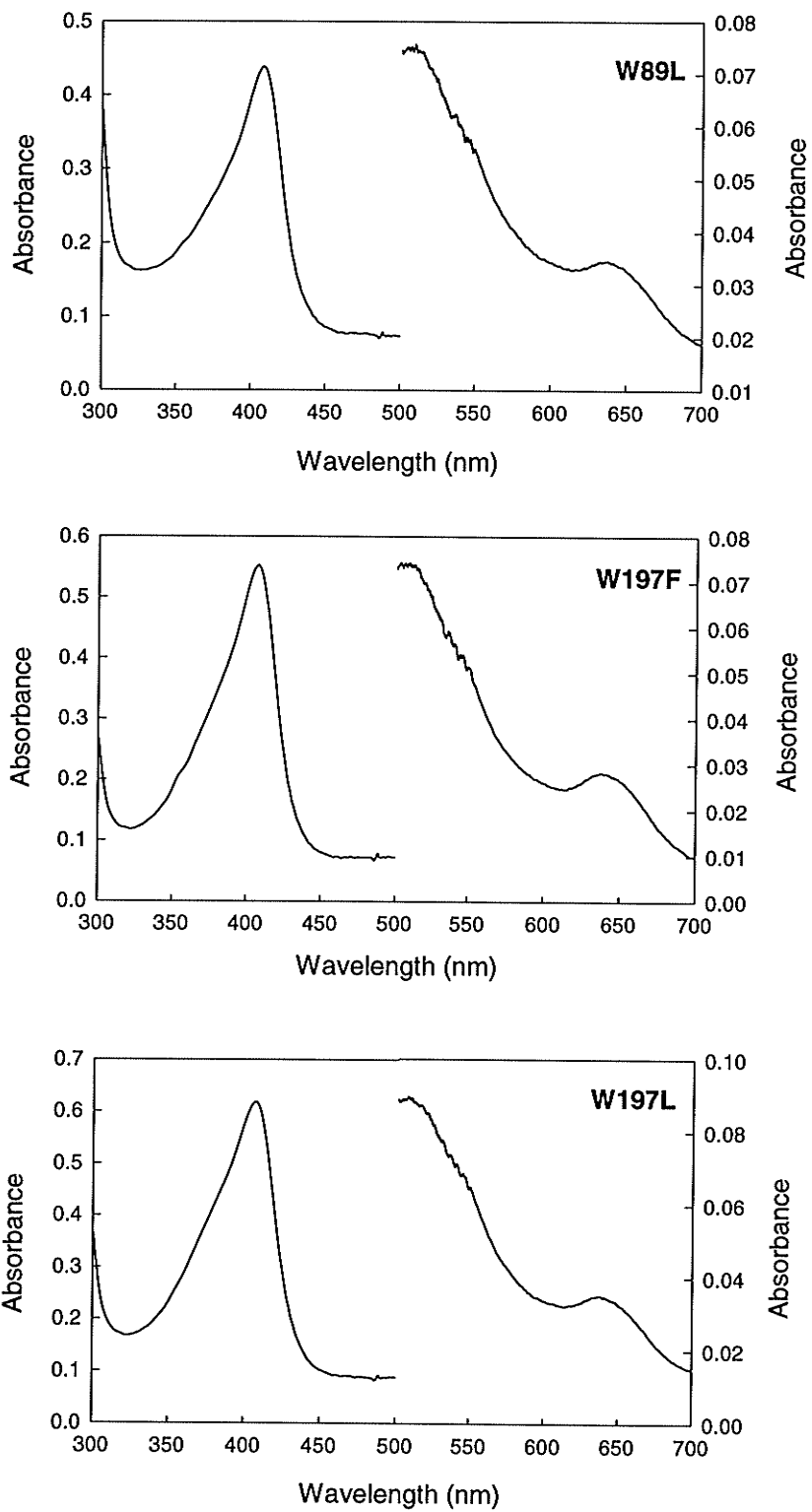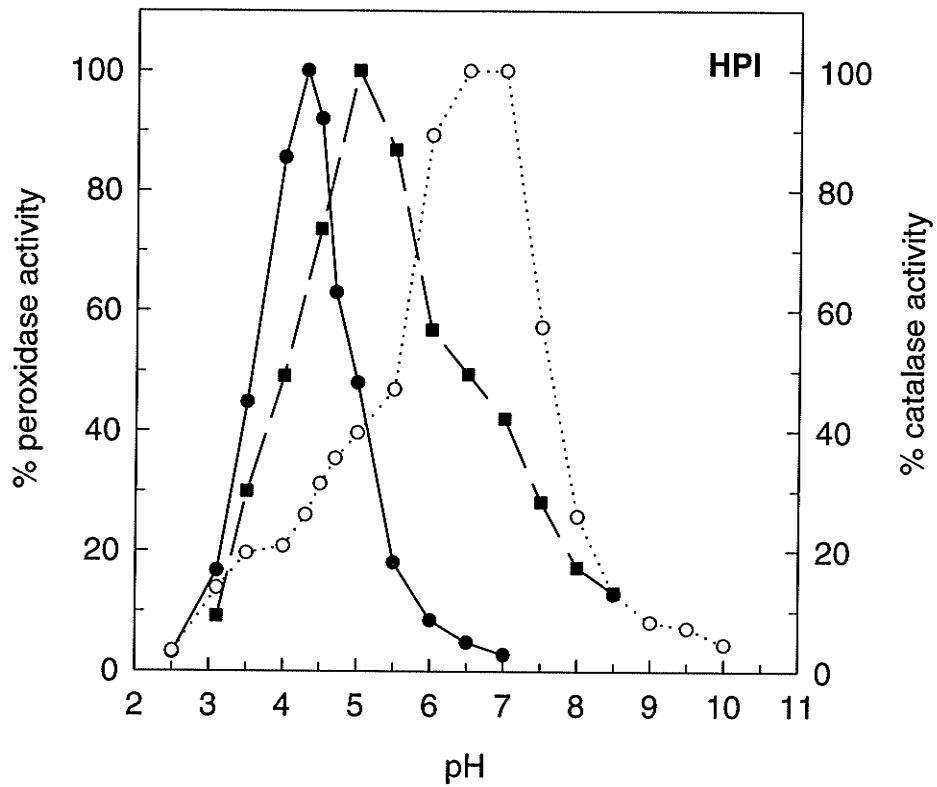to the 500-700 nm wavelength range.

Figure 3.2. (continued)

Figure 3.2. (continued)

Figure 3.3: pH profile for catalase & peroxidase activities of EcHPI & its variants.
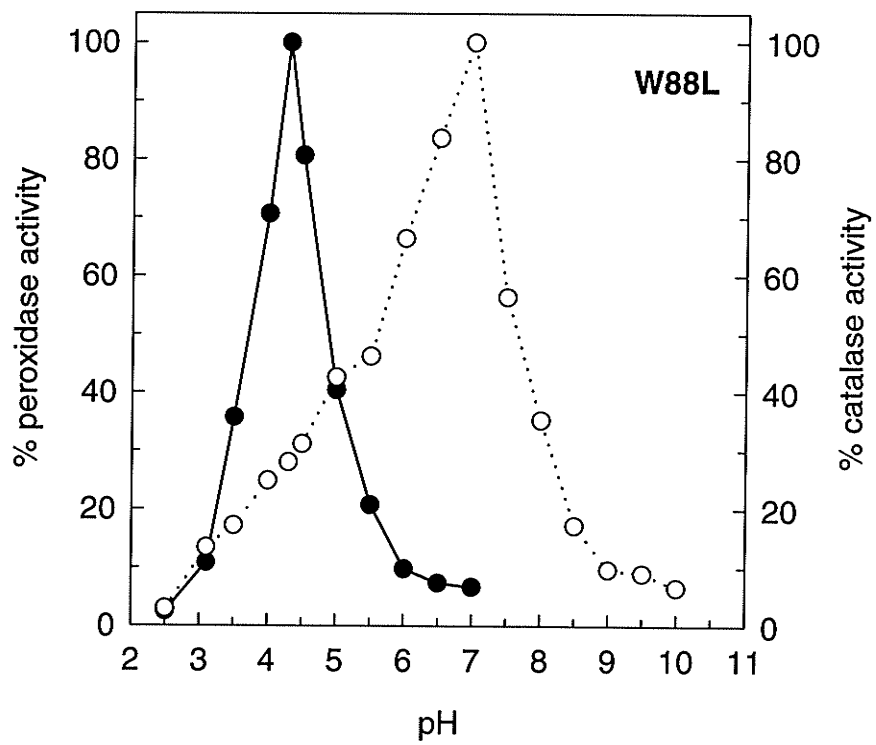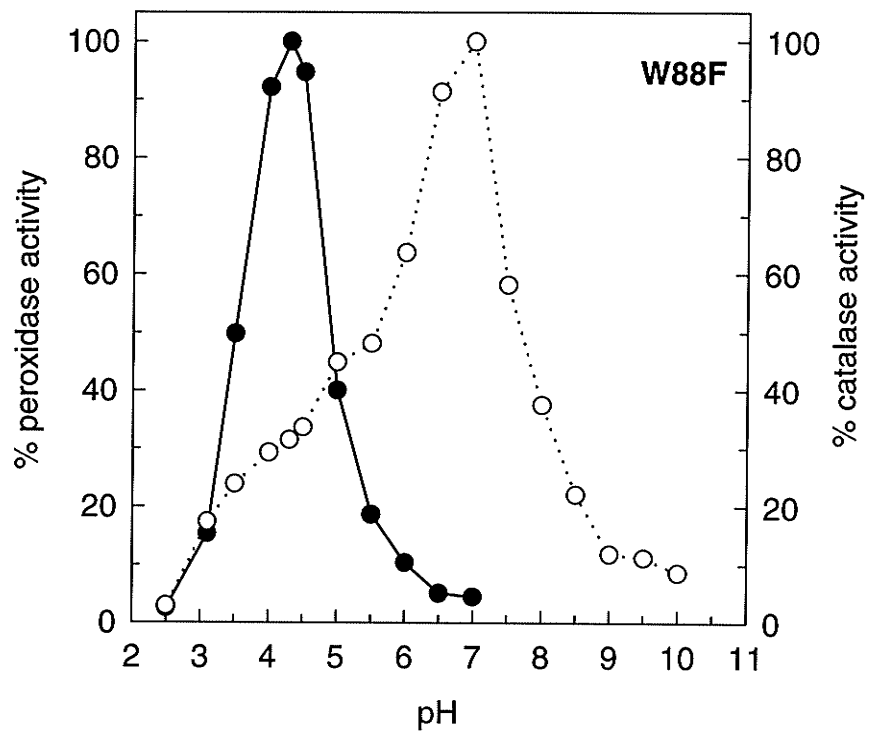···o··· catalase activity, peroxidase activity: ——●—— ABTS, ——■— · o-dianisidine.
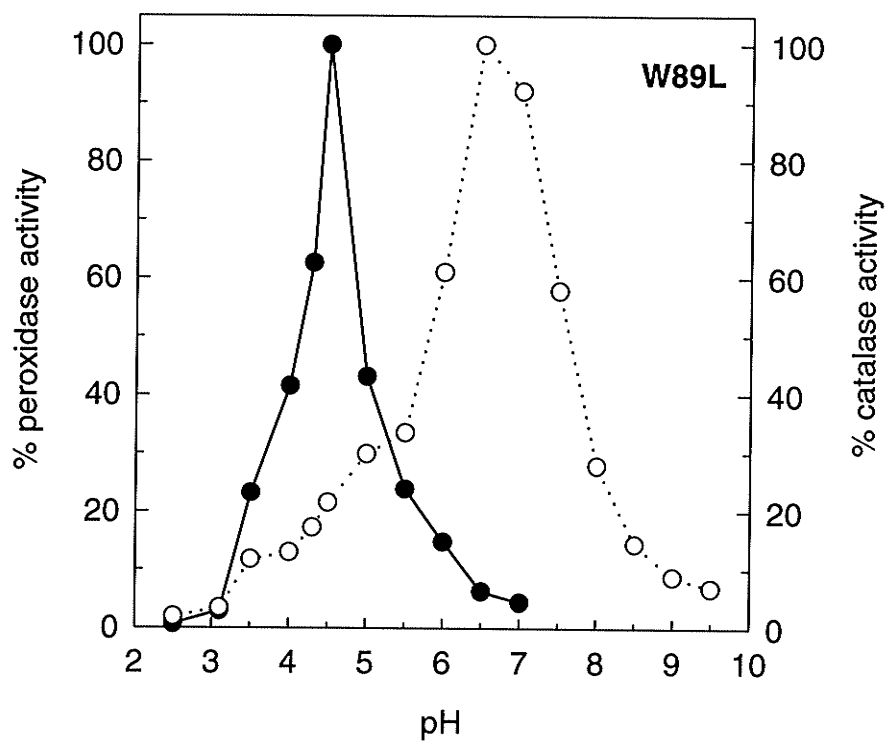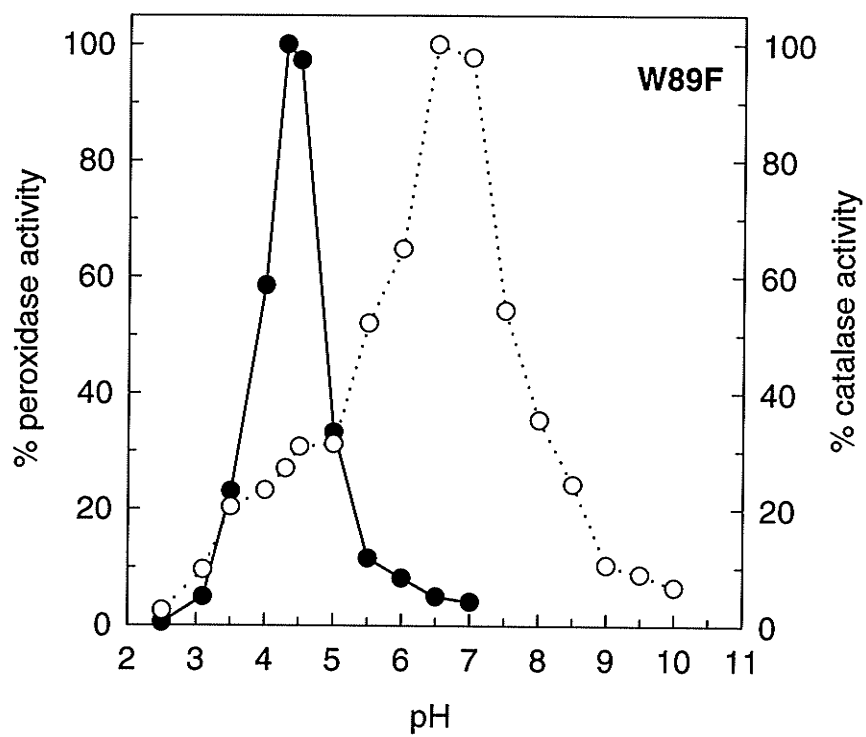
Figure 3.3. (continued)
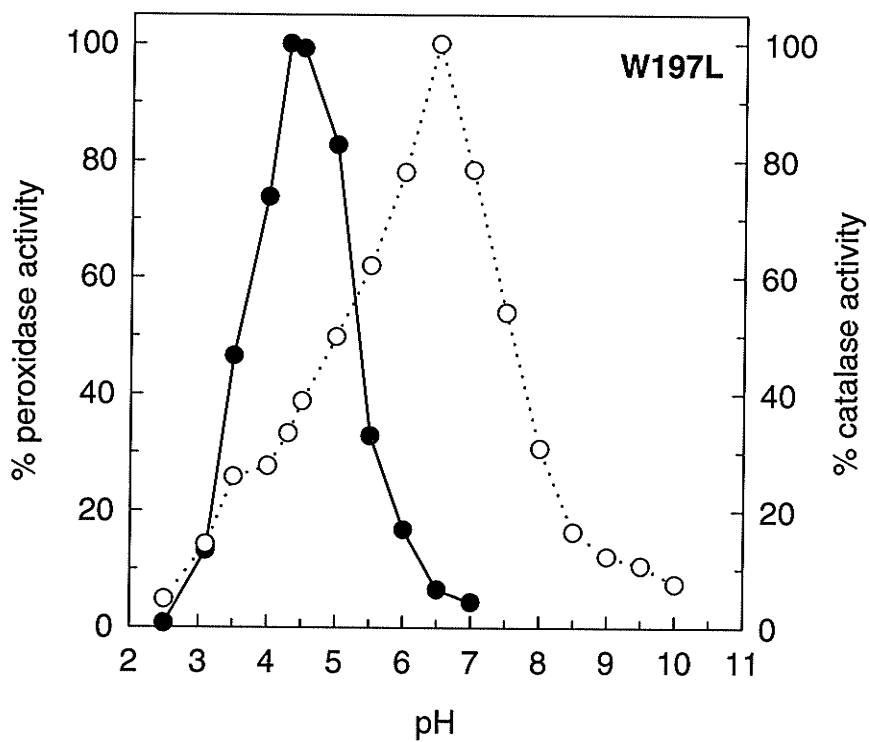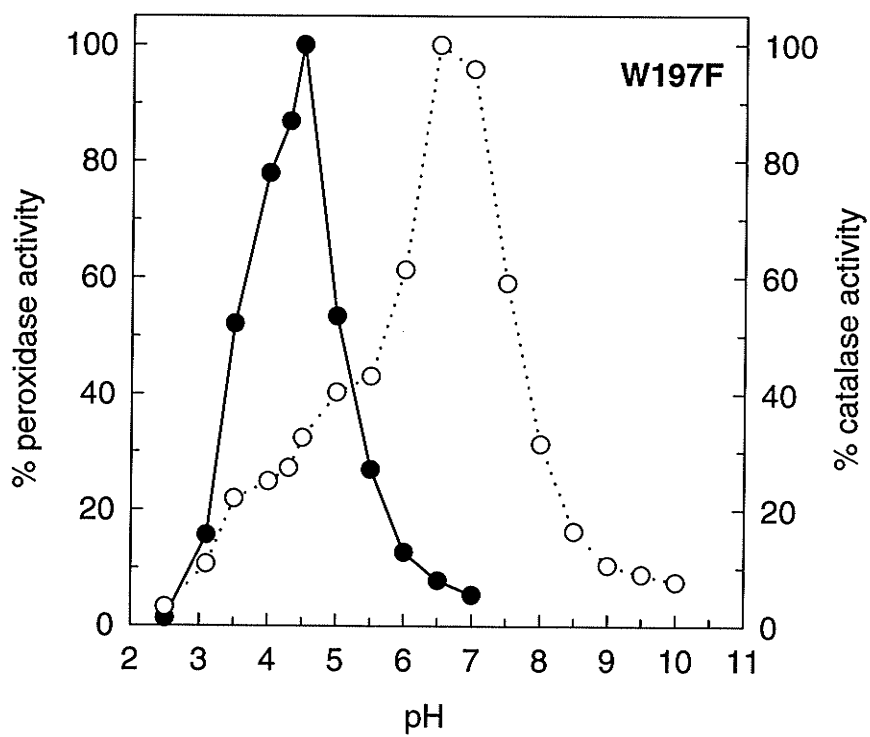
Figure 3.3. (continued)

Figure 3.3. (continued)

optima around pH 4.3-4.5, using ABTS as substrate (Figure 3.3).

3.5.2. Catalatic and peroxidatic properties

Table 3.3a summarizes the specific enzymatic activities of EcHPI and its variants

measured under optimal conditions of pH. Wild type EcHPI has a catalase specific

activity of 3200 U/mg. The catalase specific activities of the variants are reduced in

comparison to that of the wild type EcHPI, at least in part because they contain less heme

when compared to that of the wild type (Table 3.2). Variants at position Trp88 have their

catalase activities reduced by about 30% while those at Trp89 have theirs reduced by 40-

50% in comparison to that of the wild type. Among the variants at position Trp197,

W197F has about 72% and W197L only 35% of the original catalase activity in

comparison to the native enzyme.

Two different organic electron donors were evaluated as substrates for the

peroxidatic reaction namely, o-dianisidine and ABTS. EcHPI and its variants show

detectable peroxidase activity with both commonly used synthetic electron donors. EcHPI

elicited specific peroxidase activity of 22400 U/mg in the presence of ABTS and 9.1

U/mg in presence of o-dianisidine. The variants have either reduced or increased activities

in comparison to the wild type depending on the peroxidatic substrate used for analysis.

Variants at position Trp88 have only about 70-80% of ABTS peroxidatic activity but have

5% greater o-dianisidine peroxidase activity relative to the wild type. Among the

Trp89 variants, W89F shows 86% (ABTS peroxidation) and 142% (o-dianisidine

peroxidation) peroxidase activities, in relation to the wild type. W89L on the other hand

shows reduced peroxidase activities towards both substrates with about 25-60% reduction

in peroxidatic activities in comparison to the wild type EcHPI. W197F has about 5-30%

higher peroxidase specific activity, while W197L has activities quite similar to that of wild type EcHPI.

In order to make the data more meaningful the catalatic and peroxidatic specific activities of EcHPI and its variants were normalized to the heme content and represented as shown in Table3.3b. The table also shows the relative peroxidatic versus catalatic activities of the wild type and variants. Variants at position Trp197 show significant reductions in catalase activity ranging between 30-60%. However, their peroxidase activities towards ABTS were increased significantly and their activities towards o-dianisidine were unaffected in relation to the wild type. The peroxidase versus catalase activity ratios were significantly higher in the Trp197 variants. Variants at positions Trp88 and Trp89, did not show any remarkable reductions in their catalase activity, relative to the native enzyme. In addition, the peroxidase versus catalase activity ratios of Trp88 and Trp89 variants are notably higher only with o-dianisidine primarily because of relative increases in their o-dianisidine peroxidation activity.

3.5.3. Kinetic characterization

The effect of hydrogen peroxide concentration [H_2O_2] on the rate of catalase reaction of EcHPI and its variants is shown in Figure 3.4. The initial velocities (V_i) have been corrected for differing heme content and expressed as micromoles of hydrogen peroxide decomposed per minute per micromole of heme. There seems to be a good correlation between theoretical Michealis-Menten and observed catalase kinetic curves. However, this correlation seems to be stronger only at lower concentrations of hydrogen peroxide especially for the variants. Surprisingly, when the data were re-plotted as Lineweaver-Burk plots (Figure 3.4), it was found that the regression lines intercepted

Table 3.3a. Catalatic and Peroxidatic specific activities of purified HPI variants.

Variant	Catalase Activity (U/mg)	Peroxidase Activity (U/mg) (ABTS)	Peroxidase Activity (U/mg) (o-dianisidine)
Wt- HPI	3200 ± 300	22400 ± 3400	9.1 ± 1.6
W88F	2100 ± 300	17700 ± 1900	9.7 ± 1.6
W88L	2400 ± 500	15300 ± 1400	9.5 ± 1.6
W89F	1900 ± 400	19300 ± 1600	12.9 ± 1.4
W89L	1600 ± 300	8700 ± 1000	6.8 ± 1.3
W197F	2300 ± 400	28900 ± 2700	9.6 ± 0.5
W197L	1100 ± 200	23400 ± 2300	8.2 ± 0.7

Table 3.3b. Catalatic and Peroxidatic molecular activities of purified HPI variants.

Variant	Catalase Activity (U/nmol of heme*	Peroxidase activity (U/nmol of heme*)		Peroxidase/ Catalase ratio (Normalized)[++]	
		ABTS	o-dianisidine	ABTS	o-dianisidine
Wt-HPI	410	2900	1.2	1.0	1.0
W88F	330	2700	1.5	1.2	1.6
W88L	400	2600	1.6	0.9	1.4
W89F	300	3000	2.0	1.4	2.3
W89L	380	2000	1.6	0.8	1.5
W197F	290	3600	1.2	1.8	1.5
W197L	170	3500	1.2	3.0	2.6

*The normalization to heme content was based on $A_{407/280}$ ratio assuming that a ratio of 0.5 represents 0.5 heme/subunit.

[++] Given numbers are for comparison with the wild-type ratio normalized to 1.

on the X-axis at positive integer values that were very close to zero. The kinetic behavior of the catalase reaction of EcHPI and its variants has been calculated and compared using classical Michealis-Menten terms in Table 3.4. Since it is incorrect to treat catalase kinetics in Michealis-Menten terms, (and therefore the classical definition of K_m and k_{cat} should be avoided), K_m and k_{cat} have been expressed as apparent values. The data in Table 3.4 show that the apparent affinities of Trp89 and Trp197 variants to H_2O_2 are about two to three-fold lower than the wild type. While the apparent turnover rates of W89F and W197F are about 3-fold higher, those of W89L and W197L are quite similar to that of the wild type. Native EcHPI and the Trp88 variants have quite similar peroxide affinities and turnover rates.

The effect of ABTS concentration [ABTS] on the rate of peroxidase reaction of EcHPI and its variants is shown in Figure 3.5. The initial velocities have also been corrected for heme content. Table 3.5 lists the apparent peroxidatic K_m and k_{cat} calculated for purified HPI and its variants. The apparent K_m values for all the variants are not significantly different from that of the wild type enzyme. No significant trend is apparent in the apparent k_{cat} values of the variants; W88L and W89L have lower and W197L has higher turnover numbers when compared to the wild type enzyme.

3.5.4. Effect of heme inhibitors on the catalase activity

KCN and NaN_3 are common reversible heme inhibitors used in structure-function studies of heme-containing enzymes like catalases. In the present study, the inhibitory effects of KCN and NaN_3 on the catalase activity of EcHPI and its variants were compared. The top panels in Figure 3.6 show the inhibition profile of catalase activity in the presence of KCN while the bottom panels represent the catalatic inhibition profile in
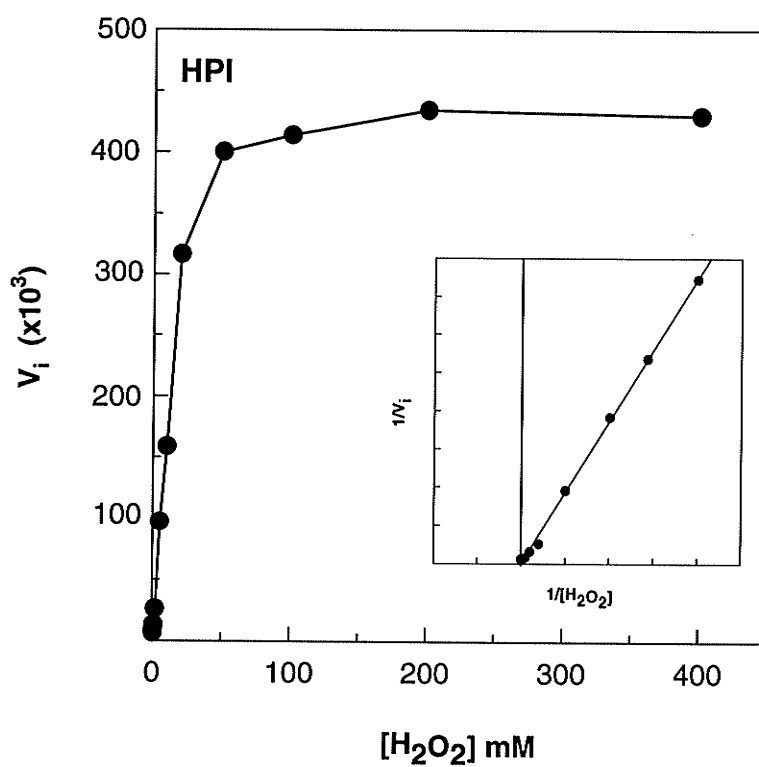
Figure 3.4: Effect of hydrogen peroxide concentrations on the initial catalatic velocities (V_i) of purified wild type EcHPI and its variants. Outer panels: Michealis-Menten (primary plots); Inset panels: Lineweaver-Burk (double reciprocal) plots.
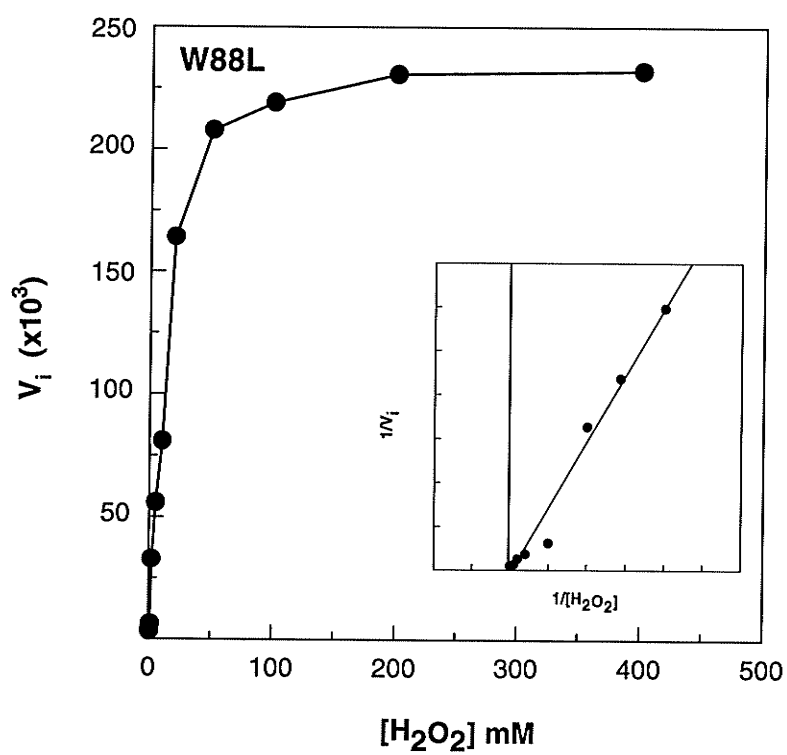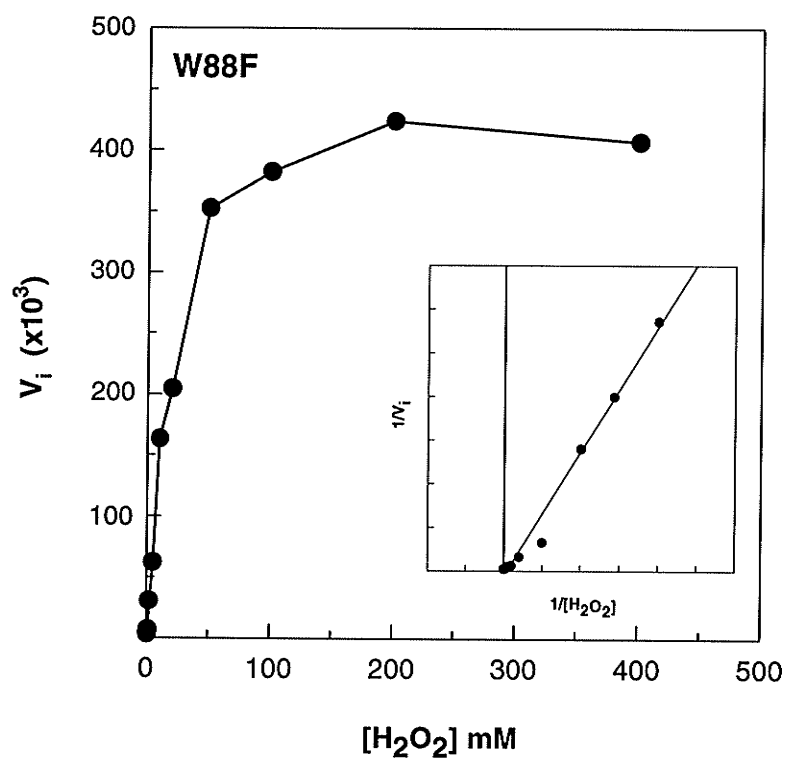
V_i = µmoles of H_2O_2 min^{-1} µmole heme^{-1}

Figure 3.4. (continued)

Figure 3.4. (continued)
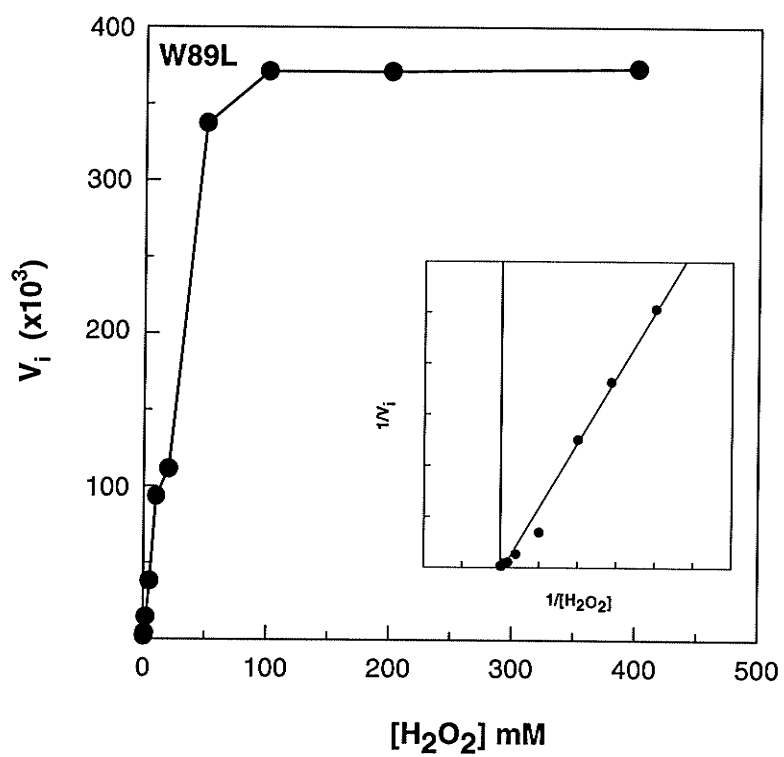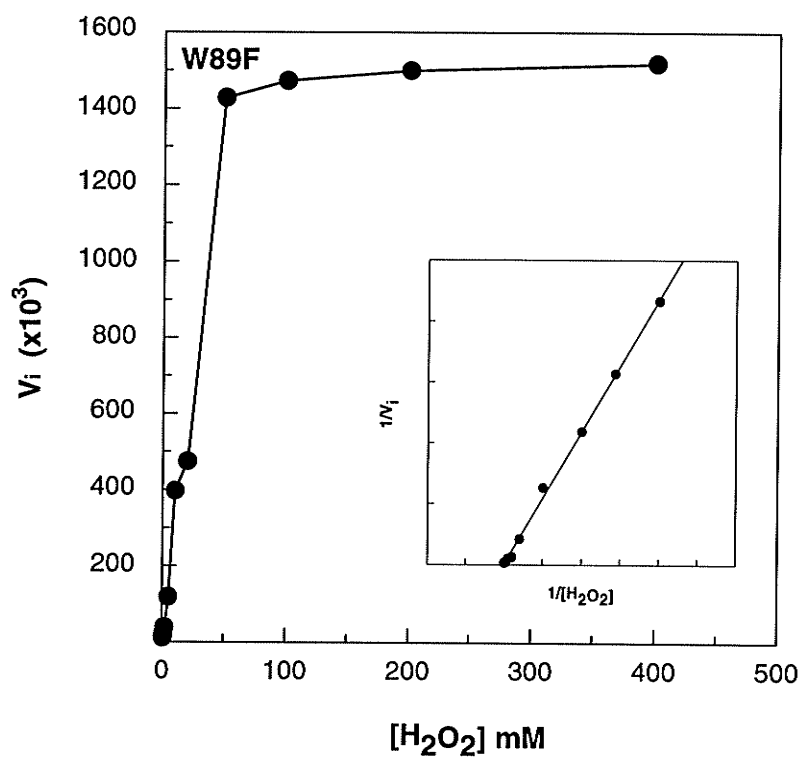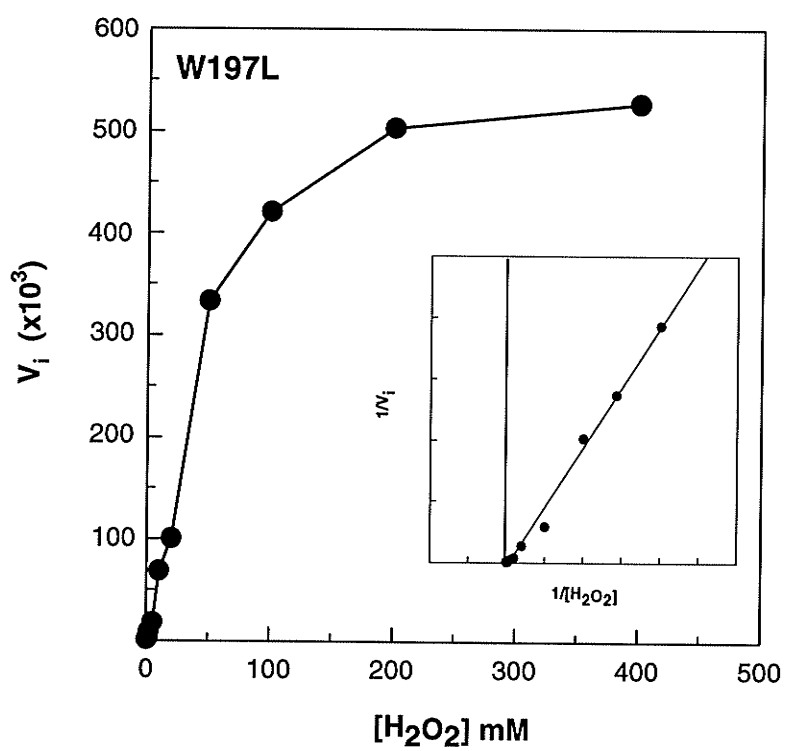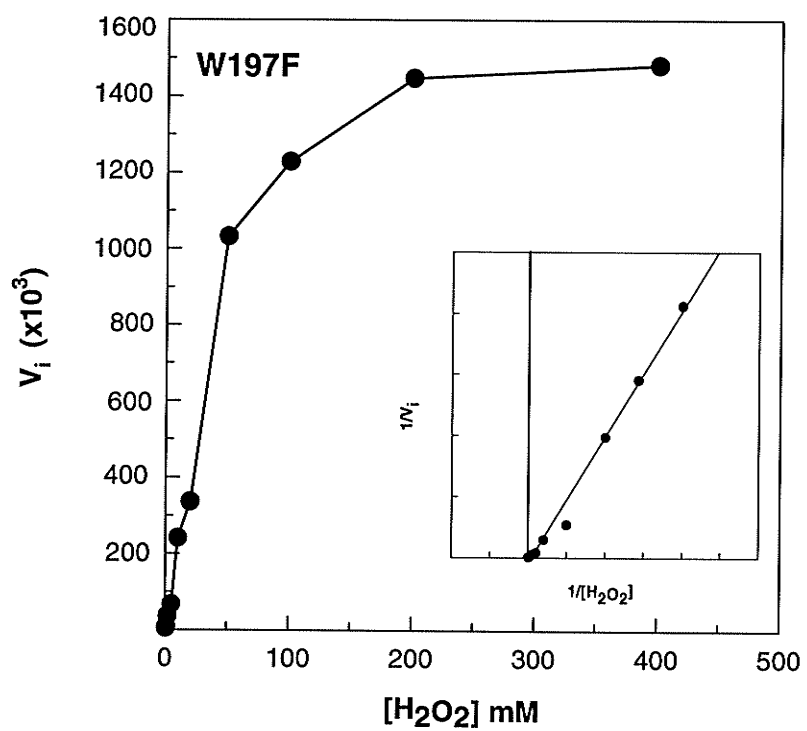
Figure 3.4. (continued)

Table 3.4. Apparent catalatic K_m and k_{cat} calculated for purified HPI and its variants.

Variant	K_m (app) (mM)	V_{max} (μmol/min)*	k_{cat} (s^{-1})*
Wt-HPI	13.7 ± 2.4	$4.3\ (\pm 0.2) \times 10^5$	$7.2\ (\pm 0.3) \times 10^3$
W88F	20.7 ± 2.8	$4.2\ (\pm 0.2) \times 10^5$	$7.1\ (\pm 0.3) \times 10^3$
W88L	14.1 ± 2.0	$2.3\ (\pm 0.1) \times 10^5$	$3.9\ (\pm 0.1) \times 10^3$
W89F	31.9 ± 9.3	$15.2\ (\pm 1.2) \times 10^5$	$25.3\ (\pm 2.1) \times 10^3$
W89L	33.5 ± 9.3	$3.7\ (\pm 0.3) \times 10^5$	$6.2\ (\pm 0.5) \times 10^3$
W197F	35.9 ± 7.2	$14.8\ (\pm 0.9) \times 10^5$	$24.7\ (\pm 1.6) \times 10^3$
W197L	39.5 ± 7.8	$5.3\ (\pm 0.3) \times 10^5$	$8.8\ (\pm 0.6) \times 10^3$

*Corrected for differing heme content based on $A_{407}/_{280}$ ratio, assuming 2 hemes/tetrameric holoenzyme.
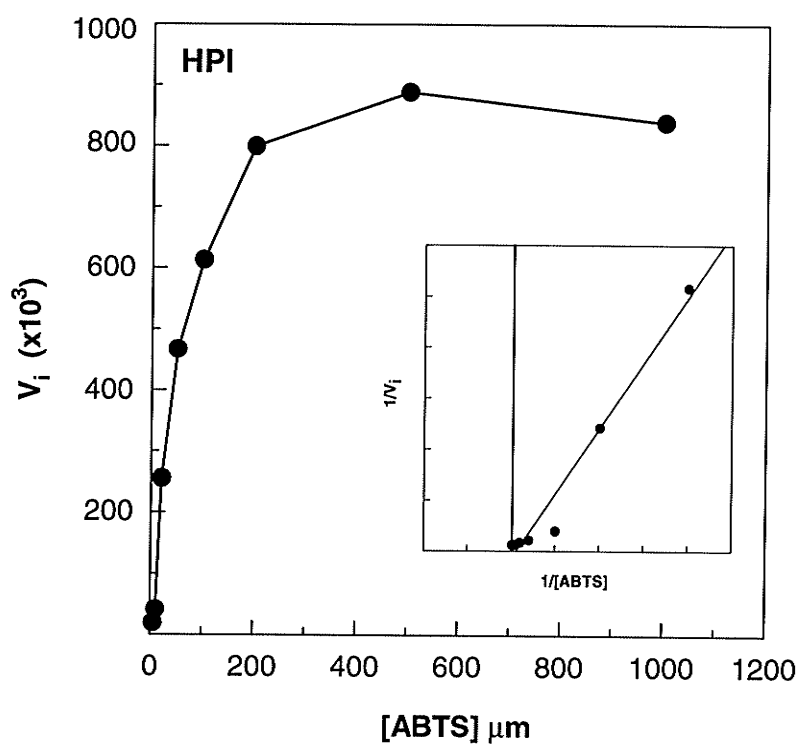
Figure 3.5: Effect of ABTS concentrations on the initial peroxidatic velocities (V_i) of purified wild type EcHPI and its variants. Outer panels: Michealis-Menten (primary plots); Inset panels: Lineweaver-Burk (double reciprocal) plots.

V_i = μmoles of ABTS min^{-1} μmole heme^{-1}
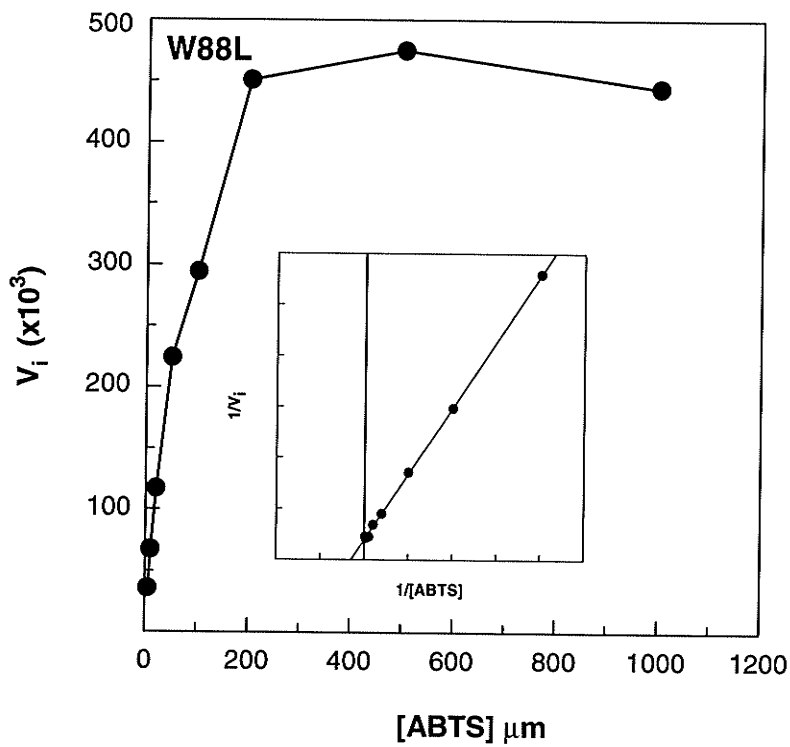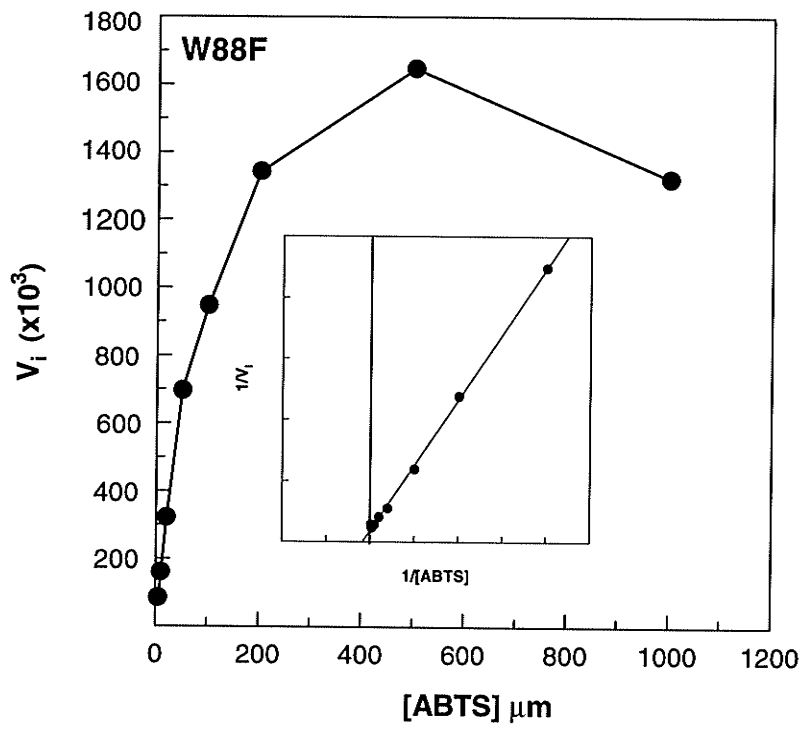
Figure 3.5. (continued)
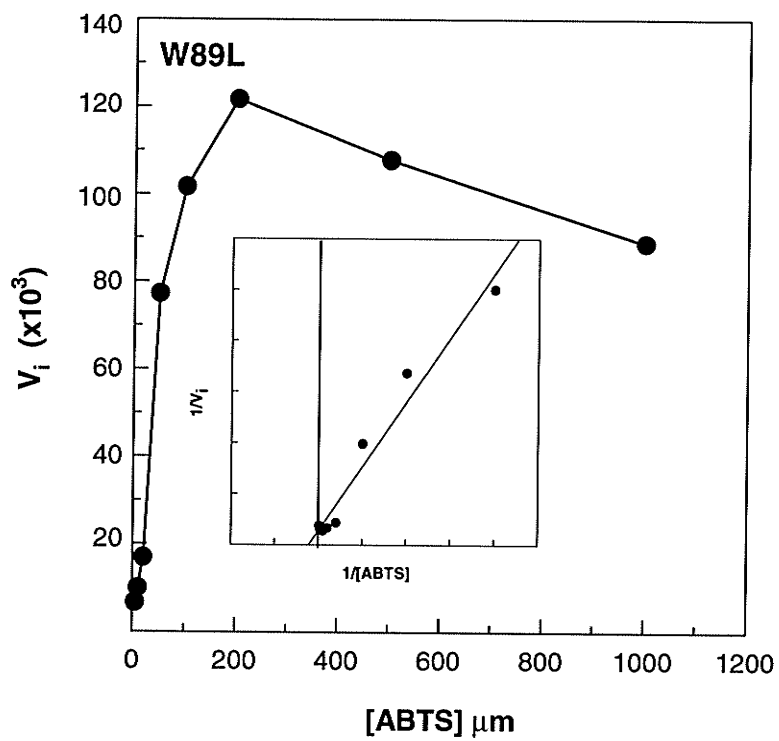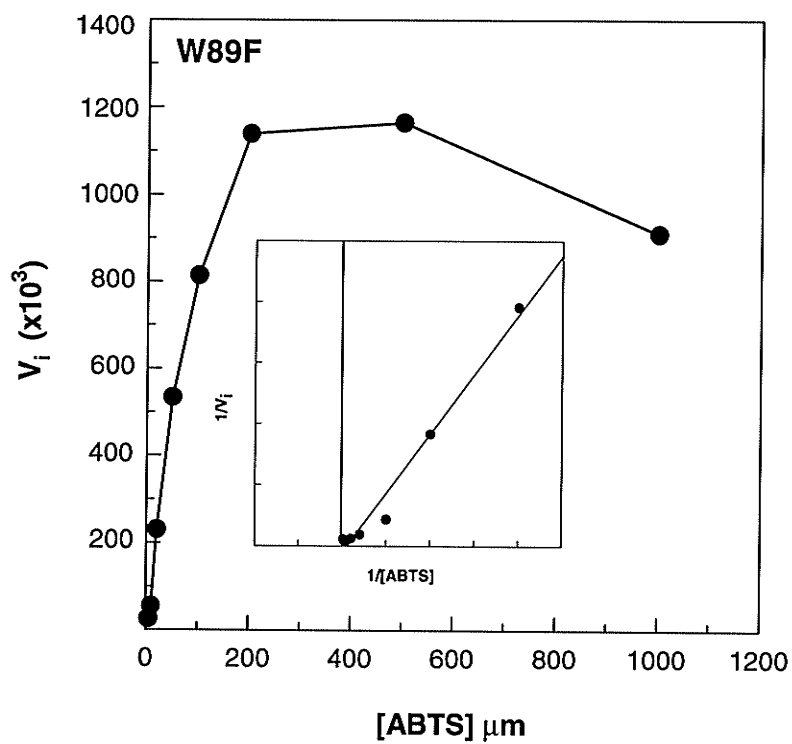
Figure 3.5. (continued)
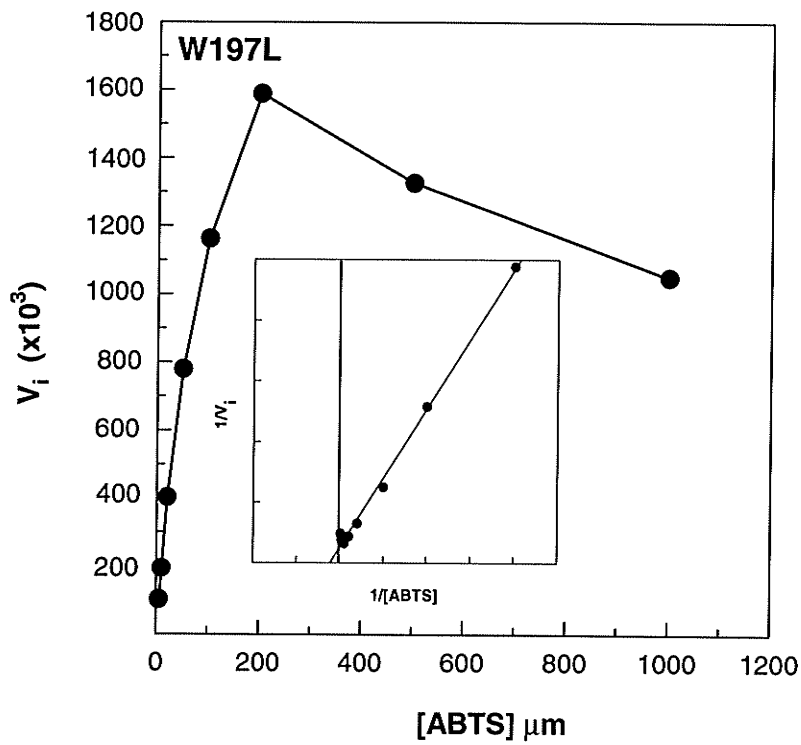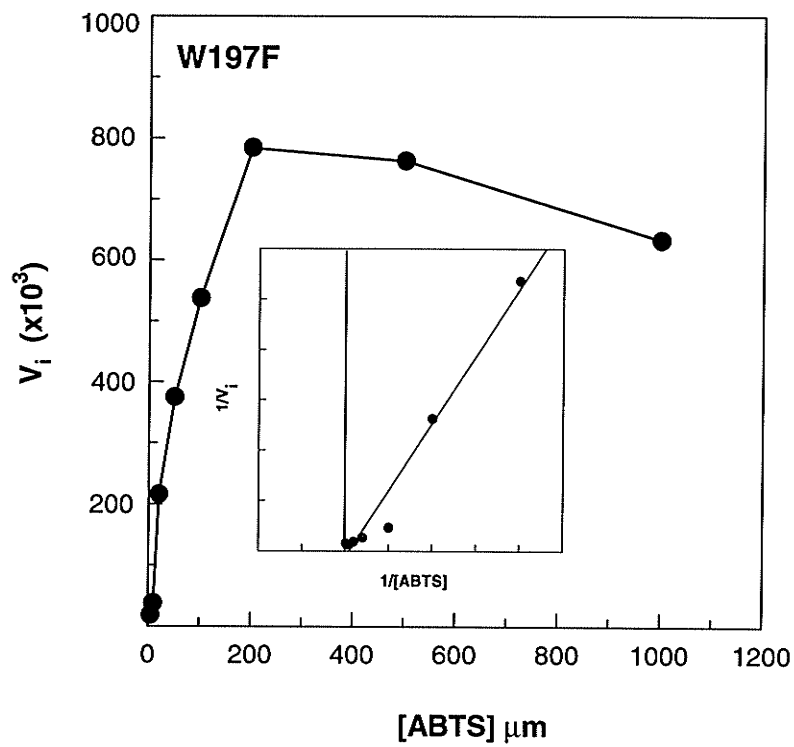
Figure 3.5. (continued)

Table 3.5. Apparent peroxidatic K_m and k_{cat} calculated for purified HPI and its variants, using ABTS as substrate.

Variant	K_m (app) (μM ABTS)	V_{max} (μmol/min)*	k_{cat} (s^{-1})*
Wt-HPI	48 ± 9.9	$8.9 (\pm 0.5) \times 10^5$	$14.8 (\pm 0.8) \times 10^3$
W88F	59 ± 16.0	$8.6 (\pm 0.6) \times 10^5$	$14.3 (\pm 1.1) \times 10^3$
W88L	53 ± 10.7	$4.7 (\pm 0.3) \times 10^5$	$7.9 (\pm 0.4) \times 10^3$
W89F	55 ± 22.5	$11.6 (\pm 1.2) \times 10^5$	$19.4 (\pm 2.1) \times 10^3$
W89L	39 ± 20.0	$1.2 (\pm 0.2) \times 10^5$	$2.0 (\pm 0.3) \times 10^3$
W197F	52 ± 19.5	$7.8 (\pm 0.8) \times 10^5$	$13.1 (\pm 1.3) \times 10^3$
W197L	51 ± 17.5	$15.9 (\pm 1.9) \times 10^5$	$26.5 (\pm 3.1) \times 10^3$

*Corrected for differing heme content based on $A_{407}/_{280}$ ratio, assuming 2 hemes/tetrameric holoenzyme.

presence of NaN$_3$. Both KCN and NaN$_3$ produced similar inhibition patterns for catalase activities of variants in relation to the wild type. The variants also had similar end-point inhibitions. Table 3.6 summarizes the effects of azide and cyanide on the catalase activities of wild type EcHPI and its variants. The numbers indicate the amount of azide or cyanide required to cause 50% inhibition of catalase activity. Wild type HPI showed 50% inhibition of catalase activity in the presence of 250 µM KCN. The variants differed by requiring slightly highly or lower concentrations for inhibition of catalase activity but such variations remained within 12-20% of that of wild type. Inhibition of half the catalase activity of the wild type in presence NaN$_3$ was achieved at a concentration of about 460 µm. The variants show only negligible differences in their inhibition profiles in presence of NaN$_3$ (+/- 10%). These studies show that there are no significant differences in the activities of the variants in the presence of either cyanide or azide compared to the native enzyme.
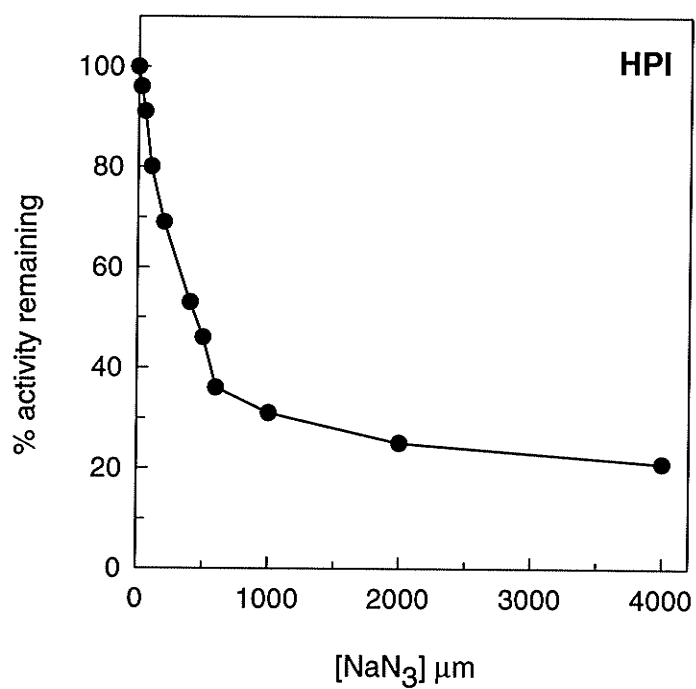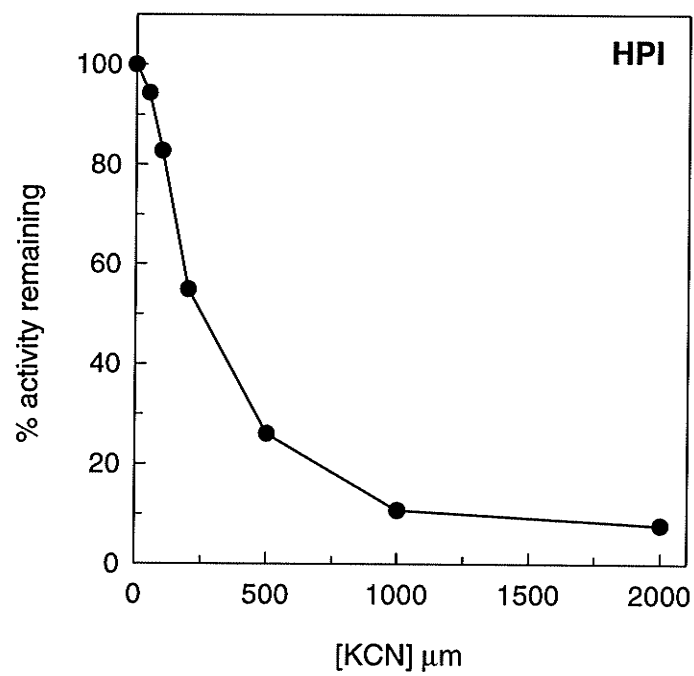
**Figure 3.6:** Inhibition of catalase activity of EcHPI and its variants by KCN (top panels) and NaN$_3$ (bottom panels).
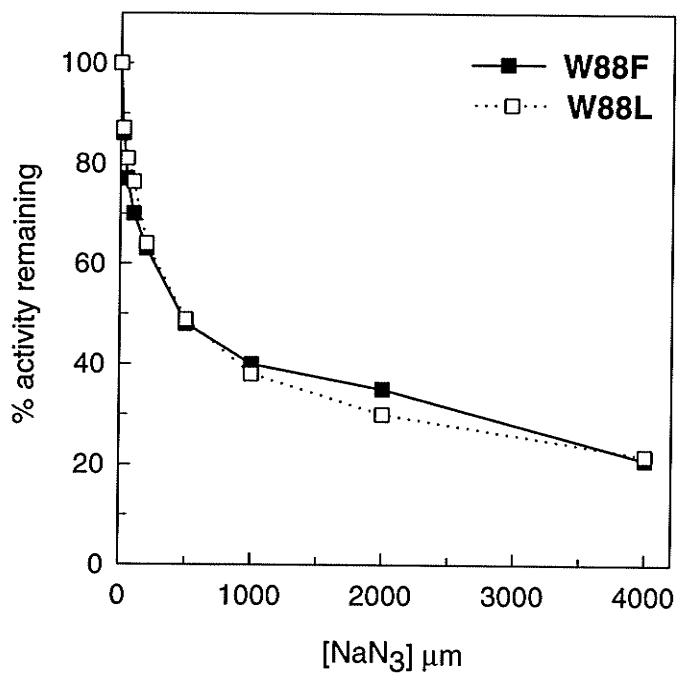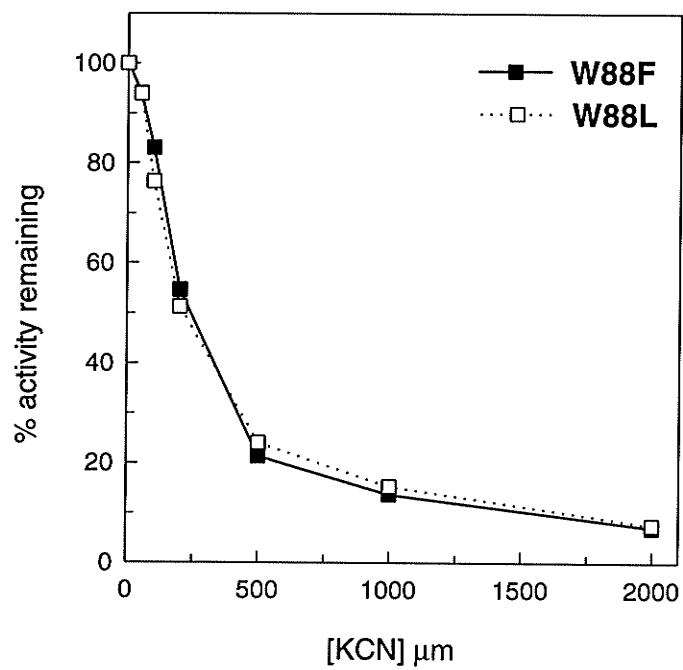
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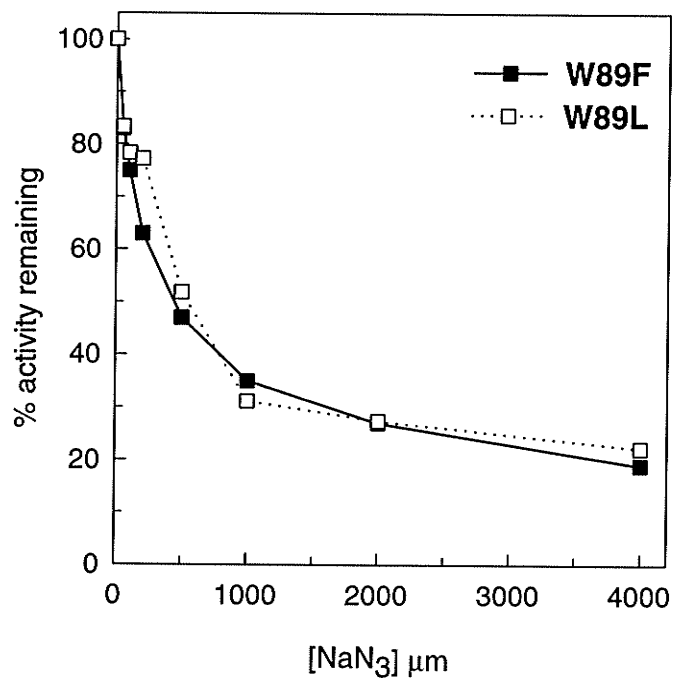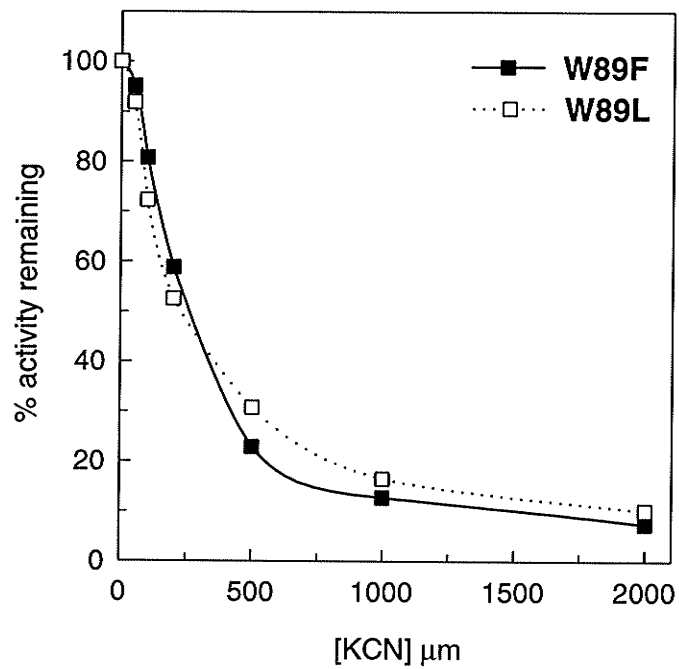
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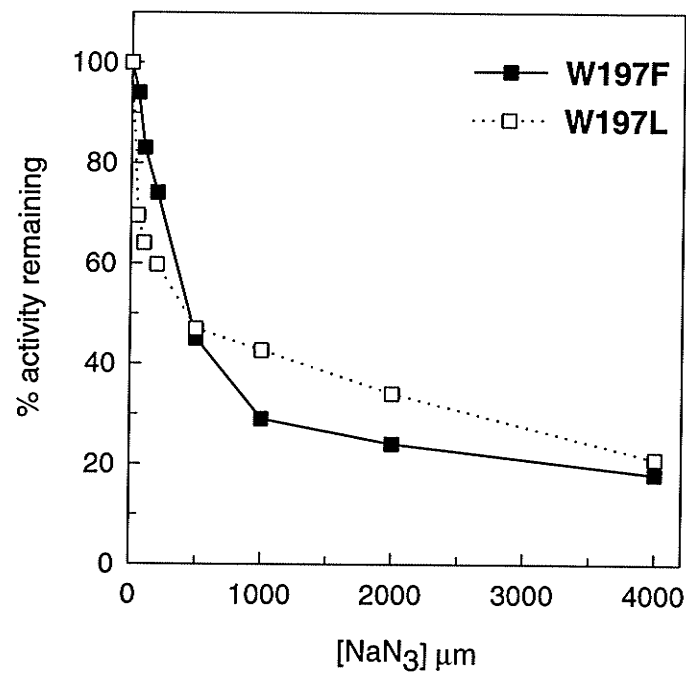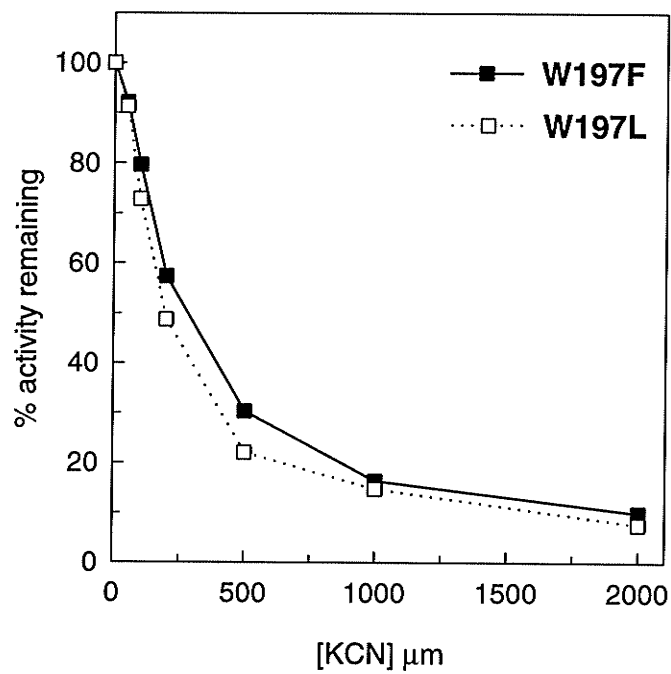
Figure 3.6. (continued)

Table 3.6. Comparison of sensitivity of HPI and its variants to cyanide (KCN) and azide (NaN$_3$).

Variant	[KCN] causing 50% inhibition (micromoles)	[NaN$_3$] causing 50% inhibition (micromoles)
Wt-HPI	250	460
W88F	250	470
W88L	225	490
W89F	280	430
W89L	240	480
W197F	280	450
W197L	200	480

4. DISCUSSION

4.1. Structure-function studies on EcHPI

Over the past few decades, protein biochemists and biophysicists have been extensively using site-directed mutagenesis studies in conjunction with information from crystal structures to understand the structure-function of various proteins. Yeast cytochrome *c* peroxidase (CCP) and horseradish peroxidase (HRP) are the best studied plant peroxidase enzymes. Various groups have carried out detailed studies on the role of individual amino acid residues in the active sites of CCP (Vitello *et al.*, 1993; Erman *et al.*, 1993) and HRP (Howes *et al.*, 1997; Gilfoyle *et al.*, 1996; Nagano *et al.*, 1996; Newmyer *et al.*, 1996). Structural and functional data from these enzymes have both facilitated and contributed to many important discoveries and achievements in the area of peroxidase research. The notable sequence similarity of catalase-peroxidases to plant peroxidases such as CCP especially in the vicinity of the heme active site has allowed certain assumptions to be made about the identity and location of a number of highly conserved active site residues in the catalase-peroxidase EcHPI (Hillar *et al.*, 2000).

Previous site-directed mutagenesis studies on EcHPI not only confirmed the important catalytic role of Trp105 but also demonstrated successfully, the modulation of the catalase versus peroxidase activities via modification of the key Trp residue in the distal heme pocket (Hillar *et al.*, 2000). Interestingly, Trp105 is conserved either as Trp in class I peroxidases (including yeast CCP residue #51) or as a Phe in all type II and type III peroxidases (including HRP residue # 41) (Nicholls et al., 2001). Site-directed mutagenesis studies on CCP where Trp51 was replaced with Phe (Fishel *et al.*, 1987), led to similar qualitative effects to those seen for W105F in EcHPI. The opposite F41W

mutation, which would result in HRP more closely resembling HPI, caused a reduction in the peroxidatic activity to 5% of wild-type (Smulevich *et al.*, 1994). Unfortunately, whether this HRP variant exhibited any catalase activity was not reported.

The specific pattern of distribution of Trp residues in and around the putative active site of EcHPI (Figure 1.2) and their conservation among other catalase-peroxidases is of great significance in light of previous findings on Trp105 and also due to the hydrophobicity and large size of the tryptophan molecule. The aim of the present study was primarily to investigate the role of some of these conserved Trp residues (Trp88, Trp89 and Trp197) while at the same time to attempt to successfully modulate the catalase versus peroxidase activities of EcHPI.

The investigation of the effect of culture growth temperature on heme binding was limited to only two temperature points (28°C and 37°C) due to experimental constraints. Native EcHPI and its variants prepared from cells grown at 28°C bind more heme and correspondingly show higher specific activities. The observed increases in the specific activities basically confirm that the measured increases in the heme/protein ratios are indeed real. Previous studies on certain variants of catalase HPII from *E. coli* have suggested that the lower temperature (28°C) provides better conditions for correct folding of the protein into a catalytically active form (Sevinc *et al.*, 1998). This effect might be responsible for the higher uptake and binding of heme by EcHPI and its variants, when *E. coli* cultures are grown at 28°C. SDS-PAGE reveals that there are no fundamental visual differences between proteins from the 28°C and 37°C cultures. However, relatively higher concentrations of EcHPI proteins were obtained from cultures grown at 37°C than the ones grown at 28°C. This could be primarily due to higher growth rates of bacteria at

37°C. The presence of more background proteins in the 37°C isolates could be rendering them more difficult to purify, which in turn is reflected in their lower heme/protein ratios.

Hemoproteins such as catalase-peroxidases exhibit two types of absorption bands within the visible and near ultraviolet region. They are the Sorêt band and the α and β - charge transfer transition bands. These absorption bands are influenced by the properties and energy levels of the five d electrons of iron (III) and the π electrons of the porphyrin, which is present in the active site of these enzymes. Spectral analysis of EcHPI and its Trp-replacement variants reveals that they have very similar spectral profiles within the visible and near ultraviolet regions. This indicates that the electronic configuration of the heme environment is not visibly altered by the mutations.

Previous pH profile studies on the catalytic activities of bacterial catalase-peroxidases from *Mycobacterium tuberculosis* (Johnsson *et al.*, 1997), *Synechocystis* PCC 6803 (Jakopitsch *et al.*, 1999), *Streptomyces reticuli* (Zou and Schrempf, 2000), *Klebsiella pneumoniae* (Hochman and Goldberg, 1991) have consistently shown results very similar to those observed in the present study. It can be easily concluded that the EcHPI enzyme shows different pH optima for catalase and peroxidase activities. The absence of any fundamental differences between the catalatic and peroxidatic pH profiles of both wild-type and its Trp-replacement variants suggest that the sensitivities of the catalytic reactions to pH are not affected by the mutations. The pH optimum for catalase activity hovers around the pH 7 mark while that for the peroxidase activity remains approximately around pH 5. The amino acid histidine present at position 106 on the distal side of the heme in active site of EcHPI has been suggested to be involved both in compound I formation and reduction (Hillar *et al.*, 2000). The pKa value of histidine is ≈ 6, which is

about half way between the pH 7 required for catalase activity and pH 5 required for peroxidase activity. Thus it can be implied that the catalase activity would involve the deprotonation of His106 while the peroxidase activity would involve the protonation of that residue.

The significant reduction in the catalase activity of W197F and W197L coupled with the corresponding increase in their peroxidase activities is indicative of their possible importance for catalatic activity of EcHPI. Variants at Trp88 and Trp89 have reduced heme binding in comparison to the wild-type. However, subsequent to correction for differing heme content they show no significant differences in their catalytic activity in relation to the wild-type. This indicates that Trp88 and Trp89 may not be important for catalytic activity of EcHPI but may have a minor role in heme binding.

Although catalases in general do not show Michealis-Menten behavior, several catalases including catalase-peroxidases seem to show true substrate saturation at much lower levels of peroxide (in the range of a few millimolar) (Nicholls et al., 2001). Previous kinetic studies on catalase-peroxidases from *Klebsiella pneumoniae* (Hochman and Goldberg, 1991), *Synechococcus* PCC 7942 (Mutsuda *et al.*, 1996), *Synechocystis* PCC 6803 (Jakopitsch *et al.*, 1999) and *Septoria tritici* (Levy *et al.*, 1992) have shown that these catalase-peroxidases exhibit Michealis-Menten behavior at lower concentrations of H_2O_2 (10-40 mM). This means that, in these enzymes the limited maximal turnover is slower and the lifetime of the putative Michealis-Menten intermediate (with the redox equivalent of two molecules of peroxide bound) is much longer (Nicholls *et al.*, 2001). In the present study on the catalatic kinetic parameters, wild-type EcHPI and most variants seem to exhibit true saturation at approximately 50-100 mM concentrations of H_2O_2, beyond which there is no apparent correlation with theoretical Michealis-Menten curves.

The apparent K_m values for EcHPI in the present study are about 2-fold higher than previously reported values for EcHPI (Hillar *et al*, 2000) and most other catalase-peroxidases in the literature (Engleder et al., 2000; Jakopitsch *et al.*, 1999; Hochman and Goldberg, 1991). However, the turnover numbers are in line with findings from previous EcHPI studies (Hillar *et al.*, 2000). The notable variation in apparent K_m values in relation to previous EcHPI findings must be taken into consideration before venturing to make direct comparisons with apparent K_m values calculated for Trp-replacement variants in the present study. Trp88 variants show very similar catalatic kinetic profiles to that of native EcHPI, implying that the mutations at this position do not alter the kinetic parameters of the enzyme. Inspite of the apparently reduced affinities of Trp89 and Trp197 variants for H_2O_2 in relation to the wild type, their turnover numbers do not show similar trends. Considering the fact that the turnover numbers calculated for the wild type are invariable unlike their apparent K_m values, it is logical to conclude that mutations at position Trp89 and Trp197 do not significantly affect their steady-state kinetic behavior in relation to the wild type.

Determination of kinetic constants for peroxidase activity of catalase-peroxidases should be ideally done by performing two types of V_i (initial velocity) measurements in tandem; one by keeping the concentration of peroxidatic substrate constant while varying $[H_2O_2]$ and the other by varying the concentration of the peroxidatic substrate while keeping $[H_2O_2]$ constant. However, due to the complex nature of these assays, most researchers express the peroxidatic K_m and k_{cat} values either in terms of hydrogen peroxide (Hochman and Goldberg 1991; Levy et al., 1992) or in terms of the organic electron donor (Hillar *et al.*, 2000). The present study discusses the peroxidatic kinetic constants in terms of the organic peroxidatic substrate ABTS, that was found to support a

2000-fold higher reaction rate than o-dianisidine. The peroxidatic kinetic constants of the Trp-replacement variants in the present study do not show any relevant trends and generally indicate the lack of any important changes in steady-state kinetic behavior towards ABTS, relative to native EcHPI.

Universal heme ligands such as cyanide and azide are competitive inhibitors of catalytic activities of hemoprotein enzymes such as catalase-peroxidase EcHPI. In the present study, both KCN and NaN_3 were used to study their inhibitory effects on the catalase activity of EcHPI variants. The apparent similarities in the sensitivities of the EcHPI and its variants to both KCN and NaN_3 show that the heme environments are not greatly distorted by mutations in the targeted Trp residues.

Physicochemical, spectral, biochemical, kinetic and heme inhibition studies together provide evidence that Trp88, Trp89 and Trp197 may be dispensable for the normal functioning of the catalase-peroxidase EcHPI. Most of the Trp-replacement variants contain relatively less heme than the native enzyme suggesting that the heme binding in EcHPI is easily disrupted. It is well known from previous mass spectral studies that EcHPI exists as a mixture of tetrameric species containing 2, 3 and 4 hemes; all subunits are capable of binding heme and incomplete occupancy probably arises from limited heme availability (Hillar *et al.*, 2000). Hence it is possible that replacement of Trp88, Trp89 or Trp197 may actually be causing shifts in the proportions of the different tetrameric species, which in turn might be affecting the heme/protein ratios of the enzyme.

4.2. Future directions

Catalase-peroxidases such as EcHPI present a unique challenge to the protein biochemist interested in understanding the bifunctional behavior of enzymes. The present

study involved only a handful of conserved Trp residues around the putative active site of EcHPI. It did not provide any substantial evidence to show that Trp88, Trp89 and Trp197 are important for catalytic activity of EcHPI. However, further studies using other peroxidatic substrates such INH (Isoniazid) and physiological hydrogen donors such NADPH and NADH are needed to fully gauge the effects of the mutations on catalysis of EcHPI. In addition future studies could also focus on other conserved Trp residues in EcHPI, that still are potential targets for site-directed mutagenesis studies.

The recent explosions in the reported crystallizations of catalase-peroxidases from *Synechococcus* (Wada *et al.*, 2002) and *Holoarcula* (Yamada *et al.*, 2001), and the C-terminal domain of EcHPI (Carpena *et al.*, 2002) have generated a renewed interest in structure-functional investigations of catalase-peroxidases. In addition, they have also opened up new opportunities for those interested in crystallizing other catalase-peroxidases enzymes. The 2.0 Å crystal structure of the catalase-peroxidase HmCP from *Holoarcula marismortui* was very recently published (Yamada *et al.*, 2002). It shows that the arrangement of the catalytic residues and the cofactor heme *b* in the active site is virtually identical to that of class I peroxidases but the heme moiety is buried inside the domain, similar to that in a typical catalase. This in turn would favor the reduction of compound I by hydrogen peroxide rather by typical bulky peroxidase substrates. The HmCP structure also reveals that, in the vicinity of the active site, novel covalent bonds are formed between the phenol group of the Tyr218 and the side chains of Trp95 (distal) and Met244. Together with the C-terminal domain, these covalent bonds fix two long loops (LL1 and LL2) on the surface of the enzyme that cover the substrate access channel to the active site (Yamada *et al.*, 2002). It has been suggested that the mutation of the distal tryptophan (Trp105 in EcHPI) might be disrupting the covalent bonding between

the side chains in the active site which would destabilize the first long loop (LL1) and remove it from the accessible channel, thereby exposing the heme to the solvent, as seen in CCP and APX.

The vital information obtained from the refined structure of HmCP has paved the way for a better understanding of the bifunctional nature of catalase-peroxidases. Future investigations on EcHPI can now be modeled on the refined HmCP structure. Tyr218 and Met244 of HmCP are conserved among all catalase-peroxidases. Corresponding residues in EcHPI are potential targets for future site-directed mutagenesis studies; so are other conserved residues that are involved in anchoring the long loops (LL1 and LL2) covering the substrate access channel. Both LL1 and LL2 in HmCP have been found to be part of intra-subunit and inter-subunit interactions between the N-terminal and C-terminal domains of HmCP, which are thought to be important for subunit association (Yamada *et al.*, 2002). Future EcHPI investigations could also focus on conserved residues in LL1 and LL2 in order to understand the precise role of these subunit interactions in structural stability of the enzyme.

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