Probing the structure and function of catalase HPII of *Escherichia coli*

by

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PROBING THE STRUCTURE AND FUNCTION OF CATALASE HPII OF *Escherichia coli*

BY

MEHMET SERDAL SEVINC

A Thesis/Practicum submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

ABSTRACT

A structure-function study of catalase HPII of *Escherichia coli* has been carried out with a focus on five regions of the enzyme. Residues in the potential NADPH-binding site were changed to their counterparts in bovine liver catalase in an attempt to promote NADPH-binding. Even with the changed residues, no NADPH bound to HPII. During this study of the putative NADPH-binding site, the Arg260Ala variant of HPII was characterized and found to be three-fold more active than wild type HPII. This was rationalized in terms of an enlarged channel leading to the active site which provides easier access for the substrate. Investigation of the effectiveness of various inhibitors of varying sizes corroborated this conclusion. A study of the C-terminal region revealed that residues 745 to 753 could be removed without affecting enzyme activity. Truncation at Arg744 or shorter caused a disruption in the folding process which prevented the accumulation of active protein. The two cysteine residues were removed without significantly affecting enzyme activity indicating that neither played a role in the catalytic process. As part of this study it was observed that Cys438 was modified by a substitution with a mass of approximately 44 Da. Subsequent work eliminated a number of possibilities leaving a hemithioacetal as the most likely modification. Three residues that were hypothesized to be involved in heme-binding were changed to their bovine counterparts in an attempt to force the heme to bind in the "flipped" orientation. This proved to be unsuccessful suggesting that heme will bind to HPII in only one orientation. The final target area was the

channel leading to the active site where Val169 was changed to a Cys. Unexpectedly, this mutant proved to be inactive and was incapable of converting the heme b to heme d. An explanation for this severe effect on activity will have to await a more extensive study of the channel. For many of the mutant proteins, it was necessary to grow the transformed cells at 28°C rather than 37°C for a reasonable yield of protein. This was attributed to proteolytic digestion of partial folding of the protein, for which the normal folding pathway has been affected by the residue change.

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LIST OF ABBREVIATIONS

A	Absorbance
Amp^r	Ampicillin resistant
BLC	Bovine liver catalase
bp	Base pair(s)
CCA	convex constrain algorithm
Da	Dalton
DEAE	Diethylaminoethyl
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
GSH	Glutathione
HPI	Hydroperoxidase I
HPII	Hydroperoxidase II
HPLC	High performance liquid chromatography
IR	Infrared
k_{cat}	Turnover number
kDa	KiloDalton
Km	Michaelis-Menten constant
LB	Luria-Bertani medium
MALDI/MS	Matrix assisted laser desorption/ionization mass spectroscopy
MSH	2-Mercaptoethanol
MW	Molecular weight
NADPH	Nicotinamide adenine dinucleotide phosphate (reduce
NMR	Nuclear magnetic resonance
PAGE	Polyacrylamide gel electrophoresis
PVC	*Penicillium vitale* catalase
SOD	Superoxide dismutase
SDS	Sodium dodecyl sulfate
V_{max}	Maximum velocity
Vi	Initial velocity
WT	Wild type
w/v	Weight per volume

1. GENERAL INTRODUCTION

1.1. Evolution of molecular oxygen and aerobic life

About two billion years ago, molecular oxygen emerged in the atmosphere of the Earth primarily due to the evolution of oxygen-producing photosynthetic organisms (Metzner, 1978; Day, 1984; Sawyer, 1987; Halliwell & Gutteridge, 1989). Evolution of molecular oxygen led to the formation of an ozone (O_3) layer in the upper part of the atmosphere so that the damaging effects of the solar ultraviolet radiation on the Earth were greatly reduced allowing the evolution of more complex organisms. Currently oxygen is the most abundant element in the crust of the Earth with a 53.8% atomic abundance while its concentration in the dry air of the atmosphere amounts to 21%.

Today most organisms, excepting those adapted to anaerobiosis, use oxygen for efficient energy production. In the early days of life the absence of molecular oxygen led to the most prevalent organisms on the Earth being "strict" anaerobic organisms. As molecular oxygen increased, most of these anaerobic organisms became extinct or had to retreat to anaerobic niches because they were unable to detoxify the products of oxygen metabolism. A variety of organisms with a range of sensitivities to oxygen gradually replaced these anaerobic organisms. Ultimately, organisms evolved that metabolized molecular oxygen as a terminal electron acceptor for more efficient energy production, and they became dependent upon molecular oxygen for survival.

1.2. Oxygen toxicity

Oxygen at greater than 21% concentration is toxic to plants, animals and aerobic bacteria such as *Escherichia coli*. For example, the growth of *E. coli* and other bacteria is slower at high concentrations of oxygen (Brown & Yein, 1978) and oxygen enhances the damaging effects of ionizing radiation in bacterial and mammalian cultures.

The unusual chemistry of oxygen plays a significant role in its reactivity. Oxygen in its most stable (triplet) state has two unpaired electrons in two different π^* orbitals with parallel spins. Theoretically, two electrons are required to reduce it to water. However, according to Pauli's principle, electrons in the same orbital will have equal or antiparallel spins (Dickerson *et al.*, 1984), which restricts the electron transfer to oxygen such that electrons are accepted one at a time. Although ground state O_2 is a free radical because of its unpaired electrons, its reactions with non-radicals are limited due to the parallel spins of these electrons.

Most of the damaging effects of oxygen can be attributed to its toxic forms which are generated as byproducts during metabolism of oxygen in biological systems. Oxygen is normally reduced to water by the addition of four electrons during aerobic respiration via the electron transport chain, but other cellular reactions can produce a variety of active oxygen species (Fridovich, 1978; Wood, 1988). In other words, reactive oxygen species, including hydroxyl radical (HO·), superoxide anion (O_2^-), hydroperoxyl radical (HOO·), hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$) can be transiently generated in a variety of ways including: the electron

transport chain, primarily at the site of coenzyme Q (Halliwell & Gutteridge, 1989); enzymatic reactions including D-amino acid oxidase; a number of dehydrogenases and glutathione reductase (Imlay & Fridovich, 1991; Farr & Kogoma, 1991); the autoxidation of a number of compounds, such as ubiquinols, catechols, thiols, flavins and ascorbate; exposure to ionizing radiation, ultraviolet light, ozone, ultrasound, lithotripsy, freeze-drying and drugs such as paraquat; macrophages in response to bacterial invasion in the mammalian body; and from other competitive organisms in the environment (Fridovich, 1978; Chance *et al.*, 1979; Ames, 1983; Cerutti, 1985; Cross *et al.*, 1987; Halliwell & Gutteridge, 1989). Unless intercepted these toxic oxygen species can have deleterious effects on the cell by reacting with macromolecules to damage DNA (Brawn & Fridovich, 1981; Demple & Linn, 1982; Levin *et al.*, 1982; Cathcart *et al.*, 1984; Hollstein, *et al.*, 1984), to oxidize protein (Brot *et al.*, 1981; Davies *et al.*, 1987), and to initiate lipid peroxidation by oxidizing membrane fatty acids (Mead, 1976; Fridovich & Porter, 1981).

1.3. Description of reactive oxygen species

All reactive oxygen molecules can cause varying degrees of damage to cells although HO· is the most toxic oxygen species. Most of these reactions are rapid, making it difficult to determine which species is actually responsible for the damage. Furthermore, some reactive species, such as superoxide are very stable and non-reactive in the absence of water casting doubt on its role in some reactions (Britton & Fridovich, 1977). Furthermore, O_2^- cannot pass through membranes (Hassan &

Fridovich, 1979) except via an "anion channel" (Halliwell & Gutteridge, 1986). Therefore, caution must be exercised when interpreting and explaining the causes of damage involving reactive oxygen species in biological systems.

Among the reactive oxygen species, superoxide anion and hydrogen peroxide are relatively less reactive molecules (Halliwell & Gutteridge, 1986). Although superoxide anion is a free radical and unstable, it is only weakly reactive in aqueous solutions (Sawyer & Valentine, 1981; Afanas'ev, 1989). The reactions of superoxide include one electron transfer, deprotonation, oxidation and nucleophilic substitution (Afanas'ev, 1989). It has little effect on proteins although it can exacerbate protein damage caused by HO· (Halliwell & Gutteridge, 1986). It can inactivate a few enzymes including *E. coli* dihydroxyacid dehydratase, and it can initiate several radical chain reactions including the oxidation of dihydroxyacetone (Diguiseppi & Fridovich, 1984). However, far more reactive species can be formed in reactions of superoxide such as the reversible protonation to the more powerful oxidizing and reducing form, hydroperoxyl radical, HOO·, which can initiate lipid peroxidation and induce chain oxidation of NADH during catalysis by glyceraldehyde-3-phosphate dehydrogenase (Chan & Bielski, 1980).

The dismutation product of O_2^-, H_2O_2 is also a weak oxidizing agent and is stable enough for commercial production. It can oxidize thiol groups in proteins, causing the inactivation of enzymes such as glyceraldehyde-3-phosphate dehydrogenase (Brodie & Reed, 1987), which results in ATP depletion and/or inhibition of glycolysis. H_2O_2 can also oxidize keto-acids such as pyruvate (Andrae *et*

al., 1985). At high concentrations, H_2O_2 can be used as a disinfectant to kill some bacteria and animal cells which are sensitive to H_2O_2. The most damaging effects of H_2O_2 can be attributed to the formation of the highly reactive molecule HO· in the Haber-Weiss reaction with metal ions (Fenton, 1894; Haber & Weiss, 1934). In contrast to O_2^-, H_2O_2 can pass through membranes rapidly, and once inside the cell can initiate a series of radical reactions with cellular compounds in the presence of iron or copper ions (Halliwell *et al.*, 1983; Fridovich, 1986). The reaction of H_2O_2 with organic molecules in the presence of Fe^{2+} was first observed by Fenton in 1894. Later, Haber and Weiss (1934) showed that this was due to the formation of HO· radical in the following chain reaction.

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + HO\cdot$$

$$HO\cdot + H_2O_2 \rightarrow H_2O + H^+ + O_2^-$$

$$O_2^- + H_2O_2 \rightarrow O_2 + OH^- + HO\cdot$$

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + 2H^+ + O_2^-$$

$$Fe^{3+} + O_2^- \rightarrow Fe^{2+} + O_2$$

In the absence of organic reagents these reactions result in added H_2O_2 decomposing to H_2O and O_2. However, the various intermediates are highly reactive and a mixture of H_2O_2 and metal ions can cause the killing of bacterial spores (Marquis & Shin, 1994) and fungal spores (Levitz & Diamond, 1984). *Staphylococcus aureus* is more sensitive to H_2O_2 when pregrown in iron-rich medium (Repine *et al.*, 1981). The strong iron chelator 1,10-phenanthroline prevents the DNA damage and cell killing caused by H_2O_2, and H_2O_2 does not attack purified DNA unless an iron catalyst is

available (Filho *et al.*, 1984). Furthermore, in the presence of excess H_2O_2, several iron-proteins are damaged causing the release of iron that may be used for a further Haber-Weiss reaction (Gutteridge, 1986). The toxicity of H_2O_2 to cells can be prevented if the cells are treated with dimethylsulphoxide which can react with intracellular HO· (Repine *et al.*, 1981). The HO· radical can react rapidly with most cellular components in reactions involving hydrogen abstraction and addition giving rise to secondary radicals of variable reactivity, which can cause further damage to cellular components.

One other toxic oxygen species is singlet oxygen which is produced when the two unpaired electrons in ground state oxygen are excited to antiparallel spins, either in the same or separate orbitals. Singlet oxygen reacts with other molecules either through bond formation or energy transfer giving rise to uncontrolled oxidation reactions, especially lipid peroxidation of membranes (Straight & Spikes, 1985). However, singlet oxygen is less reactive than HO·, and is poorly mutagenic (Dahl *et al.*, 1988).

1.4. Antioxidative defence systems

The oxygen radicals encountered by the cell either from external or internal sources are normally eliminated by antioxidative defence mechanisms before they exert their destructive effects on the cell. Both eukaryotic and prokaryotic organisms have evolved mechanisms to overcome the toxic effects of oxygen radicals (Demple & Halbrook, 1983; Christman *et al.*, 1985; Halliwell & Gutteridge, 1986, 1989; Chan

& Weiss, 1987; Imlay & Linn, 1987; Greenberg & Demple, 1989; Keyse & Tyrrell, 1989; Kim *et al.*, 1989: Walkup & Kogama, 1989; Wolff *et al.*, 1989). It is generally accepted that there is a redox equilibrium between the cellular oxidants and antioxidants inside the cell (Allen & Venkitraj, 1992) that can be disrupted by external antioxidants causing depression of the synthesis of normal antioxidants (Sohal *et al.*, 1985).

Generally an antioxidative defence system includes a combination of small molecules like ascorbate, α-tocopherol, ß-carotene, uric acid, glutathione, metal binding proteins like ferritin, transferrin, lactoferrin, and metallothioneines, antioxidative enzymes, like superoxide dismutase (SOD), catalase and peroxidase, and damage repair systems (Simic & Taylor, 1987; Halliwell & Gutteridge, 1989). However, not all organisms have all of these components. *Neisseria gonorrhaeae* does not have any SOD activity while it has exceptionally high catalase and non-specific peroxidase activities (Archibald & Duong, 1986). *Bacillus popilliae* has SOD activity (Costilow & Keele, 1972) but no catalase or peroxidase activities (Peppers & Costilow, 1965). On the other hand, *Mycoplasma pnuemoniae* does not seem to have either SOD or catalase activities, although it releases both O_2^- and H_2O_2 to damage the host cells (Almagor *et al.*, 1984). Probably the major cause of oxygen toxicity is the metal ion-catalyzed Haber-Weiss reaction which generates HO·. If the cells have systems for the efficient removal of O_2^- and H_2O_2, they will most likely prevent the formation of OH·.

1.5. Antioxidative defence system of *E. coli*

The enteric organism *E. coli* has two different mechanisms to protect the cells from oxidative damage: a predamage system and a postdamage system (Yonei *et al.*, 1987).

1.5.1. The predamage defence system

The predamage system includes the antioxidative enzymes superoxide dismutase, catalase, and peroxidase (Fridovich, 1976). Moreover, the iron-storage protein bacterioferritin of *E. coli* (Andrews *et al.*, 1989) tightly controls the available iron in the cell in order to prevent a possible Haber-Weiss reaction. *E. coli* cells also contain glutathione, which is a scavenger of HO· and singlet oxygen, although they do not have the glutathione peroxidase system for the removal of H_2O_2 (Smith & Schrift, 1979). In order to maintain a pool of reduced glutathione to keep cellular proteins in a reduced state *E. coli* has the enzyme glutathione reductase (encoded by *gotA* at 77-78 min on the *E. coli* chromosome) (Storz & Tartaglia, 1992). In addition, the enzyme alkyl hydroperoxidase reductase (encoded by *ahpC* and *ahpF* at 13 min on the *E. coli* chromosome; Jacobson *et al.*, 1989) reduces physiological alkylperoxides including thymine hydroperoxide and linoleic hydroperoxide, to their corresponding alcohols (Storz & Tartaglia, 1992). Mutants lacking this enzyme are very sensitive to killing by organic hydroperoxides (Jacobson *et al.*, 1989; Storz *et al.*, 1989).

Among the reactive oxygen species, only O_2^- and H_2O_2 are specific substrates

for the antioxidative enzymes. Superoxide dismutase converts O_2^- to H_2O_2 which is subsequently reduced to water by the action of catalase or peroxidase in the following reactions (Fridovich, 1978).

SOD (EC 1.15.1.1)	$O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$
Catalase (EC 1.11.1.6)	$H_2O_2 + H_2O_2 \rightarrow 2H_2O + O_2$
Peroxidase (EC 1.11.1.7)	$H_2O_2 + RH_2 \rightarrow 2H_2O + R$

E. coli contains four SOD isoenzymes: one manganese containing enzyme (MnSOD encoded by *sodA*; Keele *et al.*, 1970), one iron containing enzyme (FeSOD encoded by *sodB*; Yost & Fridovich, 1973), and an iron-manganase hybrid enzyme (Fe-MnSOD; Clare *et al.*, 1984), and the recently discovered CuZnSOD (Benov *et al.*, 1995; Lee & Park, 1995; Battistoni & Rotilio, 1995). The Fe and Mn containing enzymes are dimers whereas the CuZnSOD is a monomer. MnSOD is primarily produced under aerobic conditions while FeSOD is produced under both aerobic and anaerobic conditions (Hassan & Fridovich, 1977). Although SOD is one of the most important antioxidative enzymes, *E. coli* mutants without SOD can grow aerobically (Schellhorn & Hassan, 1988).

In the peroxidase reaction, H_2O_2 is converted into water at a much slower rate using an organic or halide electron donor as substrate without the formation of O_2. Catalases that have peroxidase activity are referred to as catalase-peroxidases, and they are distinctly different from monofunctional peroxidases. *E. coli* is known to

produce two different catalases, a bifunctional catalase/peroxidase (hydroperoxidase I, HPI) (Claiborne & Fridovich, 1979) and a monofunctional catalase (HPII) (Claiborne *et al.*, 1979). Their mechanism of regulation will be discussed in detail below. *E. coli* mutants defective in either or both of them can grow normally under aerobic conditions (Loewen, 1984) and a mutant deficient in both SOD and catalase activities grew aerobically, but at a reduced rate relative to the wild type strain (Schellhorn & Hassan, 1988). Even though both enzymes are essential for efficient antioxidative defence, SOD was found to be more effective than catalase in relieving the effects of oxygen toxicity on growth and mutagenesis.

1.5.2. The postdamage defence system

The post damage system involves the use of DNA repair enzymes to remove the oxidized bases from the DNA directly. These enzymes include DNA polymerase I (*polA*; Hagensee & Moses, 1986), DNA polymerase III (*polC*; Hagensee *et al.*, 1987), exonuclease III (*xthA*; Demple *et al.*, 1983) and the *recA* gene product (Carlsson & Carpenter, 1980). The mutants defective in these proteins show increased sensitivity to H_2O_2 in the order of *polA* > *xthA* > *polC* > *recA* (Hagensee & Moses, 1989). The damage caused by H_2O_2 can be repaired by one of the two possible pathways. The first pathway involves the gene products of *xthA, polC, polA* while the second pathway requires only the *polA* gene product. The RecA protein probably is not involved directly in these processes (Hagansee & Moses, 1989). There also seems to be another line of defence to remove a mispaired adenine

opposite an oxidized guanine (8-oxo-G) during DNA replication (Ahern, 1993) which involves three enzymes: MutY, MutM, and MutT.

1.6. Catalases

1.6.1. Cellular location and physiological function of catalases

Catalase was one of the first enzymes described (Gottstein, 1893; Loew, 1901; Issajew, 1904), and is found in virtually all aerobic cells including animals (Deisseroth & Dounce, 1970; Schonbaum & Chance, 1976), plants (Esaka & Asahi, 1982) and microorganisms (Herbert & Pinsent, 1948; Clayton, 1959; Vainshtein *et al.*, 1981; Murshudov *et al.*, 1992). In mammalian tissues, catalase is mostly found in blood rich tissues such as liver and kidney and to lesser extent in connective tissues. It is localized mostly in the matrix of peroxisomes (de Duve & Baudhuin, 1966; de Duve, 1974; Lazarow & Fujiki, 1985) and to a small extent in the mitochondria (Neubert *et al.*, 1962; Radi *et al.*, 1991) of eukaryotic cells. However, in erythrocytes, that like prokaryotes are devoid of peroxisomes, it is located in the cytosol (Aebi *et al.*, 1967). Plant peroxisomes contain catalases in the polytubular core or nucleoids of the organelles as well as in the matrix (Tolbert, 1978). In most eukaryotic cells, cytoplasmically translated apomonomers of catalase are transported from the cytoplasm into peroxisomes where most of the cellular H_2O_2 is produced during oxidative metabolism (Lazarow & Fujiki, 1985). In prokaryotes, catalase is normally made and retained in the cytoplasm, although it can be transported into the periplasmic space of Gram-negative bacteria (Heimberger & Eisenstark, 1988).

Catalase removes potentially harmful H_2O_2 from aerobically grown cells, but its importance in the cell is unclear because the growth of *E. coli* (Loewen, 1984) and *B. subtilis* (Loewen & Switala, 1987) strains deficient in catalase is unaffected relative to wild type cells. Generally, H_2O_2 formed in the cell is thought to be eliminated either by a catalatic reaction when H_2O_2 levels are high or by a peroxidatic reaction when H_2O_2 levels are low. However, this picture does not explain the normal growth phenotype of mutants lacking both catalases. It is necessary to assume that H_2O_2 levels generated *in vivo* are low or that cellular H_2O_2 is eliminated by a combination of mechanisms as described in section 1.4 in order to prevent formation of even more potent oxygen species. In the latter argument, catalase becomes only a small, dispensable part of the antioxidative defence system of the cell. Nevertheless, catalase provides the cell with certain advantages including enhanced survival after treatment with H_2O_2 (Yonei *et al.*, 1987; Sammartano *et al.*, 1986; Abril & Pueyo, 1990; McCann *et al.*, 1991; Volkert *et al.*, 1994), enhanced survival in stationary phase (Mulvey *et al.*, 1990), reduced rates of spontaneous and H_2O_2-induced mutations (Abril & Pueyo, 1990), and decreased sensitivity to oxygen toxicity (Dingman & Stahly, 1984).

1.6.2. Phylogeny of catalases

Since all catalases catalyze the same reaction, it might be expected that they would have similar properties. Comparison of protein sequences has revealed

variations among amino acid sequences of catalases from different species as well as within the same organism. A phylogenetic analysis based on these amino acid sequence comparisons revealed three, possibly four classes of catalases (von Ossowski *et al.*, 1993), one each for fungal, animal and plant catalases while the limited number of bacterial catalases could not be classified as a monophyletic group. More recently, 70 different catalase sequences have been compared in a phylogenetic analysis (Loewen, unpublished) revealing separate groups of plant, animal and fungal enzymes, as well as two distinct groupings of bacterial enzymes and a separate group of large subunit enzymes. These results can be explained in terms of a limited number of gene duplication events and parallel evolution of these gene families.

1.6.3. Catalase reaction

The enzyme catalase removes H_2O_2 in a two step reaction. In the first step, compound I, an unstable intermediate with an oxygen atom bound to the ferryl iron ($O=Fe^{IV}$), and a molecule of H_2O are formed in a reaction between one molecule of H_2O_2 and the heme iron. In the second step, a second molecule of H_2O_2 reacts with compound I to form a molecule of H_2O and an oxygen molecule (Chance, 1954; Fita & Rossmann, 1985).

Step 1: $H_2O_2 + Fe^{III}\text{-Enzyme} \rightarrow H_2O + O=Fe^{IV}\text{-Enzyme (compound I)}$

Step 2: $H_2O_2 + \text{compound I} \rightarrow H_2O + O_2 + Fe^{III}\text{-Enzyme}$

This can be considered to be an oxidation-reduction reaction in which one molecule of H_2O_2 acts as an electron donor and the second acts as an electron acceptor. Catalysis proceeds at a very high rate with an estimated 10^7 molecules of H_2O_2 being broken down per mole of hematin per second (Nicholls & Schonbaum, 1952).

1.6.4. Typical properties of catalases

Catalases that have been characterized to date share properties first observed in the catalases from eukaryotes. They are often homotetramers (Schroeder *et al.*, 1969) with a subunit size of approximately 60 kDa (Tanford & Lovrien, 1962; Sund *et al.*, 1967). Each subunit contains a non-covalently bound heme prosthetic group (high-spin ferric protoporphyrin IX) (Maeda *et al.*, 1973) which can be readily extracted by acetone-HCl. Unlike other hemoproteins, the fifth coordination of the heme iron in catalase is occupied by a tyrosinate residue while the sixth coordination is free in the resting state enzyme (Reid *et al.*, 1981; Fita & Rossmann 1985; Sharma *et al.*, 1989). Catalases can be isolated by chromatography on DEAE-cellulose (Thorup *et al.*, 1964), on Sepharose (Esaka & Asahi, 1982) or by gel filtration (Aebi *et al.*, 1964). Catalase activity is unchanged over a broad pH range from 5 to 10 (Chance, 1952; Schonbaum & Chance, 1976), and catalases are resistant to harsh chemical and physical treatments including: ethanol-chloroform (Nadler *et al.*, 1986; Goldberg & Hochman, 1989a); SDS, urea and heat (Meir & Yagil, 1985; Loewen & Switala, 1986). A number of compounds inhibit catalase activity including: cyanide and azide that bind reversibly to the active site heme iron (Beyer & Fridovich, 1988); 3-amino-

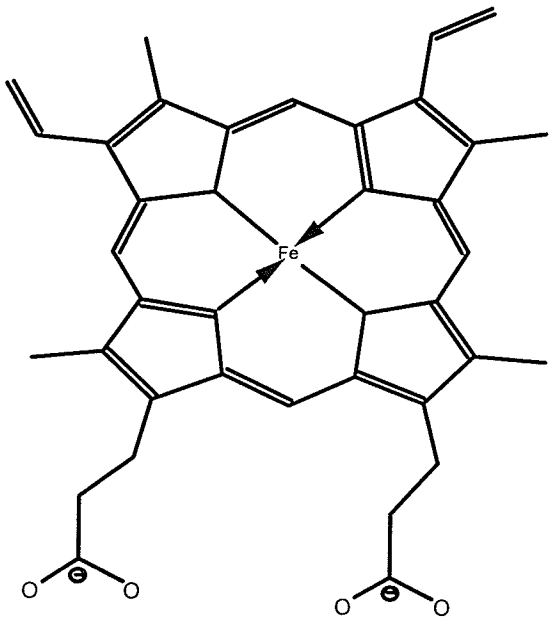
1,2,4-triazole which irreversibly forms a covalent bond with the active site His residue in the presence of H_2O_2 (Margoliash & Novogrodsky, 1958; Margoliash *et al.*, 1960; Chang & Schroeder, 1972); t-butyl hydroperoxide which acts as a suicide inhibitor through the formation of inactive compound II (Pichorner *et al.*, 1993); 3,3'-diaminobenzidine which works reversibly (Darr & Fridovich, 1985); thiol reagents which work indirectly through the formation of oxygen radicals (Takeda *et al.*, 1980); superoxide anion and H_2O_2 (Kono & Fridovich, 1982; Shimizu *et al.*, 1984; Kirkman & Gaetani, 1984; Lardinois, 1995). As mentioned earlier, the latter two compounds are also produced by pathogens like *M. pneumoniae* and cause oxidative damage in infected tissue.

A tightly bound NADPH has been detected in most typical catalases including those from human erythrocytes, bovine liver, dog (Kirkman & Gaetani, 1984), *Proteus mirabilis* (Jouve *et al.*, 1986 & 1989: Gouet *et al.*, 1995), *Micrococcus lysodeikticus (Yusifov et al., 1989)* and *Saccharomyces cerevisiae* (Hillar *et al.*, 1994). It has been suggested that NAD(P)H protects the enzyme from damage by preventing the accumulation of inactive compound II during the catalytic reaction (Kirkman *et al.*, 1987). Compound II is characterized spectrophotometrically with a reduced, red shifted Soret band and a peak at 565 nm. Glycolysation of catalases from mouse, rat and guinea pig (Pegg *et al.*, 1986) and from *Aspergillus niger* (Wasserman & Hultin, 1981; Kikuchi-Torii *et al.*, 1982) has also been reported.

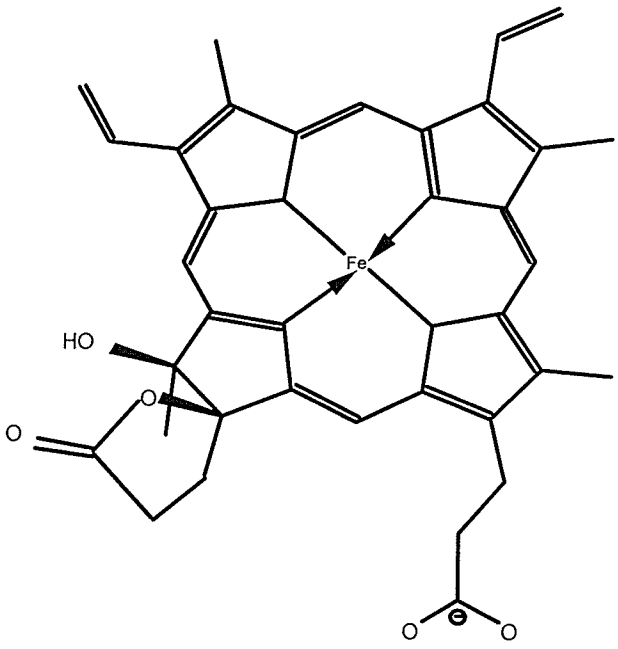
1.6.5. Deviations from the 'typical' properties and classification of bacterial catalases

The above characteristics are shared by most catalases but as more catalases have been characterized, an increasing diversity of properties has become apparent. Variations in the number and size of subunits, in the nature of the prosthetic group, in the presence of associated groups, and in the sensitivity to chemical agents have been reported. This has become particularly evident in bacteria which frequently produce more than one catalase. Bacterial catalases have recently been classified into four distinct groups based on their sequence and physical properties (Loewen, 1996). The first group includes 'typical' monofunctional catalases, both hexameric from *Bacillus subtilis* (Bol & Yasbin, 1991) and *Lactobacillus sake* (Knauf *et al.*, 1992), tetrameric from *Micrococcus luteus* (Herbert & Pinsent, 1948), *Brucella abortus* (Sha *et al.*, 1994), *Proteus mirabilis* (Jouva *et al.*, 1983 and 1984; Buzy *et al.*, 1995) and *Klebsiella pneumonia* (Hochman & Goldberg, 1991), and heterodimeric from *Pseudomonas syringae* (Klotz *et al.*, 1995). The second group includes monofunctional catalases with 'atypical' properties including larger molecular weights in excess of 80 kDa and a heme *d* group. The heme *d* from *E. coli* has recently been characterized as having *cis*-hydroxychlorin γ-spirolactone (Murshodove *et al.*, 1996) modification on ring III. The structures of heme *b* and heme *d* from HPII are shown in Figure 1.1. Examples of 'atypical' monofunctional catalases include the hexameric enzyme from *B. subtilis* (Loewen & Switala, 1988), HPII of *E. coli* (Claiborne *et al.*, 1979; Loewen & Switala, 1986, von Ossowski *et al.*, 1991), a dimeric catalase from

Figure 1.1. Structures of heme *b* (protoheme-IX) and heme *d* (*cis*-hydroxychlorin γ-spirolactone) (adapted from Loewen *et al.*, 1993).



Heme b



Heme d

K. pneumonia (Goldberg & Hochmann, 1989b) and one of the three catalases produced by *Bacillus firmus* (Hick, 1995). The third group includes catalases with an associated peroxidase activity from *Bacillus* YN-2000 (Yamoto *et al.*, 1990), *K. pneumonia* (Hochmann & Goldberg, 1991), *E. coli* (HPI) (Claiborne & Fridovich, 1979; Triggs-Raine *et al.*, 1988), *Mycobacterium smegmatis* (Marcinkeviciene *et al.*, 1995), *Rhodobacter capsulata* (Hochmann & Shemesh, 1987; Forkl *et al.*, 1993), *Halobacterium halobium* (Brown-Peterson & Salin, 1993), *Bacillus stearothermophilus* (Loprasert *et al.*, 1989), and *Mycobacterium tuberculosis* (Zhang *et al.*, 1992; Heym *et al.*, 1993; Nagy *et al.*, 1995). The fourth group includes catalases with non-heme redox centers that are resistant to the typical catalase inhibitors azide and cyanide. These include hexameric and tetrameric catalases from *Lactobacillus plantarum* (Kono & Fridovich, 1983) and *Thermoleophilum album* (Allgood & Perry, 1986), respectively.

1.6.6. Structure of 'typical' catalases

Catalase was one of the first enzymes to be crystallized, the bovine liver enzyme (BLC) was reported in 1937 (Sumner & Dounce, 1937) and the *Micrococcus lysodeikticus* enzyme was reported in 1948 (Herbert & Pinsent, 1948). The crystal structure of a number of catalases have now been solved including the enzymes from bovine liver (BLC), (Murthy *et al.*, 1981; Reid *et al.*, 1981; Melik-Adamyan *et al.*, 1986), *Penicillium vitale* (PVC) (Vainshtein *et al.*, 1986; Melik-Adamyan *et al.*, 1986; Murshudov *et al.*, 1996), *Micrococcus lysodeikticus* (Yusifov *et al.*, 1989;

Murshudov *et al.*, 1992), *Proteus mirabilis* (Gouet, 1993: Gouet *et al.*, 1995) and *E. coli* (HPII) (Bravo *et al.*, 1995).

BLC was the first structure to be solved at 2.5 Å resolution (Murthy *et al.*, 1981; Reid *et al.*, 1981) later resolved to 2.0 Å (Fita & Rossman, 1985). Each subunit contains 506 amino acids folded into four distinct domains. The N-terminal domain (residues 1-70) forms an extended arm from the globular core and interacts with the surrounding subunits. The core domain (residues 76 to 320) forms a β-barrel with two four-stranded, anti-parallel β-sheets (β_1 to β_4 and β_5 to β_8) intercalated with α-helices (α_3 to α_7). This domain includes active site residues His74, Ser113 and Asn147 and the fifth heme ligand Tyr357. The wrapping domain (residues 321-436) is mostly unordered with respect to secondary structure with the exception of the essential helix (α_9). The C-terminal domain (residues 437 to 506) contains four α-helices (α_{10} to α_{13}) on the surface of the molecule. The site of NADPH binding in BLC is located in the "hinge" region within the subunits and away from the active site and heme pocket (Kirkman & Gaetani, 1984; Fita & Rossman, 1985). The structure of BLC has been used to as a model to determine the structures of other catalases. Generally in all examined catalases, the heme group in the active site of the enzyme is situated at the bottom of a hydrophobic channel, about 20 Å from the surface.

1.7. Catalases of *Escherichia coli*

Two different catalases, HPI and HPII, are produced by *E. coli*. Purification and characterization of these enzymes revealed that they are very different from each

other catalytically, structurally, antigenically and in terms of expression (Claiborne *et al.*, 1979; Loewen *et al.*, 1985a).

1.7.1. Properties of HPI

HPI is a catalase-peroxidase encoded by *katG* located at 89.2 min on the *E. coli* chromosome (Loewen *et al.*, 1985b). It is a homotetramer of 726 amino acid (80,014 Da, Triggs *et al.*, 1988) subunits with two protoheme IX groups (Claiborne & Fridovich, 1979). Its pH optima for catalatic and peroxidatic activities are 7.5 and 6.5 respectively, and the apparent Km for H_2O_2 is 3.9 mM at pH 7.5. In non-denaturing gels, purified HPI separates into two isoenzymes: HPI-A and HPI-B (Loewen *et al.*, 1985a; Meir & Yagil, 1985), but the difference between the two forms is not known. Unlike typical catalases, HPI is insensitive to 3-amino 1,2,4-triazole and is heat-labile (Meir & Yagil, 1985). The primary sequence (Triggs-Raine *et al.*, 1988), physical and enzymatic properties of HPI are very similar to those of the *katG* gene products of *Salmonella typhimurium* (Loewen & Stauffer, 1990) and *Mycobacterium tuberculosis* (Zhang *et al.*, 1992) as well as to the catalase-peroxidase from *Bacillus stearothermophilus* (Loprasert *et al.*, 1989). HPI is synthesized primarily in exponential phase cells (Loewen *et al.*, 1985a) and its synthesis can be induced by H_2O_2 or ascorbate (Richter & Loewen, 1982; Loewen *et al.*, 1985a). Welinder (1991) has noted sequence similarities between HPI and plant peroxidases and has suggested a peroxidative origin for HPI. However, the plant peroxidases are smaller monomeric enzymes, suggesting that a gene duplication mechanism may have

been involved in HPI evolution. The crystal structure of HPI has not been solved.

1.7.1.1. Regulation of HPI synthesis

HPI is induced together with 29 other proteins when cells are exposed to high concentrations of H_2O_2 (Christman *et al.*, 1985; Morgan *et al.*, 1986). Nine of these 30 proteins, including HPI, alkyl hydroperoxidase reductase, and glutathione reductase were shown to be regulated directly by the *oxyR* gene product mapped at 89.6 min on the *E. coli* chromosome (Christman *et al.*, 1985; Morgan *et al.*, 1986; Storz & Tartaglia, 1992). OxyR, a 34 kDa protein, is a member of the LysR family of regulatory proteins that can act as either transcriptional activators or repressors and include the *E. coli* LysR, IlvY, CysB proteins, the *Salmonella typhimurium* MetR, and CysB proteins, the *Rhizobium* NodD protein, the *Enterobacter cloacae* AmpR protein, and the *Pseudomonas aeruginosa* TrpI protein (Christman *et al.*, 1989; Schell, 1993). The oxidation of OxyR acts as a sensor of environmentally induced oxidative stress to produce a transcriptional activator of genes involved in the protection against oxygen toxicity (Storz *et al.*, 1990). The addition of H_2O_2 to the medium or the formation of other reactive oxygen species from dissolved oxygen both result in the oxidation of OxyR and its conversion to a transcriptional activator (Storz *et al.*, 1990). OxyR also negatively regulates its own synthesis such that a five-fold increase of β-galactosidase from an *oxyR::lacZ* fusion was observed in a Δ*oxyR* mutant as compared to a wild type strain (Christman *et al.*, 1989). Although the overproduction of HPI alone is sufficient to overcome the harmful effects of H_2O_2

(Greenberg & Demple, 1988), the other 8 proteins controlled by OxyR must have protective roles, but they have not been well characterized. A mutation in the middle of the OxyR binding site upstream of *katG* caused overproduction of HPI (J.T. Greenberg *et al.*, unpublished results cited in Loewen, 1992) even in a Δ*oxyR* mutant suggesting that there may be another activator protein for *katG* expression. In addition to induction by OxyR, KatF or stationary phase sigma factor, σ^S also controls HPI expression in exponential phase cells but the significance of this observation is not clear (Ivanova *et al.*, 1994).

1.7.2. Properties of HPII

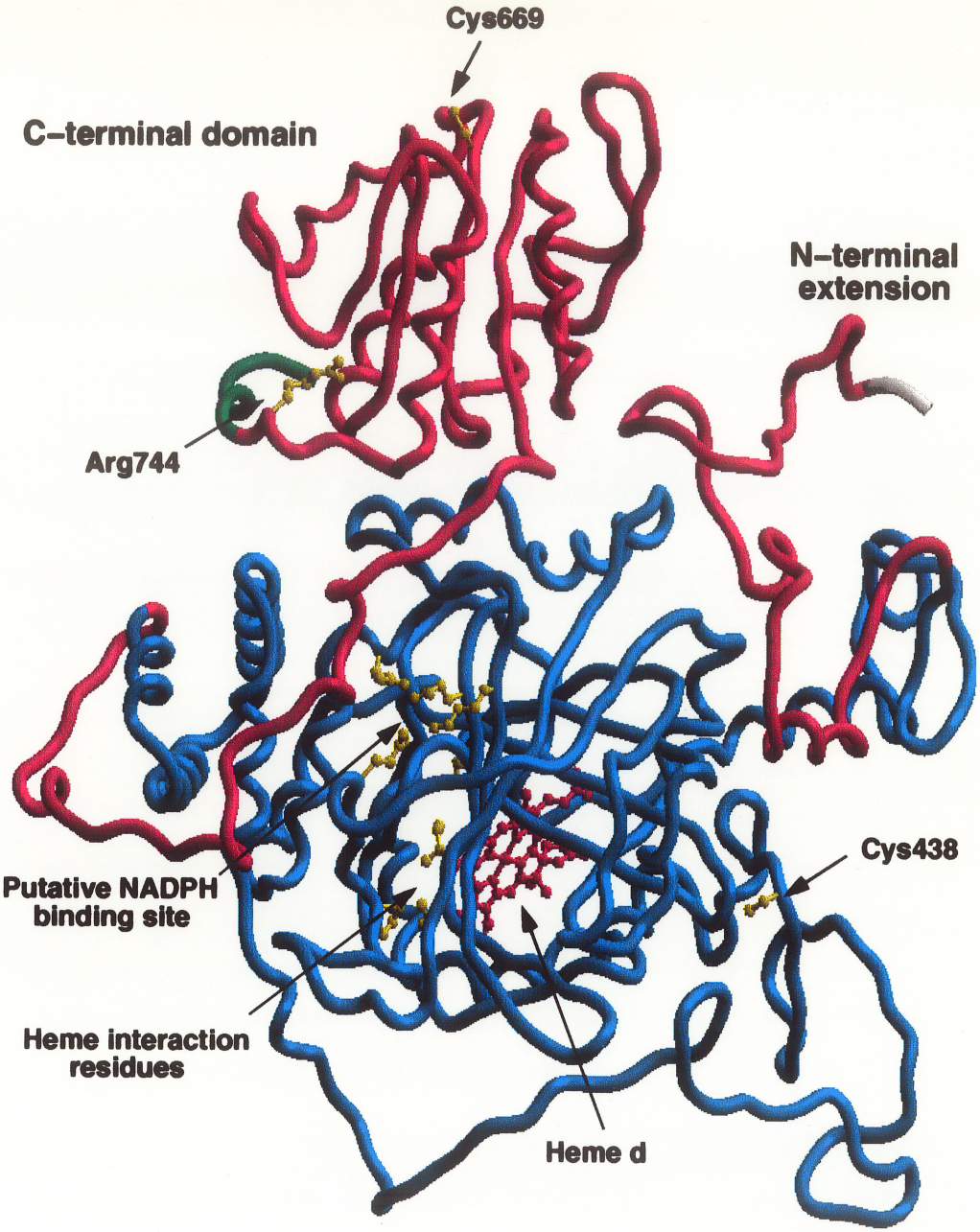
HPII is encoded by *katE* located at 37.8 min on the *E. coli* chromosome (Loewen, 1984), and regulated by σ^S (KatF) (Loewen & Triggs, 1984). Despite earlier evidence that the enzyme might be hexameric, the recent X-ray crystallographic analysis of HPII has revealed a tetrameric structure (Bravo *et al.*, 1995). The nucleotide sequence of *katE* predicts a protein of 753 residues with a calculated mass of 84.2 kDa (von Ossowski *et al.*, 1991). HPII contains a heme *d* derivative (Chiu *et al.*, 1989) that has recently been characterized as a *cis*-hydroxychlorin γ-spirolactone (Murshudov *et al.*, 1996) that is formed in a reaction catalyzed by HPII utilizing heme *b* and H_2O_2 as substrates (Loewen *et al.*, 1993). Like typical catalases, HPII is sensitive to 3-amino 1,2,4-triazole (Meir & Yagil, 1985; Loewen & Switala, 1986), is stable in 0.1% SDS or 7 M urea, at 70 °C (Meir & Yagil, 1985; Loewen & Switala, 1986) and is active over a broad pH range (pH 4-

11) with two optima at pH 6.8 (18.2 mM) and pH 10.5 (10 mM) (Meir & Yagil, 1985; Loewen & Switala, 1986). Unlike HPI, HPII is produced primarily in stationary phase, and in response to nutrient deprivation or growth on tricarboxylic acid cycle intermediates and is not induced by H_2O_2 or ascorbate (Loewen *et al.*, 1985a).

1.7.2.1. Structure of HPII

The crystal structure of HPII has recently been determined at 2.8 Å resolution by X-ray diffraction techniques (Bravo *et al.*, 1995). Figure 1.2. shows a cartoon of the subunit structure of HPII in which four domains are identified. The N-terminal domain contains residues 1 to 79 in a disordered structure other than two small α-helices involving residues 52-57 and 62-71. This domain is unique to HPII and it seems to function by providing closer associations between subunits and by blocking a lateral entrance to the active site. It is folded underneath the hinge or wrapping domain of adjacent subunit producing a very stable, tightly folded quaternary structure that may explain the enhanced stability of the protein (Bravo, 1996). The core domain from 80 to 434 is highly conserved among all catalases in both sequence and structure including the active site residues His128, Ser167, Asn201 and the fifth heme ligand Tyr415. A wrapping domain is formed by residues 434-600 in a structure similar to that of BLC, although extended in length, which serves as a link to the C-terminal domain and loops over top of the N-terminus. The remaining residues (600-753) at the C-terminus are contained in a structure unique to HPII and PVC that

Figure 1.2. Subunit structure of HPII highlighting the main features related to this study. The C-terminal and N-terminal extensions are colored in red while the core sequence that is conserved in all catalases is colored blue. The C-terminal residues are colored green. The side chains of various residues that are studied in this work are highlighted as is the heme (adapted from Bravo *et al.*, 1995).


Cys669
C–terminal domain
N–terminal extension
Arg744
Putative NADPH binding site
Cys438
Heme interaction residues
Heme d

contains four α-helices and eight β-strands. This domain resembles the flavodoxin domain found in a number of nucleotide binding enzymes. However, neither PVC nor HPII has been shown to bind nucleotides, and the function of this domain remains unknown. Although, it has been speculated that it may contribute to the enhanced stability of the enzyme under harsh chemical and physical conditions as well as interfering with compound II formation thereby making NADPH-binding redundant (Loewen, 1996).

The heme group in both HPII and PVC is modified to heme *d* and rotated 180 degrees (axially flipped) by comparison with the heme of BLC and other typical bacterial catalases (Murshudov *et al.*, 1996). The inversion of the heme is most likely controlled by making contact with the heme, and both PVC and HPII have similar residues in this role. In PVC, the contact residues are Ile41, Val209, Pro291 and Leu342, which are conserved in HPII as Ile114, Ile274, Pro356 and Leu407. These differ significantly from their bovine counterparts Met60, Ser216, Leu298 and Met349. The oxygen atoms of spirolactone and hydroxyl group on ring III are located on the proximal side of the heme opposite and at considerable distance from the active site residues (Loewen 1996) raising the question of how the modification is catalyzed.

HPII does not bind NADPH but two of the four residues involved in NADPH-binding in BLC are conserved in HPII as Arg260 and Lys294. The other two residues are changed to Glu270 from Asp and Glu362 from His. The potential NADPH-binding domain is remote from the active site, closer to the surface with the

four residues of HPII being in a similar spatial relationship as the corresponding residues from BLC. Minor differences are apparent however including the absence of NADPH in HPII where the resulting void is filled by closer interaction of the oppositely charged side chains of Lys/Arg and Glu/Glu.

HPII contains only two cysteine residues, C438 and C669. The residue C438 is found to be conserved among catalases from different species, including bovine liver catalase (von Ossowski *et al.*, 1993) and C438 is situated on the surface of the enzyme, on the opposite side of the subunit from the entrance to the channel leading to the active site (Bravo *et al.*, 1995). Residue C669 is not conserved and is located in the C-terminal domain over 63 Å distant from C438. Both residues are located on the surface of the subunit but there is no evidence for disulfide linkage.

1.7.2.2. Regulation of HPII synthesis

HPII is produced together with about 54 other proteins in stationary phase and in response to starvation (Matin, 1990; Lange & Hengge-Aronis, 1991a,b). The synthesis of HPII and 31 other proteins (McCann *et al.*, 1991) is under the control of the *rpo*S gene mapping at 59 min on the *E. coli* chromosome (Loewen & Triggs, 1984). Sequence analysis of the *rpoS* gene revealed a 1,086 bp open reading frame encoding a 41.5 kDa protein with sequence similarity to known sigma transcription factors (Mulvey & Loewen, 1989).

When *E. coli* was grown in minimal medium, both *katE* and *rpoS* were induced in exponential phase whereas in rich medium, *katE* was expressed maximally

only in stationary phase, and *rpoS* was turned on and increased gradually during exponential phase into stationary phase (Mulvey *et al.*, 1990). In addition to starvation causing induction of *katE* and *rpoS*, weak acids, like acetate and benzoate, had a similar effect in minimal medium (Schellhorn & Stones, 1992) but not in rich medium (Mulvey *et al.*, 1990). Strains defective in RpoS protein died off faster during incubation in stationary phase and during starvation (Mulvey *et al.*, 1990; McCann *et al.*, 1991) suggesting that RpoS protein is a key factor in the activation of genes that are essential for adaptation to nutrient depletion and survival during dormancy.

Although RpoS was required for *katE* expression, a low level of *rpoS* expression during exponential phase was not enough to turn on *katE*. Furthermore, in stationary phase when *rpoS* expression reached its maximum, only one third of the maximum synthesis of KatE was achieved while the rest was synthesized in the late stationary phase (Mulvey *et al.*, 1990). These findings suggest that there may be some other factor(s) required for *katE* expression although one has never been identified.

Proton motive force may play a role in *rpoS* expression because inhibition of electron transport and reduction of the pH gradient across the membrane with uncouplers was found to prevent expression (Loewen, 1992). Conversely, in rich medium, the uncouplers turned on the *rpoS* expression suggesting that the cells in rich medium had an optimally charged membrane that normally prevented *katF* expression. As starvation took place in late stationary phase a drop in the membrane potential

occurred which caused the turn on of *rpoS* expression.

RpoS (σ^S) acts as an alternate sigma factor to regulate the expression of a group of genes with diverse functions, including protection against DNA damage, cell morphology, virulence mediation, osmoprotection, thermotolerance, glycogen synthesis and many others that are reviewed in detail by Loewen & Hengge-Aronis (1994). The synthesis of σ^S is regulated by several factors, including ppGpp (Gentry *et al.*, 1993; Lange *et al.*, 1995), homoserine lactone (Huisman & Kolter, 1994), UDP-glucose (Bochreinger *et al.*, 1995), histone-like protein H-NS (Barth *et al.*, 1995).

1.8. Object of the thesis

The goal of this project is to study the structure-function relationship in HPII catalase of *Escherichia coli* by changing key residues using site-directed mutagenesis. Earlier work by I. von Ossowski (1993) had targeted His-128, Asn-201, Ser-167 and Tyr-415 in the active site. This project has continued this study by focusing on a large number of residues, including the presumptive NADPH binding counterparts of BLC, the cysteine residues, the long C-terminus, the heme binding residues and the channel leading to the active site. It is hoped that this project will broaden our understanding of the role of individual amino acids in the topography of proteins with respect to catalysis.

2. MATERIALS AND METHODS

2.1. *Escherichia coli* strains, plasmids and bacteriophage

The list of the *E. coli* strains, plasmids and bacteriophage are given in Table 2.1. The *katE* gene encoding HPII catalase was originally cloned into pKS+ (Stratagene) to generate the plasmid pAMkatE72 (von Ossowski *et al.*, 1991). Strain CJ236 harboured plasmid clone pAMkatE72 and the subclones pKS$^-$H-E and pKS$^+$E-C (Figure 2.1), and was used for generation of the uracil-containing single-stranded DNA employed in site-directed mutagenesis. Strain NM522 was used for most cloning, plasmid maintenance and production of single-stranded DNA for sequencing. Helper phage R408 was used for the infection of strains CJ236 and NM522 to generate single stranded DNA. Strain UM255 was used for expression of catalase proteins from various plasmids. Strain JM109 was used for production of plasmid DNA for double-strand DNA sequencing.

2.2. Media, growth conditions and storage of cultures

E. coli cultures were routinely grown in LB (Luria-Bertani) medium that contained 10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl. Solid media contained 14 g/L agar. Ampicillin was added to 250 μg/ml for the growth of the plasmid-hosting cells. In addition, chloramphenicol was added to 40 μg/ml to maintain the presence of F' episome for the growth of strain CJ236. Cultures were grown at 37°C or 28°C. *E. coli* cultures were normally stored at -70°C in 8% (v/v) dimethyl

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

	Genotype		Source
Strains			
CJ236	*dut*1 *ung*1 *thi*-1 *rel*A1/pCJ105/·*cam*^{r}F'		Kunkel *et al.*, 1987
NM522	*sup*E Δ(*lac-pro*AB) *hsd*-5 [F' *pro*AB *lac*I^q *lac*ZΔ15]		Mead *et al.*, 1985
UM255	*pro leu rps*L *hsd*M *hsd*R *end*I *lac*Y *kat*G2 *kat*E12::Tn*10* *rec*A		Mulvey *et al.*, 1988
JM109	*rec*A1 *sup*E44 *end*A1 *hsd*R17 *gyr*A96 *rel*A1 *thi* Δ(*lac-pro*AB)		Yanisch-Perron *et al.*, 1985
Plasmids			
pAMkatE72 (pKS$^+$P-C, *katE* clone)		Amp^r	von Ossowski *et al.*, 1991
pKS$^-$E-H (subclone II)		Amp^r	von Ossowski *et al.*, 1991
pKS$^+$E-C (subclone III)		Amp^r	von Ossowski *et al.*, 1991
Bacteriophage			
R408 (helper phage)			Stratagene Cloning Systems

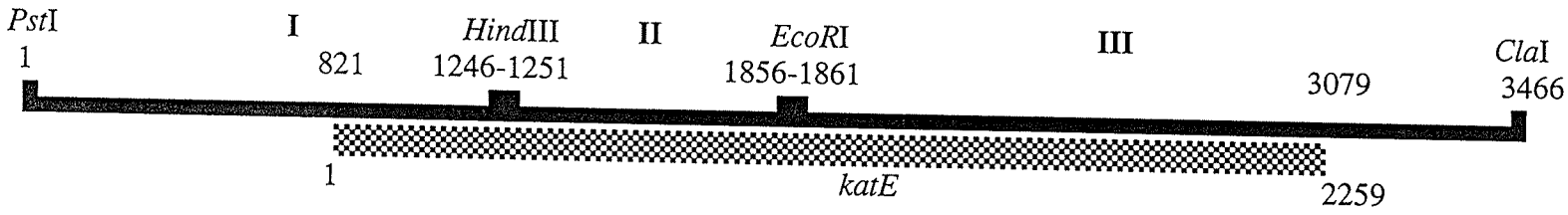


Figure 2.1. Simplified restriction map of the cloned 3466 bp chromosomal fragment in pAMKatE72. Three subclones are shown: **I,** pKS$^+$P-H; **II**, pKS$^-$H-E; **III**, pKS$^+$E-C. The 2259 nucleotide long *katE* open reading frame (▒) is shown as a part of the chromosomal insert (▬). (redrawn from von Ossowski *et al.*, 1991).

sulfoxide. Bacteriophage R408 was stored at +4°C in culture supernatant.

2.3. Site-directed mutagenesis strategy

Targeted base changes on *katE* were generated according to the *in vitro* mutagenesis protocol described by Kunkel *et al.* (1987). A simple restriction map of *katE* that also indicates the locations of subclones is shown in Figure 2.1. The subclones rather than the whole gene were mutagenized to limit the amount of sequencing needed for mutant characterization. Target bases for mutagenesis were selected from the DNA sequence of *katE* shown in Figure 2.2. The sequences of oligonucleotides used in mutagenesis construction are listed in Table 2.2. Mutagenesis was performed by annealing the phosphorylated oligonucleotides to the uracil-containing single-stranded DNA obtained from the appropriate Bluescript phagemid subclone, and synthesizing the complementary strand *in vitro* with unmodified T7 DNA polymerase (New England Biolabs) and T4 DNA ligase (Pharmacia). The DNA was subsequently transformed into NM522 where the uracil-containing DNA strand was degraded by host enzymes. Potential mutant subclones were screened for the presence of the desired mutation by sequence analysis. Once the correct mutant was identified, the complete sequence of the subclone was determined in order to ensure that no other base changes had occurred. Mutant subclones were then used to reconstruct the complete *katE* gene, which was transformed into UM255 for determination of enzyme activity and visualization of protein in crude extracts by SDS-PAGE. Clones that expressed significant levels of

Figure 2.2. The DNA sequence of *katE* within the chromosomal insert in pAMkatE72. The sequences of the mutagenic and sequencing primers and the restriction enzymes used in cloning are also shown. The sites for the putative promoter sequences (**-35** and **-10**), the transcriptional start (**G**), the potential Shine-Dalgarno (**S.D.**) ribosome binding and the potential transcriptional termination (**T.T.**) are underlined. (data modified and revised from von Ossowski *et al.*, 1991)

Pst I
CTGCAGCCTTTCTTTAAAAGAGTCGAAAGCCAGGCTTTTAATATTTAAATCACCATAATT 60

ACTCTGTATTAAGTTTGTAGAAAACATCTCCCGCCTCATATTGTTAACAAAATTATTATC 120

TCATTTAAATCTAAGTCATTTACAATATAAGTTTAAGAGCGACGCCACAGGATGAACTAT 180

CAAAAATAGCTCATCATGATTAGCAAAACTTAACCATTTTAAAATAAATAAACAATTAAA 240

GAAAAAAGATCACTTATTTATAGCAATAGATCGTCAAAGGCAGCTTTTTGTTACAGGTGG 300

TTTGAATGAATGTAGCAACGAAATACAGAATTTCAGGTCATGTAACTCCCGGCAAACCGG 360

GAGGTATGTAATCCTTACTCAGTCACTTCCCCTTCCTGGCGGATCTGATTTGCCCAACGT 420

TGGGCAGATTCAGGCACAGTAAACGCCGGTGAGCGCAGAAATGACTCTCCCATCAGTACA 480

AACGCAACATATTTGCCACGCAGCATCCAGACATCACGAAACGAATCCATCTTTATCGCA 540

TGTTCTGGCGGCGCGGGTTCCGTGCGTGGGACATAGCTAATAATCTGGCGGTTTTGCTGG 600

CGGAGCGGTTTCTTCATTACTGGCTTCACTAAACGCATATTAAAAATCAGAAAAACTGTA 660

GTTTAGCCGATTTAGCCCCTGTACGTCCCGCTTTGCGTGTATTTCATAACACCGTTTCCA 720
-35 -10

GAATAGTCTCCGAAGCGGGATCTGGCTGGTGGTCTATAGTTAGAGAGTTTTTTGACCAAA 780

ACAGCGGCCCTTTCAGTAATAAATTAAGGAGACGAGTTCA ATG TCG CAA CAT AAC 835
S.D. *met ser gln his asn*
1

GAA AAG AAC CCA CAT CAG CAC CAG TCA CCA CTA CAC GAT TCC AGC 880
glu lys asn pro his gln his gln ser pro leu his asp ser ser 20

GAA GCG AAA CCG GGG ATG GAC TCA CTG GCA CCT GAG GAC GGC TCT 925
glu ala lys pro gly met asp ser leu ala pro glu asp gly ser 35

CAT CGT CCA GCG GCT GAA CCA ACA CCG CCA GGT GCA CAA CCT ACC 970
his arg pro ala ala glu pro thr pro pro gly ala gln pro thr 50

GCC CCA GGG AGC CTG AAA GCC CCT GAT ACG CGT AAC GAA AAA CTT 1015
ala pro gly ser leu lys ala pro asp thr ala pro gly ser leu 65

AAT TCT CTG GAA GAC GTA CGC AAA GGC AGT GAA AAT TAT GCG CTG 1060
asn ser leu glu asp val arg lys gly ser glu asn tyr ala leu 80

```
ACC ACT AAT CAG GGC GTG CGC ATC GCC GAC GAT CAA AAC TCA CTG   1105
thr thr asn gln gly val arg ile ala asp asp gln asn ser leu 95


 ----------------------> C
CGT GCC GGT AGC CGT GGT CCA ACG CTG CTG GAA GAT TTT ATT CTG   1150
arg ala gly ser arg gly pro thr leu leu glu asp phe ile leu 110


CGC GAG AAA ATC ACC CAC TTT GAC CAT GAG CGC ATT CCG GAA CGT   1195
arg glu lys ile thr his phe asp his glu arg ile pro glu arg 125


ATT GTT CAT GCA CGC GGA TCA GCC GCT CAC GGT TAT TTC CAG CCA   1240
ile val his ala arg gly ser ala ala his gly tyr phe gln pro 140

      Hind III
TAT AAA AGC TTA AGC GAT ATT ACC AAA GCG GAT TTC CTC TCA GAT   1285
tyr lys ser leu ser asp ile thr lys ala asp phe leu ser asp 155

                                                  TGT (V169C)
                                    ------------------->
CCG AAC AAA ATC ACC CCA GTA TTT GTA CGT TTC TCT ACC GTT CAG   1330
pro asn lys ile thr pro val phe val arg phe ser thr val gln 170

------->                            ----------------------> D
GGT GGT GCT GGC TCT GCT GAT ACC GTG CGT GAT ATC CGT GGC TTT   1375
gly gly ala gly ser ala asp thr val arg asp ile arg gly phe 185

GCC ACC AAG TTC TAT ACC GAA GAG GGT ATT TTT GAC CTC GTT GGC   1420
ala thr lys phe tyr thr glu glu gly ile phe asp leu val gly 200

          ----------------------> D2
AAT AAC ACG CCA ATC TTC TTT ATC CAG GAT GCG CAT AAA TTC CCC   1465
asn asn thr pro ile phe phe ile gln asp ala his lys phe pro 215


GAT TTT GTT CAT GCG GTA AAA CCA GAA CCG CAC TGG GCA ATT CCA   1510
asp phe val his ala val lys pro glu pro his trp ala ile pro 230


CAA GGG CAA AGT GCC CAC GAT ACT TTC TGG GAT TAT GTT TCT CTG   1555
gln gly gln ser ala his asp thr phe trp asp tyr val ser leu 245

                                              (R260A) GCC
                                          ------------->
CAA CCT GAA ACT CTG CAC AAC GTG ATG TGG GCG ATG TCG GAT CGC   1600
gln pro glu thr leu his asn val met trp ala met ser asp arg 260

     --------------------> E
                      (E270D) GAT
                  ------------------------->
                                       (I274S) AGT
----------->                        ------------------->
GGC ATC CCC CGC AGT TAC CGC ACC ATG GAA GGC TTC GGT ATT CAC   1645
gly ile pro arg ser tyr arg thr met glu gly phe gly ile his 275

------->                                                   -
ACC TTC CGC CTG ATT AAT GCC GAA GGG AAG GCA ACG TTT GTA CGT   1690
thr phe arg leu ile asn ala glu gly lys ala thr phe val arg 290
```

(K294A) GCA

```
---------------------------->
TTC CAC TGG AAA CCA CTG GCA GGT AAA GCC TCA CTC GTT TGG GAT   1735
phe his trp lys pro leu ala gly lys ala ser leu val trp asp 305

GAA GCA CAA AAA CTC ACC GGA CGT GAC CCG GAC TTC CAC CGC CGC   1780
glu ala gln lys leu thr gly arg asp pro asp phe his arg arg 320

GAG TTG TGG GAA GCC ATT GAA GCA GGC GAT TTT CCG GAA TAC GAA   1825
glu leu trp glu ala ile glu ala gly asp phe pro glu tyr glu 335

                                        ----------------------> F
                                           EcoR I
CTG GGC TTC CAG TTG ATT CCT GAA GAA GAT GAA TTC AAG TTC GAC   1870
leu gly phe gln leu ile pro glu glu asp glu phe lys phe asp 350
```

CTA (P356L)

```
          --------------------------->          CAC (E362H)
                                      ---------------------------->
TTC GAT CTT CTC GAT CCA ACC AAA CTT ATC CCG GAA GAA CTG GTG   1915
phe asp leu leu asp pro thr lys leu ile pro glu glu leu val 365

CCC GTT CAG CGT GTC GGC AAA ATG GTG CTC AAT CGC AAC CCG GAT   1960
pro val gln arg val gly lys met val leu asn arg asn pro asp 380

AAC TTC TTT GCT GAA AAC GAA CAG GCG GCT TTC CAT CCT GGG CAT   2005
asn phe phe ala glu asn glu gln ala ala phe his pro gly his 395

                                          ATG (L407M)
                                      ---------------------------->
ATC GTG CCG GGA CTG GAC TTC ACC AAC GAT CCG CTG TTG CAG GGA   2050
ile val pro gly leu asp phe thr asn asp pro leu leu gln gly 410

CGT TTG TTC TCC TAT ACC GAT ACA CAA ATC AGT CGT CTT GGT GGG   2095
arg leu phe ser tyr thr asp thr gln ile ser arg leu gly gly 425

                                                  GCC (C438A)
            G                          TCC (C438S)
          ---------------------->    ---------------------->
CCG AAT TTC CAT GAG ATT CCG ATT AAC CGT CCG ACC TGC CCT TAC   2140
pro asn phe his glu ile pro ile asn arg pro thr cys pro tyr 440

--->                          SphI
CAT AAT TTC CAG CGT GAC GGC ATG CAT CGC ATG GGG ATC GAC ACT   2185
his asn phe gln arg asp gly met his arg met gly ile asp thr 455

AAC CCG GCG AAT TAC GAA CCG AAC TCG ATT AAC GAT AAC TGG CCG   2230
asn pro ala asn tyr glu pro asn ser ile asn asp asn trp pro 470

CGC GAA ACA CCG CCG GGG CCG AAA CGC GGC GGT TTT GAA TCA TAC   2275
arg glu thr pro pro gly pro lys arg gly gly phe glu ser tyr 485
```

```
CAG GAG CGC GTG GAA GGC AAT AAA GTT CGC GAG CGC AGC CCA TCG   2320
gln glu arg val glu gly asn lys val arg glu arg ser pro ser 500

                                                  ---------->
TTT GGC GAA TAT TAT TCC CAT CCG CGT CTG TTC TGG CTA AGT CAG   2365
phe gly glu tyr tyr ser his pro arg leu phe trp leu ser gln 515

---------> H
ACG CCA TTT GAG CAG CGC CAT ATT GTC GAT GGT TTC AGT TTT GAG   2410
thr pro phe glu gln arg his ile val asp gly phe ser phe glu 530

TTA AGC AAA GTC GTT CGT CCG TAT ATT CGT GAG CGC GTT GTT GAC   2455
leu ser lys val val arg pro tyr ile arg glu arg val val asp 545

CAG CTG GCG CAT ATT GAT CTC ACT CTG GCC CAG GCG GTG GCG AAA   2500
gln leu ala his ile asp leu thr leu ala gln ala val ala lys 560

AAT CTC GGT ATC GAA CTG ACT GAC GAC CAG CTG AAT ATC ACC CCA   2545
asn leu gly ile glu leu thr asp asp gln leu asn ile thr pro 575

CCT CCG GAC GTC AAC GGT CTG AAA AAG GAT CCA TCC TTA AGT TTG   2590
pro pro asp val asn gly leu lys lys asp pro ser leu ser leu 590

                      I ----------------------> TGA (V603STOP)
                                  ---------------------->
TAC GCC ATT CCT GAC GGT GAT GTG AAA GGT CGC GTG GTA GCG ATT   2635
tyr ala ile pro asp gly asp val lys gly arg val val ala ile 605

-->
TTA CTT AAT GAT GAA GTG AGA TCG GCA GAC CTT CTG GCC ATT CTC   2680
leu leu asn asp glu val arg ser ala asp leu leu ala ile leu 620

AAG GCG CTG AAG GCC AAA GCG[1] GTT CAT GCC AAA CTG CTC TAC TCC   2725
lys ala leu lys ala lys ala  val his ala lys leu leu tyr ser 635

CGA ATG GGT GAA GTG ACT GCG GAT GAC GGT[2] ACG GTG TTG CCT ATA   2770
arg met gly glu val thr ala asp asp gly  thr val leu pro ile 650

GCC GCT ACC TTT GCC GGT GCA CCT TCG CTG ACG GTC GAT GCG GTC   2815
ala ala thr phe ala gly ala pro ser leu thr val asp ala val 665

            GCC (C669A)
            TCC (C669S)
-------------------------->
ATT GTC CCT TGC GGC AAT ATC GCG GAT ATC GCT GAC AAC GGC GAT   2860
ile val pro cys gly asn ile ala asp ile ala asp asn gly asp 680

            ----------------------> J
GCC AAC TAC TAC CTG ATG GAA GCC TAC AAA CAC CTT AAA CCG ATT   2905
ala asn tyr tyr leu met glu ala tyr lys his leu lys pro ile 695

GCG CTG GCG GGT GAC GCG CGC AAG TTT AAA GCA ACA ATC AAG ATC   2950
ala leu ala gly asp ala arg lys phe lys ala thr ile lys ile 710
```

```
GCT GAC CAG GGT GAA GAA GGG ATT GTG GAA GCT GAC AGC GCT GAC   2995
ala asp gln gly glu glu gly ile val glu ala asp ser ala asp 725

                                                      ------>
GGT AGT TTT ATG GAT GAA CTG CTA ACG CTG ATG GCA GCA CAC CGC   3040
gly ser phe met asp glu leu leu thr leu met ala ala his arg 740

                                (K750STOP)
                        T AAG ...     TAA     ... GC
                        ---------------------------->
                      (K747ST)
              CGC ...     TAG     ... AAA
              ---------------------------->
                    (P746ST)
          TCA ...     TGA     ... GAC
          ---------------------------->
                (I745ST)
      TGG ...     TAA     ... ATT
      ---------------------------->
            (R744K/I745ST)
GTG ...     AAA TAA ... AAG
---------------------------->
            (R744K)
GTG ...     AAA     ... AAG
---------------------------->
            (R744A/I745ST)
GTG ...     GCC TAA ... AAG
---------------------------->
            (R744A)
GTG ...     GCC     ... AAG
---------------------------->
            (R744ST)
GTG ...     TGA     ... AAG
---------------------------->
  (W742ST)
    TAG
---------------------->
GTG TGG TCA CGC ATT CCT AAG ATT GAC AAA ATT CCT GCC TGATGGG   3086
val trp ser arg ile pro lys ile asp lys ile pro ala 753

AGCGCGCAATTGCGCCGCCTCAATGATTTACATAGTGCGCTTTGTTTATGCCGGATGCGC   3146

GTGAACGCCTTATCCGGCCTACAAAACTGTGCAAATTCAATATATTGCAGGAAACACGTA   3206

GGCCTGATAAGCGAAGCCATCAGGCAGTTTTGCGTTTGTCAGCAGTCTCAAGCGGCGGCA   3266
  T.T.              T.T.

GTTACGCCGCCTTTGTAGGAATTAATCGCCGGATGCAAGGTTCACGCCGATCTGGCAAAC   3326

ATCCTCACTTACACATCCCGATAACTCCCCAACCGATAACCACGCTGAGCGATAGCACCT   3386

TTCAACGACGCTGATGTCAACACATCCAGCTCCGTTAAGCGTGGGAAACAGTAAGCACTC   3446

                Cla I
TGACGGATAGTATTATCGAT   3466
```

[1]GCG (*ala*) instead of GGC (*gly*) and [2]GGT (*gly*) instead of GGA (*gly*) were observed.

Table 2.2. Sequences of the oligonucleotides used for site-directed mutagensis of *katE*.

Primer name	Position within *katE*	Oligonucleotide sequence	Subclone mutagenized
V169C (GTT → TGT)	1316-1336	5'-TTCTCTACC**TGT**CAGGGTGGT	II
R260A (CGC → GCC)	1591-1609	5'-GATGTCGGAT**GCC**GGCATCCCC	II
E270D (GAA → GAT)	1619-1639	5'-CGCACCATC**GAT**GGCTTCGGT	II
I274S (ATT → AGT)	1631-1651	5'-GGCTTCGGT**AGT**CACACCTTC	II
K294A (AAA → GCA)	1690-1711	5'-TTTCCACTGG**GCA**CCACTGGCA	II
P356L (CCA → CTA)	1877-1894	5'-CTTCTCGAT**CTA**ACCAAACTT	III
E362H (GAA → CAC)	1894-1915	5'-CTTATCCCG**CAC**GAACTGGTG	III
L407M (CTG → ATG)	2030-2050	5'-AACGATCCG**ATG**TTGCAGGGA	III
C438A (TGC → GCC)	2123-2143	5'-CGTCCGACC**GCC**CCTTACCAT	III
C438S (TGC → TCC)	2123-2143	5'-CGTCCGACC**TCC**CCTTACCAT	III
V603Stop (GTA → TGA)	2618-2638	5'-GGTCGCGTG**TGA**GCGATTTTA	III
C669A (TGC → GCC)	2816-2836	5'-ATTGTCCCT**GCC**GGCAATATC	III
C669S (TGC → TCC)	2816-2836	5'-ATTGTCCCT**TCC**GGCAATATC	III
W742Stop (TGG → TAG)	3035-3055	5'-CACCGCGTG**TAG**TCACGCATT	III
R744Stop (CGC → TGA)	3041-3061	5'-GTGTGGTCA**TGA**ATTCCTAAG	III
R744A (CGC → GCC)	3041-3061	5'-GTGTGGTCA**GCC**ATTCCTAAG	III
R744A/I745Stop (CGC → GCC/ATT → TAA)	3041-3061	5'-GTGTGGTCA**GCCTAA**CCTAAG	III
R744K (CGC → AAA)	3041-3061	5'-GTGTGGTCA**AAA**ATTCCTAAG	III
R744K/I745Stop (CGC → AAA/ATT → TAA)	3041-3061	5'-GTGTGGTCA**AAATAA**CCTAAG	III
I745Stop (ATT → TAA)	3044-3064	5'-TGGTCACGC**TAA**CCTAAGATT	III
P746Stop (CCT → TGA)	3047-3067	5'-TCACGCATT**TGA**AAGATTGAC	III
K747Stop (AAG → TAG)	3050-3070	5'-CGCATTCCT**TAG**ATTGACAAA	III
K750Stop (AAA → TAA)	3058-3078	5'-TAAGATTGAC**TAA**ATTCCTGC	III

HPII-like protein were then grown in large scale (4-8 liters) for purification and characterization of the enzyme.

2.3.a. DNA isolation and purification

Plasmid DNA was isolated according to Sambrook *et al.* (1989). The protocol was modified as follows. Plasmid containing cells from a 3-5 ml culture were pelleted by centrifugation and resuspended in a buffer containing 50 mM Tris, pH 7.5, 10 mM Na-EDTA and 100 μg/ml RNAse. The cells then lysed in 1% SDS (w/v) and 0.2 M NaOH solution and neutralized with 2.55 M potassium acetate, pH 4.8. After 10-15 min incubation at room temperature, the mixture was centrifuged twice to eliminate all the pellet. The supernatant was then precipitated in isopropanol and washed 3 times with 70% (v/v) ethanol. The pellet was resuspended in HPLC grade distilled water and stored at -20 °C until further use.

Single-stranded DNA for site-directed mutagenesis and sequencing of DNA was isolated and purified according to Vieira & Messing (1987). Plasmid containing cells from a 5 ml culture in exponential phase was infected with 10-20 μl of helper phage (10^{11}-10^{12} per ml) and grown overnight. After harvesting by centrifugation, a solution of 300 μl of 1.5M NaCl and 20% PEG (Polyethylene glycol, 6000) was added for per ml of supernatant and mixed. The mixture was incubated at room temperature for 15 min and centrifuged to pellet the phage particles. The pellet was resuspended in TE buffer (10 mM Tris, pH 8.0 and 1 mM Na-EDTA). The mixture was extracted first with an equal volume of buffer-saturated phenol and then with an

equal volume of water-saturated chloroform. Then, with an equal volume of 7.5 M ammonium acetate, pH 7.5 plus 4 volumes of ice-cold absolute ethanol were added and the mixture was kept at -20°C for 30 min. Single-stranded DNA was precipitated by centrifugation and washed 3 times with 70% (v/v) ethanol. The dried pellet was kept at -20°C until further use.

DNA bands excised from agarose electrophoresis gels were recovered and purified using the GENECLEAN DNA extraction kit (Bio/Can Scientific Inc.).

2.3.b. Preparation of synthetic oligonuclotides

Mutant oligonucleotides were synthesized using a PCR-Mate DNA synthesizer (Applied Biosystems Inc.). Extraction of the oligonucleotides from the cartridges was carried out using 0.8 ml concentrated ammonium hydroxide. The extracted solution was incubated at 55°C overnight, lyophilized, resuspended in HPLC grade distilled water and stored at -20 °C until further use. Concentration of DNA was determined spectrophotometrically using the conversion of 1 absorbance unit at 260 nm = 40 μg/ml single-stranded DNA (Sambrook *et al.*, 1989).

Oligonucleotides used for site-directed mutagenesis were phosphorylated at the 5' end using T4 kinase (GIBCO-BRL) according to the method of Ausubel *et al.* (1989). Approximately 100 ng of single-stranded DNA in 25 μl, 1 mM ATP and 10 u of T4 kinase were incubated in the manufacturer's buffer at 37°C for 30 min. T4 kinase was inactivated by incubation at 65°C for 5 min.

2.3.c. Transformation

Transformation of *E. coli* cells with the various plasmids was carried out according to the method of Chung *et al.* (1989). Exponential phase cells were harvested and made competent by resuspending in ice-cold 0.1 M calcium chloride for 15 min. DNA was added followed by incubation for 30-45 min and heat-shock at 42°C for 90 sec. The cells were then added to 0.5 ml LB medium and incubated at 37°C for 1 h without aeration. The mixture was plated on ampicillin-containing LB plates.

2.3.d. Restriction endonuclease digestion of DNA

Plasmid DNAs were digested with restriction endonucleases (GIBCO-BRL) in the manufacturer's suggested buffer. All reactions were performed in 10-20 μl volumes with 0.5-2.0 μg DNA which were incubated for 1-3 h at 37°C. The 5'-phosphate groups of vector DNA obtained from single restriction endonuclease digestions were removed by 1-2 units/μl calf intestinal alkaline phosphatase added during the cleavage reaction (Boehringer Mannheim).

2.3.e. Agarose gel electrophoresis

Electrophoresis of the restriction endonuclease digested DNA was carried out according to Sambrook *et al.* (1989). Agarose gels were prepared in TAE buffer (40 mM Tris-acetate and 1 mM EDTA, pH 8.0) containing 1% (w/v) agarose and 0.1 μg/ml ethidium bromide in Bio-Rad Mini Sub DNA Cell horizontal electrophoresis

trays (6.5 cm x 10 cm). Electrophoresis was carried out at 50-80 mA constant current until the blue dye in the STOP buffer migrated two-thirds of the distance. Samples of 10 μl were mixed with 1 μl STOP buffer (40% (v/v) glycerol, 10 mM EDTA pH 8.0, 0.25% (w/v) bromphenol blue). "1 Kilo base DNA ladder" (GIBCO-BRL) was used as molecular weight size markers. DNA bands were visualized with ultraviolet light and photographed using a red filter and Polaroid Type 667 black and white film, or using the Bio-Rad Gel Doc 1000.

2.3.f. Ligation

Ligation of the insert DNA into the vector DNA was carried out according to Sambrook *et al.* (1989). Purified DNA was mixed in a 2 to 1 ratio of insert to vector in 20 μl volume with 1 unit of T4 ligase (GIBCO-BRL) and the manufacturer's buffer. The mixture was incubated overnight at 15°C. A sample without the insert DNA was used as a control.

2.3.g. DNA sequencing

Sequencing of DNA was carried out according to Sanger *et al.* (1977). Sequencing of DNA was carried out manually with either single or double stranded templates using the primers shown in Table 2.3. Sequencing reactions were prepared using Pharmacia T7 Sequencing kit and 5-15 μCi [α-32P]dATP (DuPont). Separation of the reaction mixtures was on 8% (w/v) polyacrylamide gels containing 7 M urea, 0.13 M Tris, 0.13 M boric acid and 10 mM EDTA. Dried gels were exposed to an

Table 2.3. Sequences of the oligonucleotides used for sequence confirmation of various mutant *katE* genes.

Primer	Position within *katE*	Oligonucleotide sequence
C	1107-1123	5'-GTGCCGGTAGCCGTGGT
D	1356-1372	5'-TGCGTGATATCCGTGGC
D2	1430-1447	5'-CCAATCTTCTTTATCCAG
E	1606-1620	5'-CCCCCGCAGTTACCG
F	1854-1870	5'-ATGAATTCAAGTTCGAC
G	3004-3020	5'-CCATGAGATTCCGATT
H	2357-2373	5'-CTAAGTCAGACGCCATT
I	2609-2625	5'-GATGTGAAAGGTCGCGT
J	2870-2886	5'-TACCTGATGGAAGCCTA

film (Kodak X-OMAT AR) to visualize the DNA bands.

2.4. Purification of HPII catalase

For small scale crude extracts to be used for HPII assay, the plasmid containing cells were grown in 30 ml LB medium in 125 ml flasks at 37°C and 28°C. The cells were pelleted, resuspended in 1 ml SM buffer and disrupted by sonication (4 x 30 sec with medium probe at 35%). After removing unbroken cells and debris by centrifugation, the supernatant was used for enzyme assay and visualization of proteins by electrophoresis on sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE).

For large scale preparations, mutant proteins were harvested from UM255 cells harbouring plasmids containing mutated *katE* genes grown in LB medium supplemented with 100 μg/ml ampicillin for 20-22 h at 28°C with shaking in 8-20 2 l flasks (500 ml per flask). Isolation of HPII was done according to Loewen & Switala (1986) with some modifications as follows. The cells were pelleted and resuspended in 200-300 ml of 50 mM potassium phosphate buffer, pH 7.0 containing 5 mM EDTA. The cells were disrupted using a French press at 20 000 psi. The unbroken cells and debris were pelleted by centrifugation, and streptomycin was added to a final concentration of 2.5% (w/v). The resulting precipitate was removed by centrifugation and discarded. Ammonium sulfate precipitations were carried out at 35, 50 and 60% saturation with solid ammonium sulfate. The pellets from 50% and 60% ammonium sulfate precipitations were combined in about 50-80 ml of 50 mM potassium

phosphate buffer, pH 7.0 (buffer A). The mixture was heat-treated at 60 °C for 1 h and dialysed against 2 l of buffer A overnight. The sample was loaded onto a 2.5 x 23 cm column of DEAE-cellulose A-500 (Amicon Matrex Cullufine, CHISSO Corp. Japan) equilibrated in buffer A. The column was washed with buffer A until the A_{280} of column fractions was below 0.050. HPII was then eluted by a 500 mM NaCl linear gradient in buffer A. Appropriate fractions were collected and concentrated using a protein concentrator (Amicon). The concentrated sample was dialysed against 2 l of the same buffer overnight. The purity of catalase was estimated spectrophotometrically from A_{407}/A_{280} ratio (heme/protein) and by SDS-PAGE. If this ratio was below 0.8 a second heat-treatment was performed. Finally, small aliquots of the enzyme were stored at -70°C.

Initially protein samples were extracted 3 times with ethanol/CCl_4 (50%/30%, v/v, Nadler *et al.*, 1986) before loading onto the column. However, this treatment was found to interfere with protein crystallization, and was later replaced with heat-treatment at 60 °C for 1 h. All proteins characterized in this work were isolated with heat-treatment. In some cases, the treatment was also repeated after final concentration of the sample. The significance of the treatment lies in its properties of eliminating most of the impurities in HPII preparations without loss of activity.

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

Denaturing SDS-PAGE was carried out according to Weber *et al.* (1972). The concentration of acrylamide was 10% for the crude extract samples containing 100-

200 μg protein or 8% for the samples containing 10 μg purified protein. Protein samples of 10 μl were mixed with an equal volume of sample buffer (3.4 mg/ml NaH_2PO_4, 10.2 mg/ml Na_2HPO_4, 10 mg/ml SDS, 1.28 M 2-mercaptoethanol, 0.36 g/ml urea and 0.15% bromophenol blue) and boiled for 3 min prior to loading. Samples were run with 30-50 mA constant currant in a vertical BIO-RAD Protean II electrophoresis system. Samples were stained overnight in staining solution (0.5 g/l Coomassie Brilliant Blue R-250 (Sigma), 30% ethanol and 10% acetic acid) and destained with repeated change of the destaining solution (15% methanol and 7% acetic acid until the background color was clear. After soaking in a final destaining solution of 7% acetic acid and 1% glycerol, the gel was dried on a 3MM paper (Whatman) at 60°C in a slab gel dryer (Savant).

2.6. Catalase assay and protein quantification

Catalase activity was determined by the method of Roth & Jensen (1967) in a Gilson oxygraph equipped with a Clark electrode. One unit of catalase is defined as the amount of enzyme that decomposes 1 μmol H_2O_2 in 1 min in a 60 mM H_2O_2 at 37°C, pH 7.0. Appropriately diluted samples were incubated for 0.5-1.0 min at 37°C in 1.8 ml SM buffer (100 mM NaCl, 1 mM $MgSO_4$, 20 mM Tris HCl, pH 7.6 and 100 mg/L gelatin) followed by the addition of 50 μl H_2O_2 (to 60 mM final concentration). For the determination of catalase activity from whole cells, LB medium was used for resuspension instead of SM buffer. Catalase activity as units/ml was determined from the slope of the evolving of oxygen. Specific activity was

expressed as units/ml per mg purified protein or units/ml per mg dry cell weight. Protein concentration (mg/ml) was estimated spectrophotometrically based on A_{280}/A_{260} ratios (Layne, 1957). Protein samples in a 1 cm path length cell were scanned from 200 nm to 350 nm either in 50 mM potassium phosphate buffer, pH 7.0 or SM buffer at room temperature. Specific activity in whole cells (unit/mg dry cell weight) was determined by converting the cell turbidity values at 600 nm to Klett values (A_{600} x 168.6 - 34.7) where Klett 100 = 0.16 mg dry cell weight. Specific activity was always determined as the average of minimum two or more determinations.

2.7. Determination of enzyme spectra

Absorption spectra of enzymes in 50 mM potassium phosphate buffer, pH 7.0 were obtained using a Milton Roy MR3000 spectrophotometer with 1 cm pathlength quartz cells at room temperature. Data were transferred to Freelance (Lotus) computer program for preparation of spectra.

2.8. Hemochromogen characterization

Heme extraction and characterization was carried out according to Loewen *et al.* (1993). One mg HPII protein in 50 mM potassium phosphate, pH 7.0 was extracted in 1 ml acetone containing 0.13% HCl and neutralized with 8 μl 1 M Na_2CO_3 and lyophilized. Samples were redissolved in a solution of 24% methanol, 24% acetonitrile, 2% acetic acid and 50% water, and separated on 4.6 x 250 mm column packed with Whatman 5 μM ODS III (C18 coating) eluted with a gradient of

24:24:48:4 to 48:48:0:4 acetonitrile/methanol/water/acetic acid in a LKB HPLC system with detection at 370 nm. Data were transferred to Lotus 123 and Freelance (Lotus) for preparation of elution profiles.

2.9. Determination of sulfhydryl groups

The number of sulfhydryl groups in HPII catalase was determined spectrophotometrically using DTNB [5.5'-dithiobis-(2-nitrobenzoic acid)] (Ellman, 1959). Sulfhydryl groups on non-denatured protein were quantitated using 10-30 μl samples (5-10 μM protein in 50 mM potassium phosphate buffer, pH 7.0) diluted to 1 ml with 50 mM potassium phosphate buffer, pH 8.0 to which was added 20 μl DTNB (2-3 fold excess in molar ratio). After 5-10 min at room temperature, A_{412} values were determined. The amount of sulfhydryl group was calculated using an extinction coefficient for DTNB of ϵ_{412} = 13 600. For NaOH treated samples, 10-30 μl of protein was treated with an equal volume of 0.5 N NaOH incubated at room temperature for 3 h. The mixture was neutralized with an equal volume of 0.5 N HCl prior to the assay (Wardell, 1974).

DTNB assay was also performed after treating HPII with 2-mercaptoethanol (MSH) in which 10-20 μM HPII was incubated with 1 M MSH for 3 hours at room temperature, followed by repeated dialysis to remove excess MSH. For the reaction of HPII with hydroxylamine (Lipmann & Turtle, 1945), 5-10 μM HPII in 330 μl 50 mM potassium phosphate buffer, pH 7.0 was incubated with 165 μl neutralized hydroxylamine (28% hydroxylamine plus 14 % NaOH) for 10 min at room

temperature, followed by the addition of solutions of 165 μl 3N HCl, 165 μl 12% tricarboxylic acid and 165 μl $FeCl_3$ (5% in 0.1 N HCl). The absorbance was determined at 540 nm using acetyl CoA and acetaldehyde as controls. Reaction of HPII with methylamine (Motoshima *et al.*, 1988) was carried out incubating 5-10 μM HPII (in 50 mM potassium phosphate buffer, pH 7.0) with 200 mM methylamine in 50 μl volume for 1 h at room temperature, followed by the DTNB assay using acetyl CoA as a control. Both hydroxylamine and methylamine assays were performed with denatured (NaOH-treated or boiled) as well as non-denatured HPII. Treatment of HPII with sodium borohydride involved incubating 5-10 μM enzyme and acetyl CoA (positive control) with $NaBH_4$ (4 mg) in 25 μl volume for 15 min at 37°C, followed by DTNB assay. The pH was maintained between 7.8 to 8.0 in all DTNB assays to eliminate any background effect.

2.10. Inhibition studies

The effect of various inhibitors on HPII was studied following enzyme activity and spectral changes. Various concentrations of NaCN, NaN_3, hydroxylamine, *O*-methylhydroxylamine and *O*-ethylhdroxylamine were incubated with HPII at 37°C for 1 min in SM buffer prior to the addition of H_2O_2 in the reaction cell. The thiol reagents 2-mercaptoethanol, cysteine, dithiothreitol and glutathione, all at 5 mM concentrations, were incubated with HPII at 37°C and aliquots were removed at specified intervals for assay. To test the reversibility of inhibition, HPII was

incubated with the same reagents and dialysed against 2 liters of 50 mM potassium phosphate buffer, pH 7.0. Aliquots were removed at 3 h, 6 h and 16 h intervals for assay. For spectral analysis, HPII was incubated with the various inhibitors for 3 h at 37°C after which spectra from 200 nm to 750 nm were obtained.

2.11. CNBr digestion and MALDI/MS

200 μg HPII catalase in 400 μl distilled water was incubated overnight with 2 μl of 5 M CNBr in acetonitrile in the presence of 0.1 M HCl. Following lyophilization, the mixture was analyzed by matrix assisted laser desorption/ionization mass spectroscopy (MALDI/MS) using reflecting time-of-flight mass spectrometer operated in positive ion linear mode. Desorption/ionization was achieved by a pulsed ultraviolet laser beam (N_2 laser, λ=337 nm). The acceleration voltage was 30 kV and the laser power density was $\approx 10^6$ W/cm^2. The protein sample (0.1 g/l) was prepared in a solution containing saturated α-cyano-4-hydroxycinnamic acid (4HCCA) in 2 (0.1% trifluoroacetic acid) : 1 (acetonitrile); of which 2 μl was applied to the sample matrix, and air-dried prior to desorption/ionization. For better target uniformity the crushed matrix method in which the matrix was crushed with glass slide was employed (Xiang & Beavis, 1994). The mass spectrum of each sample was the average of >100 laser shots using ubiquitin (8565 Da) for calibration.

2.12. Circular dichroism spectroscopy

Circular dichroism spectroscopy of wild type HPII and mutant enzymes was

carried out by using a Jasco-500A spectropolarimeter equipped with a thermostat set at 37 °C and a water-jacketed cell of 0.5 cm pathlength. The instrument was calibrated with d(+)-1-camphorsulfonic acid as described by Hennessey & Johnson (1982). Secondary structure of catalase proteins was analyzed using the convex constraint algorithm (CCA) of Perczel *et al.* (1992). The processed data obtained from the circular dichroism spectroscopy of each protein was appended to the reference data set of 25 proteins supplied with CCA program. In this analysis, samples in 50 mM potassium phosphate buffer, pH 7.0 were scanned at 0.25 nm intervals from 195 nm to 240 nm in 1 mm pathlength cuvette. The three pure components corresponding α-helix, β-sheet, and unordered structures were assigned based on the CCA analysis of the wild type HPII (Figure 2.3) and used to analyze the secondary structure of mutants.

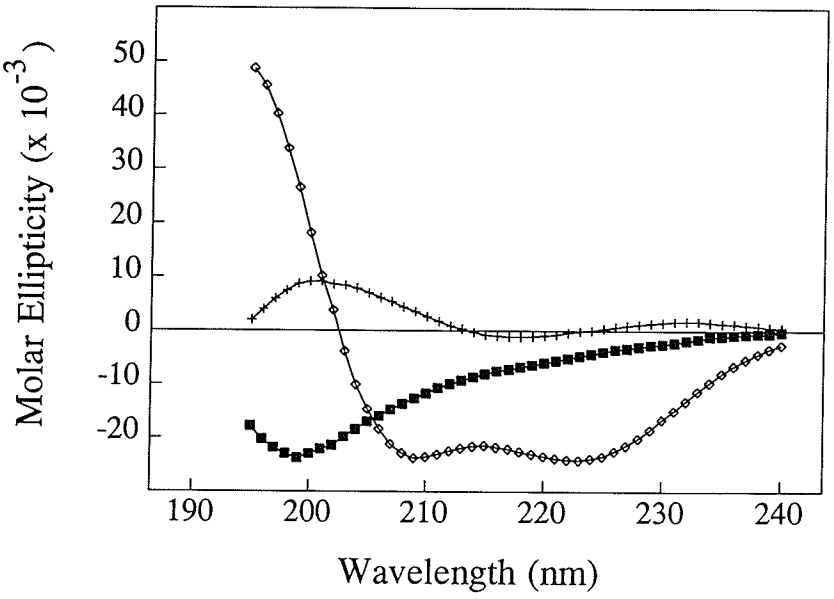


Figure 2.3. The three pure component curves of HPII catalase generated by the convex constraint algorithm analysis of the circular dichroim spectra. Curves corresponds to (+) beta-sheet, (◇) alpha-helix and (▪) unordered structures.

3. RESULTS

3.1. Construction and characterization of the presumptive NADPH-binding site mutants

3.1.1. Introduction

The binding of NADPH to catalase was first discovered in bovine liver catalase (Kirkman & Gaetani, 1984) and subsequently in some bacterial and yeast catalases (Jouve *et al.*, 1986 & 1989; Yusifov *et al.*, 1989; Hillar *et al.*, 1994). The X-ray crystal structure of the bovine (Fita & Rossman, 1985) and *Proteus mirabilis* (Gouet *et al.*, 1995) enzymes revealed no connection between the NADPH-binding domain and the active site. Furthermore, NADPH-binding was not essential for catalysis. However, it was shown that the bound-NADPH protects the enzyme from conversion to inactive compound II and even reverses the inactivation process (Kirkman *et al.*, 1987). The protective role of NADPH was proposed to occur by donation of either two electrons (Hillar & Nicholls, 1992) or one electron (Almarrson *et al.*, 1993) to a presumptive free radical formed during catalysis. Not all catalases bind NADPH and, in particular, attempts to demonstrate the binding of NADPH to HPII catalase failed (Hillar *et al.*, 1994).

By comparison with the sequence of the bovine catalase, four residues in HPII were identified as potential NADPH binding residues, namely R260, E270, K294 and E362 corresponding to bovine residues R202, D212, K236 and H304, respectively.

In order to determine if the changes in residue were responsible for the absence of NADPH-binding in HPII, the HPII residues E270 and E362 were changed to the equivalents in the bovine enzyme, Asp and His respectively. In addition the conserved residues R260 and K294 were changed to Ala to study their role. The organization of these four presumptive NADPH-binding residues in HPII is shown in Figure 3.1.1.

3.1.2. Mutants in the putative NADPH-binding site

Site-directed mutagenesis was used to construct E270D, E362H and E270D/E362H in which the bovine residues were incorporated into HPII. The production of mutant proteins was affected by growth temperature with maximal production being realized at 28°C. At 37°C, the activities of E270D and E362H proteins were 17% and 35% of the wild type level respectively while the double mutant was barely detectable. (Table 3.1.1). The purity of the proteins was analyzed by SDS-PAGE revealing protein that was estimated to be more than 95% pure (Figure 3.1.2). The specific activities, apparent Km, V_{max} and k_{cat} values were determined (Table 3.1.2), all of which were slightly increased relative to the wild type enzyme. The saturation curves of mutants and the wild type enzyme were similar to each other and resembled the Michaelis-Menten hyperbolic pattern (Figure 3.1.3). The visible spectra (Figure 3.1.4) of the mutants were also similar to the wild type enzyme with a Soret band at 407 nm and a peak characteristic of heme *d* at 590 nm. Furthermore, the HPLC chromatograms (Figure 3.1.5) of the extracted heme from these mutants

Figure 3.1.1. The presumptive NADPH-binding residues of HPII. The protein backbone is shown in blue and only the residues studied in this work are shown.

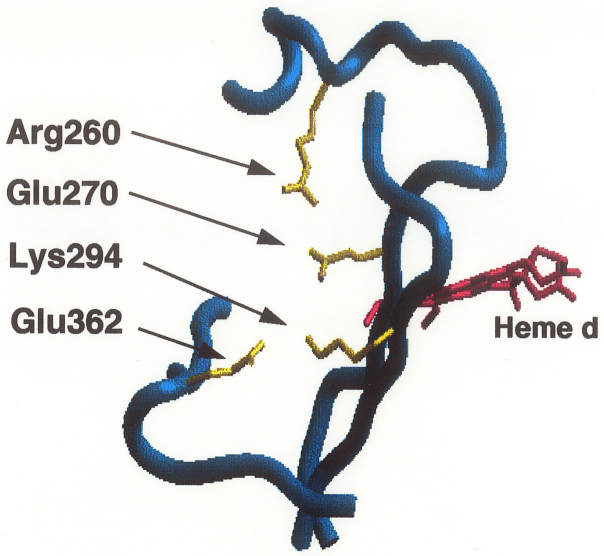

Arg260
Glu270
Lys294
Glu362
Heme d

Table 3.1.1. Comparison of the effect of the culture growth temperature on the production of the wild type and various mutant proteins of HPII. Cultures of *E. coli* strain UM255 containing plasmids encoding mutant HPII proteins were shaken at 37°C for 16-17 h or at 28°C for 20-22 h in LB medium supplemented with ampicillin.

Mutant	Catalase Activity (units/mg dry cell weight) 37°C	28°C	Ratio of activities 28°C/37°C
E270D	71 ± 6	268 ± 47	3.8
E362H	145 ± 9	305 ± 61	2.1
E270D/E362H	10 ± 1	115 ± 23	11.5
R260A	88 ± 16	273 ± 28	3.1
K294A	91 ± 16	297 ± 43	3.3
R260A/K294A	1.7 ± 1	27 ± 7	15.9
Wild Type	425 ± 51	388 ± 59	0.9

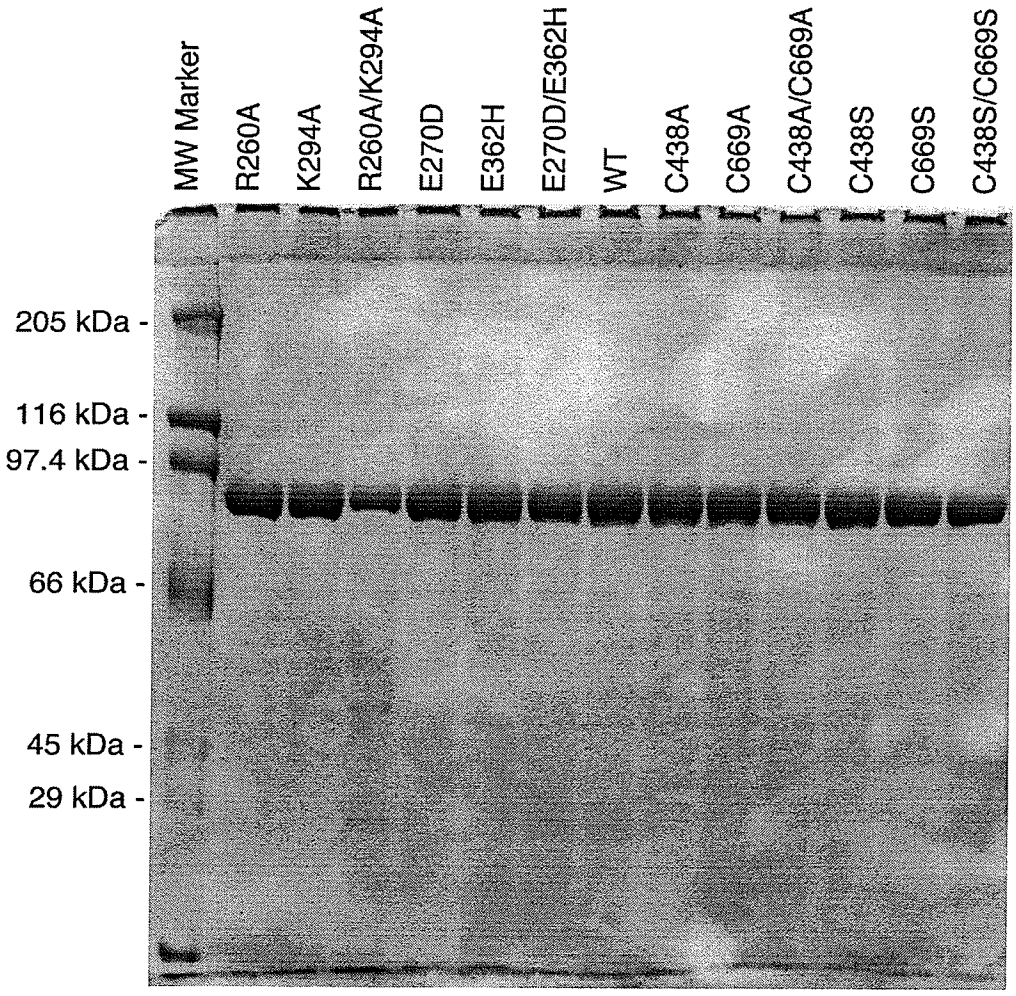


Figure 3.1.2. SDS-polyacrylamide gel electrophoresis analysis of various mutants of HPII following purification. Samples of approximately 10 μg were electrophoresed in an 8% gel and stained with Coomassie Brillant Blue.

Table 3.1.2. Comparison of the kinetic and physical properties of purified wild type and HPII mutant proteins.

Mutant	Specific Activity (u/mg)	A_{407}/A_{280}	Apparent Km (mM)	Apparent V_{max} (mol/min)	Apparent k_{cat} (sec^{-1})
E270D	16 137 ± 950	0.854	27.1 ± 3.3	18.4 ± 0.9	2.6 ± 0.1 x 10^7
E362H	18 325 ± 601	0.861	32.9 ± 1.6	21.8 ± 2.6	3.1 ± 0.4 x 10^7
E270D/E362H	16 859 ± 1 427	0.722	33.7 ± 3.4	22.2 ± 0.5	3.1 ± 0.1 x 10^7
R260A	35 891 ± 1 408	0.845	58.4 ± 8.4	67.9 ± 9.1	9.2 ± 0.9 x 10^7
K294A	18 625 ± 596	0.892	28.4 ± 4.0	23.9 ± 3.1	3.4 ± 0.4 x 10^7
R260A/K294A	12 880 ± 68	0.404	87.1 ± 3.8	36.5 ± 2.0	5.1 ± 0.3 x 10^7
Wild Type	14 322 ± 690	0.912	27.8 ± 5.4	17.8 ± 2.9	2.5 ± 0.4 x 10^7

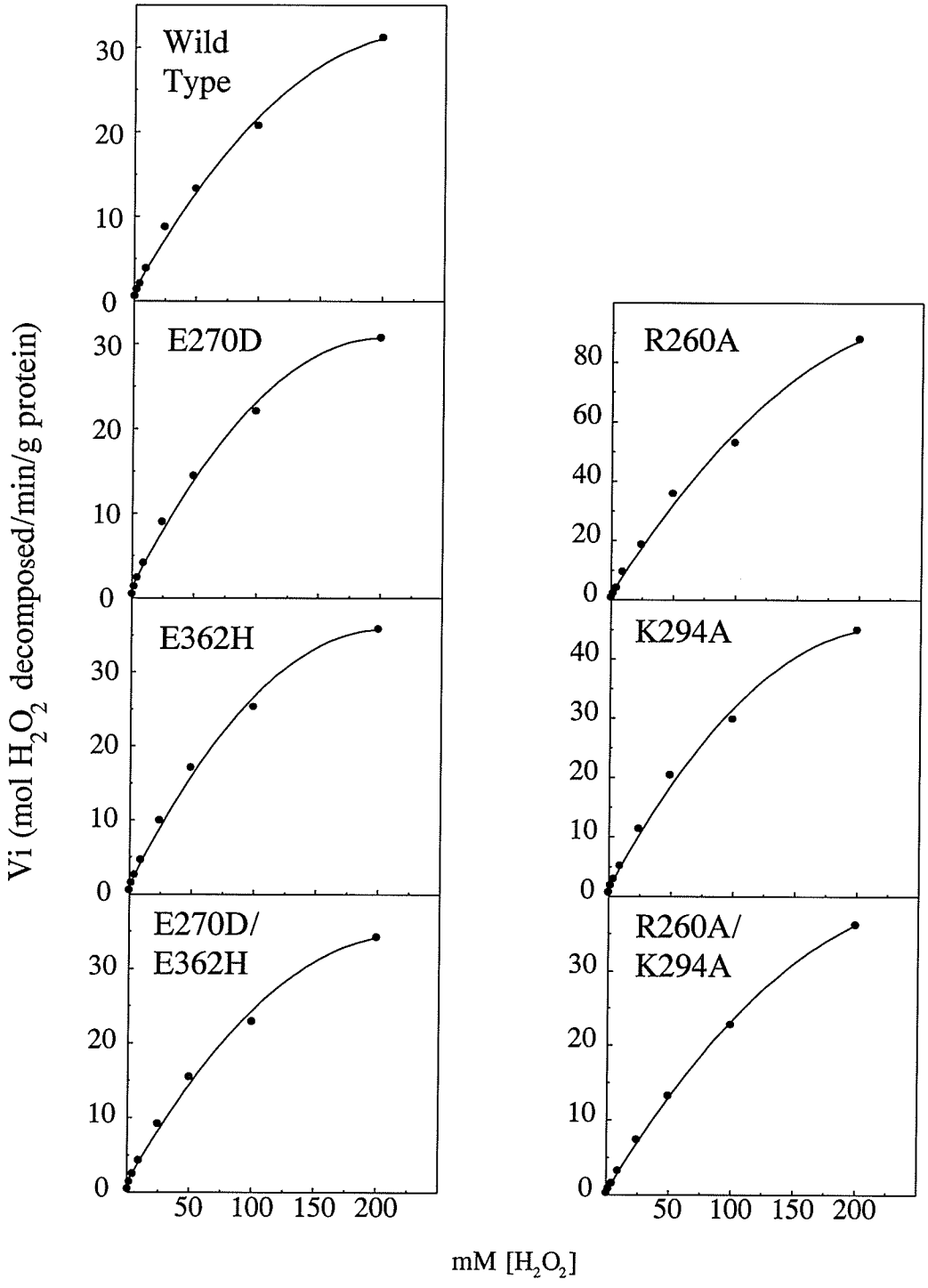


Figure 3.1.3. Comparison of the effect of hydrogen peroxide concentrations on the initial velocities (Vi) of wild type HPII and various mutants.

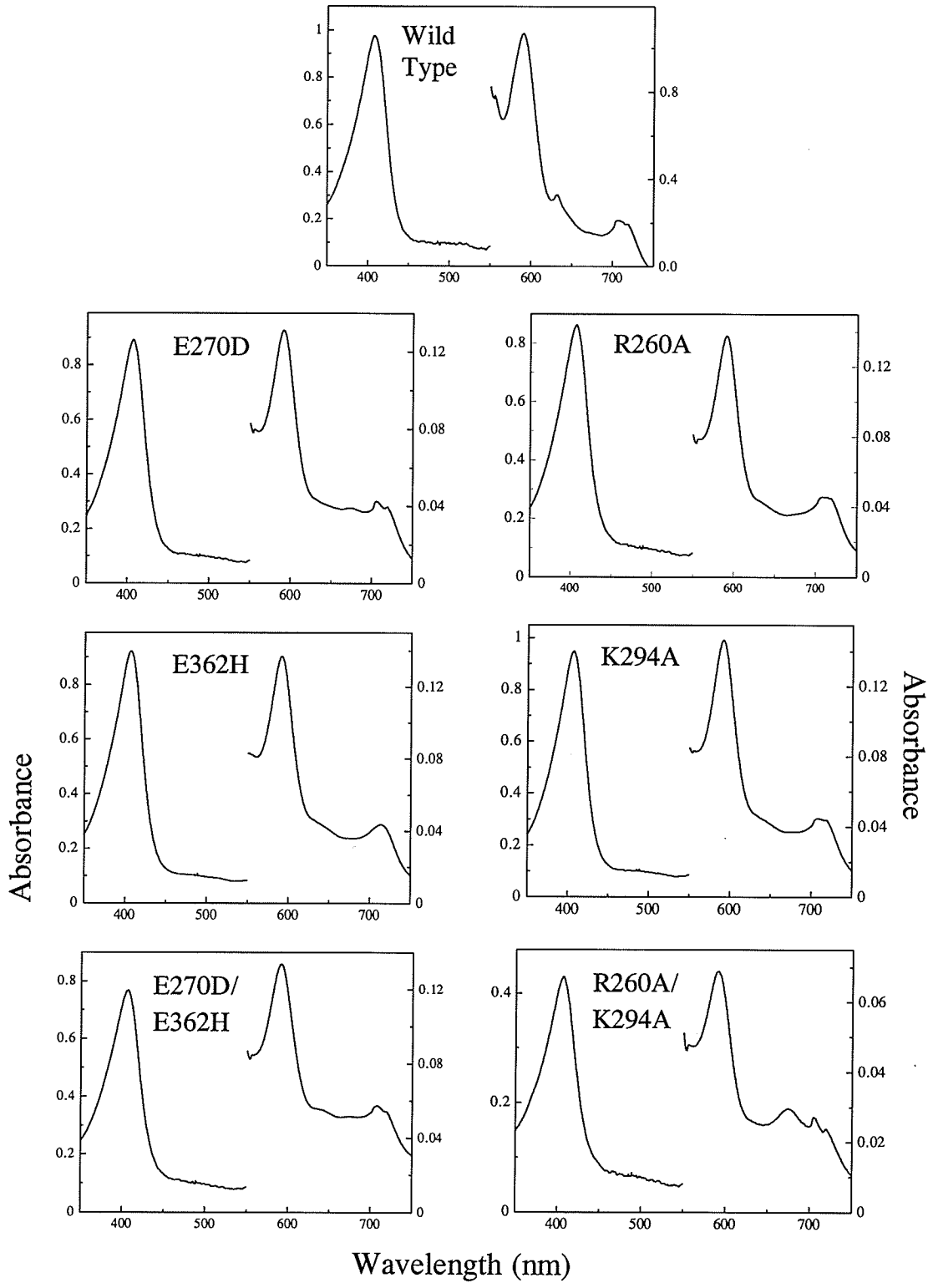


Figure 3.1.4. Visible spectra of wild type HPII and various mutants. The left axis is for the range from 350 to 550 nm while the right axis is for the range from 550 to 750 nm.

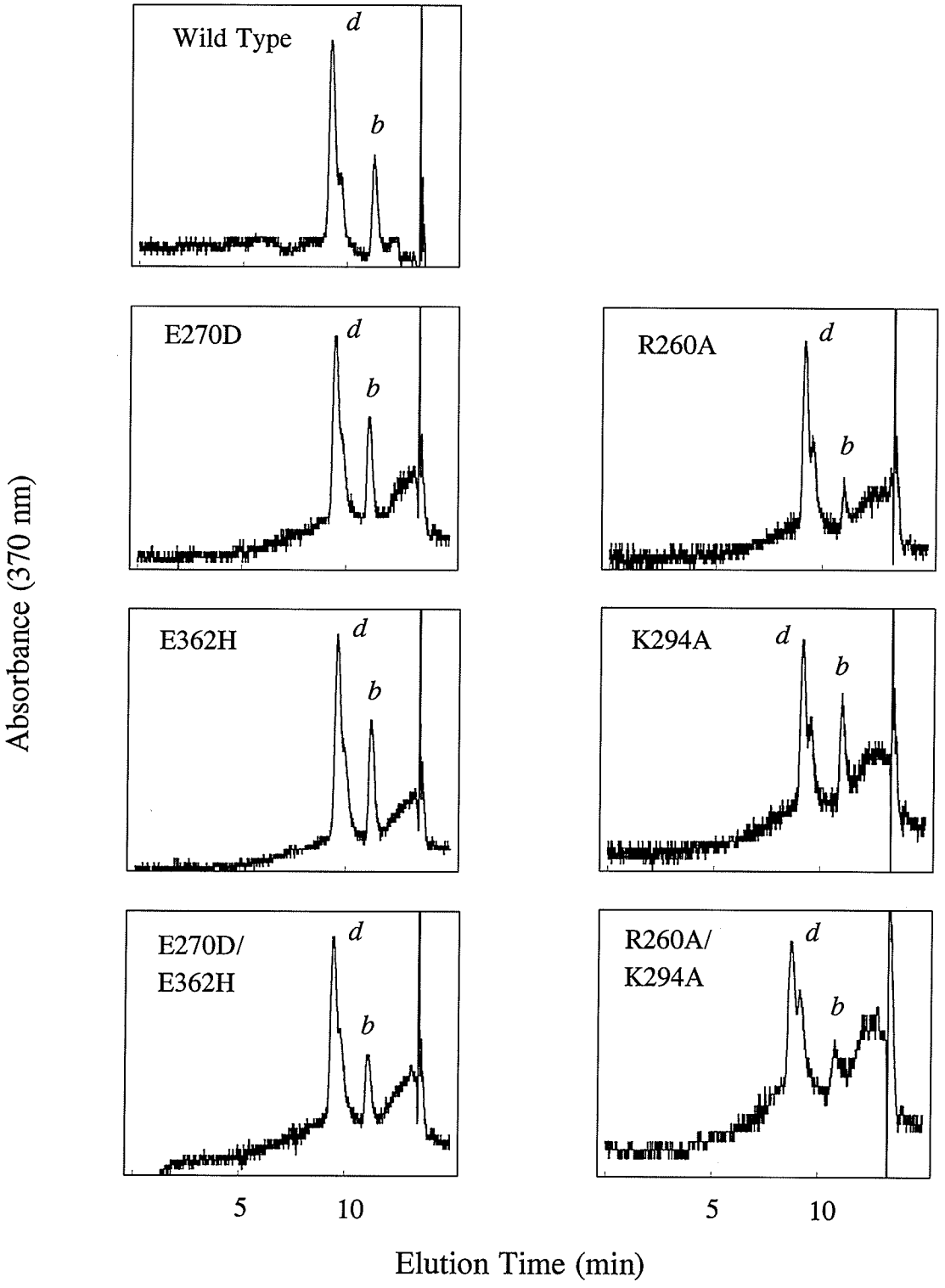


Figure 3.1.5. Elution profiles from C18 reverse phase HPLC chromatography of heme extracted from various HPII mutants. The letters '*b*' and '*d*' denote the locations of heme *b* and heme *d* respectively.

were also similar to the wild type enzyme with a predominant heme *d* peak and a smaller heme *b* peak. The mutants were analyzed for NADPH-binding fluorometrically using bovine catalase as the control, and no fluorescence was detected with wild type HPII or the mutant enzymes in contrast to the bovine enzyme (Table 3.1.3). These results indicate that the simple presence of the four bovine NADPH-binding sites in HPII was not enough to allow NADPH-binding.

3.1.3. Conversion of presumptive NADPH-binding sites to Ala

In order to study the roles of R260 and K294 on HPII, two residues that are conserved in the bovine enzyme, they were changed to Ala in single and double mutants to generate R260A, K294A and R260A/K294A containing HPIIs. The production of these mutant proteins was also temperature-dependent (Table 3.1.1) with optimum yields being obtained at 28°C and yields of only 20% (R260A and K294A) or 0% (R260A/K294A) being obtained at 37°C. The purified mutant proteins were shown to be >95% pure by SDS-PAGE (Figure 3.1.2), and the A_{407}/A_{280} ratio confirmed a normal heme to protein ratio for the R260A and K294A mutants and 50% of normal heme in the double mutant (Table 3.1.2). Most significantly the R260A mutant enzyme had a 2.5-fold higher specific activity than wild type HPII, whereas the K294A mutant enzyme and double mutant enzyme were only 30% more active or less active respectively than the wild type (Table 3.1.2). Kinetic analysis revealed that all three mutant enzymes, had similar saturation curves to the wild type HPII (Figure 3.1.3). Consistent with the elevated specific activity, apparent Km, V_{max}

Table 3.1.3. Determination of the fluorescence emission of catalase proteins. Catalase proteins (0.8 μM each) and pure NADPH (1.0 μM) were scanned in 20 mM Tris-HCl, pH 8.0 buffer from 340 nm to 560 nm at room temperature and the fluorescence emmisions at 430 nm (F_{430}) were determined.

Catalase Proteins	**F_{430} (arbitrary units)**
Blank (buffer)	0.2
+ HPII-Wild Type	0.2
+ HPII-E270D	0.1
+ HPII-E362H	0.2
+ HPII-E270D/E362H	0.2
+ Bovine liver catalase	2.7
+ NADPH	3.5

and k_{cat} values were also significantly higher for the R260A enzyme than the K294A enzyme (Table 3.1.2, Figure 3.1.3). The visible spectra of the three mutant enzymes were similar to that of HPII consistent with heme *d* being present (Figure 3.1.4) which was confirmed by the HPLC analysis of the extracted heme (Figure 3.1.5).

3.1.4. Characterization of mutant R260A

Residue R260 is situated about 20 Å away from the active site with its side chain oriented away from the heme channel. With the side chain shortened and the positive charge removed in the R260A mutation there would have to be an adjustment in the protein to partially fill the resulting void and this adjustment may result in an enlargement of the channel leading to the active site, allowing easier access of large inhibitors and substrates. This in turn might explain the enhanced catalysis of R260A reported in Table 3.1.2. In order to test this hypothesis, the interaction of R260A with different inhibitors was studied.

3.1.4.a. Effects of hydroxylamine and its derivatives

The inhibitory effects of hydroxylamine (H_2NOH) and its derivatives *O*-methylhydroxylamine (NH_2OCH_3) and *O*-ethylhydroxylamine ($NH_2OC_2H_5$) on the R260A enzyme were studied (Figure 3.1.6). Inhibition by hydroxylamine was effective at nM concentrations whereas mM concentrations of methylhydroxylamine and ethylhydroxylamine were required to achieve the same level of inhibition (Figure 3.1.6 and Table 3.1.4). This can be explained in terms of the larger size of the

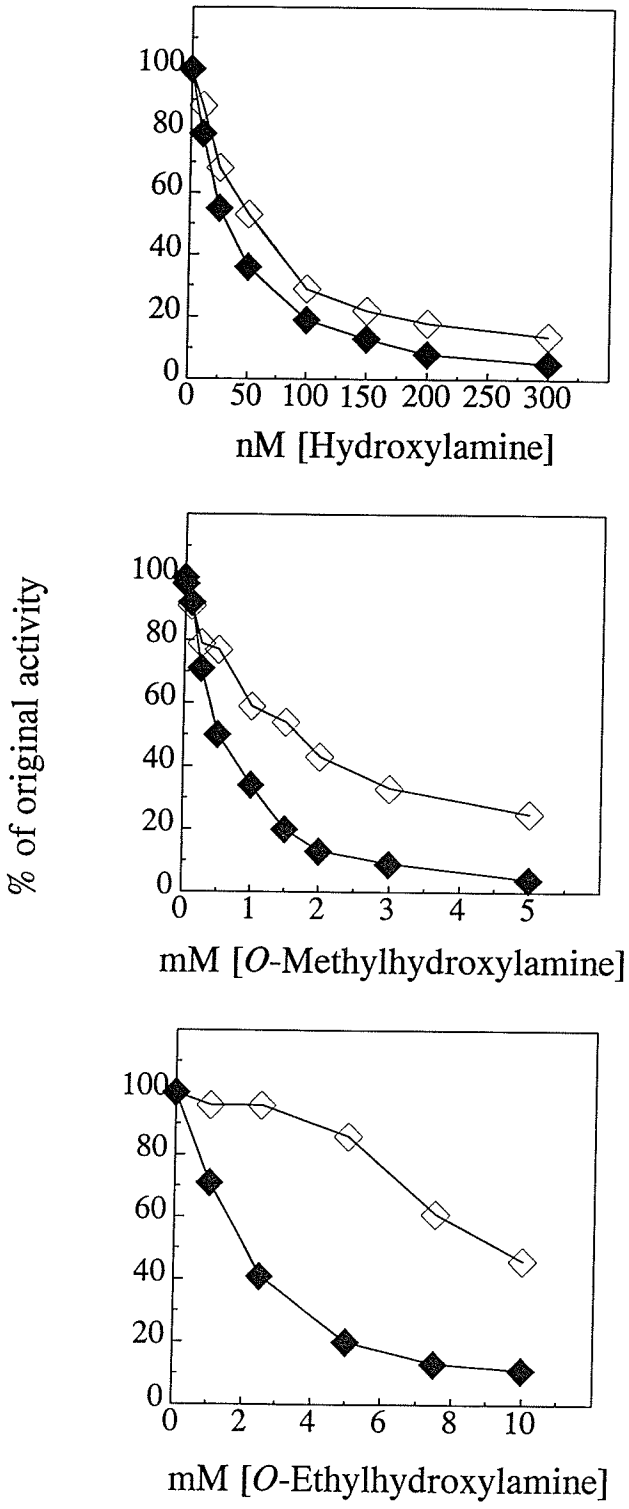


Figure 3.1.6. Comparison of the effects of various hydroxylamine derivatives on mutant R260A (◆) and wild type HPII (◇) activities. Each enzyme was incubated with the inhibitor for 1 min in SM buffer at 37°C prior to the assay.

Table 3.1.4. Determination of the 50% inhibitory concentrations of some catalase inhibitors for the R260A enzyme and wild type HPII (enzyme and the inhibitor were incubated for 1 min at 37°C in SM buffer prior to the assay).

Inhibitory compound	**μM needed for 50% inhibition**	
	Wild Type	**R260A**
Sodium cyanide (NaCN)	14	16
Sodium azide (NaN_3)	300	50
Hydroxylamine (H_2NOH)	0.055	0.030
O-Methylhydroxylamine (NH_2OCH_3)	2 300	500
O-Ethylhydroxylamine ($NH_2OC_2H_5$)	8 600	2 000

alkylhydroxylamines which made their access to the channel more difficult. Consistent with this conclusion and the hypothesis of an enlarged channel in the R260A enzyme, it was more sensitive to the larger alkylhydroxylamines. The inhibition caused by all of the hydroxylamine derivatives could be reversed by 3 h of dialysis indicating that a covalent modification was not involved. Also consistent with this conclusion was the lack of change in the visible spectra of the mutant enzymes following reaction with hydroxylamine (Figure 3.1.7).

3.1.4.b. Effects of cyanide and azide

Cyanide and azide were effective inhibitors of HPII and the R260A enzyme at μM concentrations. The major difference between the two inhibitors was the fact that HPII and the R260A enzyme were equally sensitive to cyanide (Figure 3.1.8, Table 3.1.4) whereas the R260A enzyme was more sensitive to azide. This difference can be rationalized in terms of a larger channel in the R260A enzyme allowing easier access to larger azide (MW 42 compared to 26 for cyanide). Like the hydroxylamine inhibition, azide and cyanide inhibition was completely reversible upon dialysis. Nevertheless, both compounds caused some changes in the visible spectra including a red shift in both the Soret band and A_{590} peak by cyanide and an increase in the A_{630} peak by azide (Figure 3.1.9). These data support the idea that the heme channel of HPII has evolved to accommodate molecules the size of its substrate, H_2O_2, and this seems to exclude larger molecules.

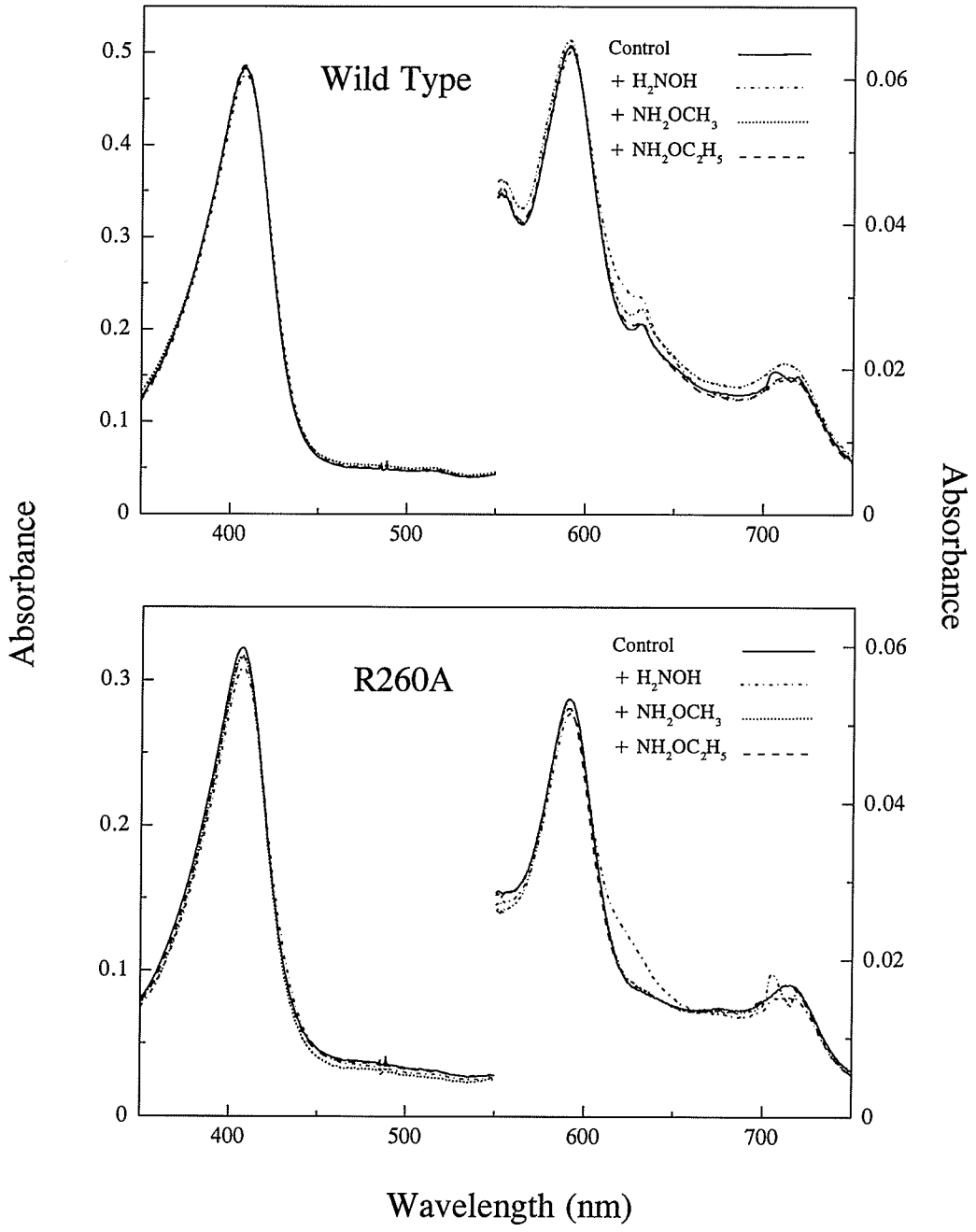


Figure 3.1.7. Visible spectra of wild type HPII and mutant R260A with and without various hydroxylamine derivatives. Hydroxylamine and its methyl and ethyl derivatives at 0.5 mM, 25 mM and 50 mM respectively were incubated with each enzyme for 15 min at room temperature prior to spectral analysis. The left axis is for the range from 350 nm to 550 nm while the right axis is for the range from 550 to 750 nm.

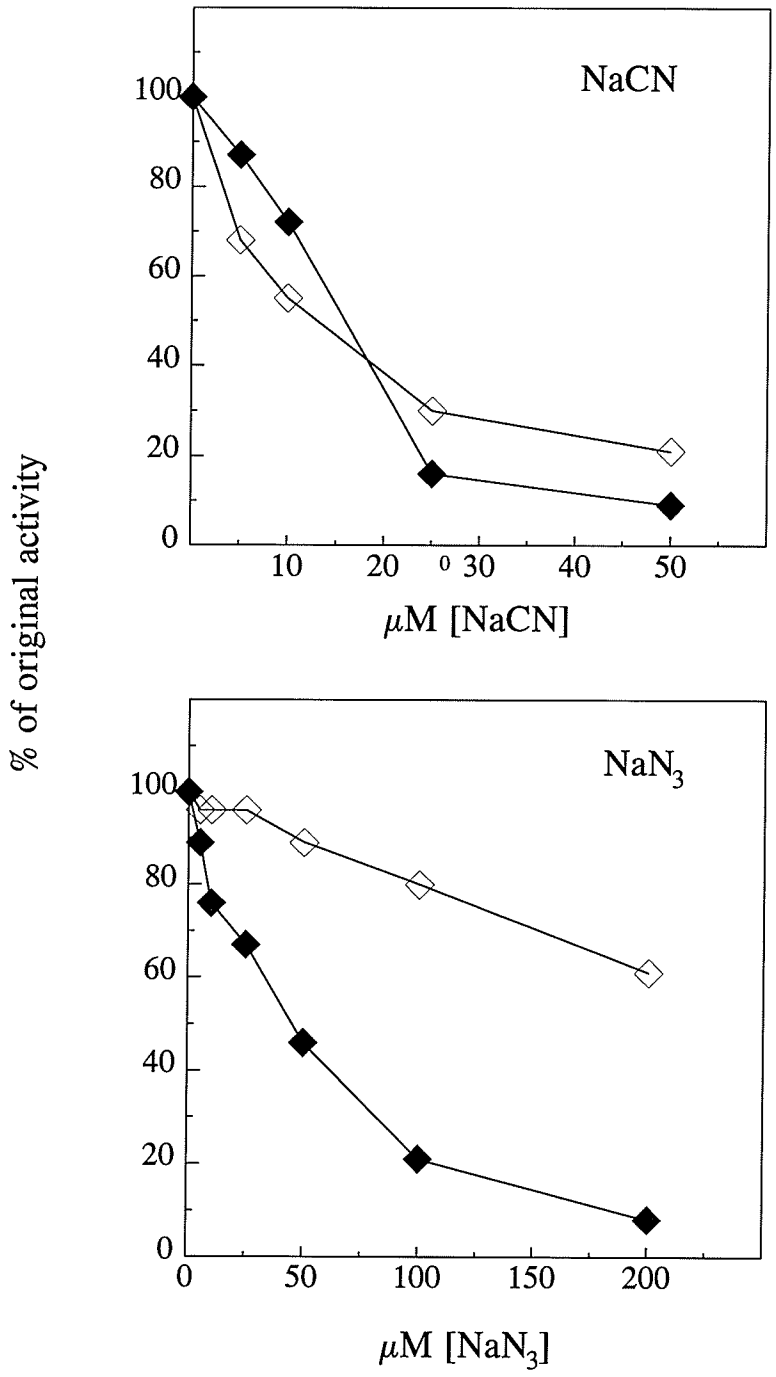


Figure 3.1.8. Comparison of the effects of sodium cyanide and sodium azide on mutant R260A (◆) and wild type HPII (◇) activities. Each enzyme was incubated with the inhibitor for 1 min in SM buffer at 37°C prior to the assay.

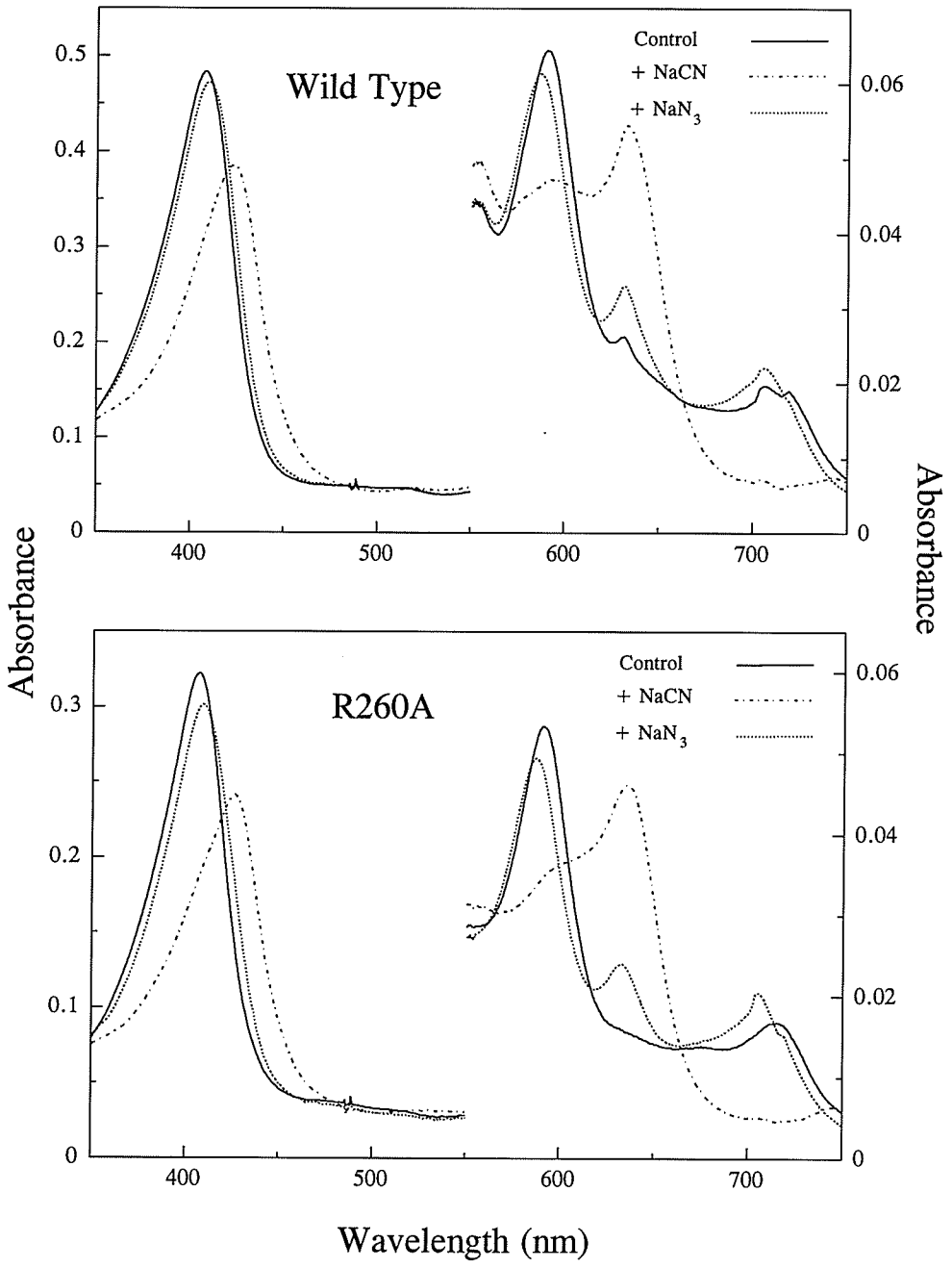


Figure 3.1.9. Visible spectra of wild type HPII and mutant R260A in the presence and absence of NaCN and NaN_3. Each enzyme was incubated with 0.5 mM NaCN and 1 mM NaN_3 at room temperature for 15 min prior to spectral analysis. The left axis is for the range from 350 to 550 nm while the right axis is for the range from 550 to 750 nm.

3.1.4.c. Effects of sulfhydryl reagents

The effect of sulfhydryl compounds, 2-mercaptoethanol, dithiothreitol, cysteine and glutathione on HPII and the R260A enzyme was studied generating the results shown in Figure 3.1.10. 2-Mercaptoethanol had a similar effect on both enzymes causing 40% inhibition. Cysteine was more effective on the R260A enzyme causing 80% inhibition compared to 40% on the wild type. Similarly, neither dithiothreitol nor glutathione had any effect on HPII but they caused 80% and 40% inhibition respectively of the R260A enzyme. Inhibition caused by all of these reagents was irreversible with no recovery of activity after 16 h dialysis. All four sulfhydryl compounds caused a reduction of the Soret band and the A_{590} peak of both enzymes (Figure 3.1.11) while 2-mercaptoethanol and cysteine caused an increase in the A_{630} peak which was greater for the R260A enzyme than HPII.

3.1.4.d. Circular dichroism spectroscopy analysis of R260A

Circular dichroism spectroscopy was performed to determine if the R260A mutation had resulted in any measurable changes in the secondary structure of the protein (Figure 3.1.12). Surprisingly there was an apparent increase in the β-sheet content from 25.7% to 29.1% at the expense of α-helix which decreased from 22.7% to 19.7% (Table 3.1.5). The unordered structural content remained unchanged. Crystal structure analysis will be required to confirm such a significant change in the secondary structure.

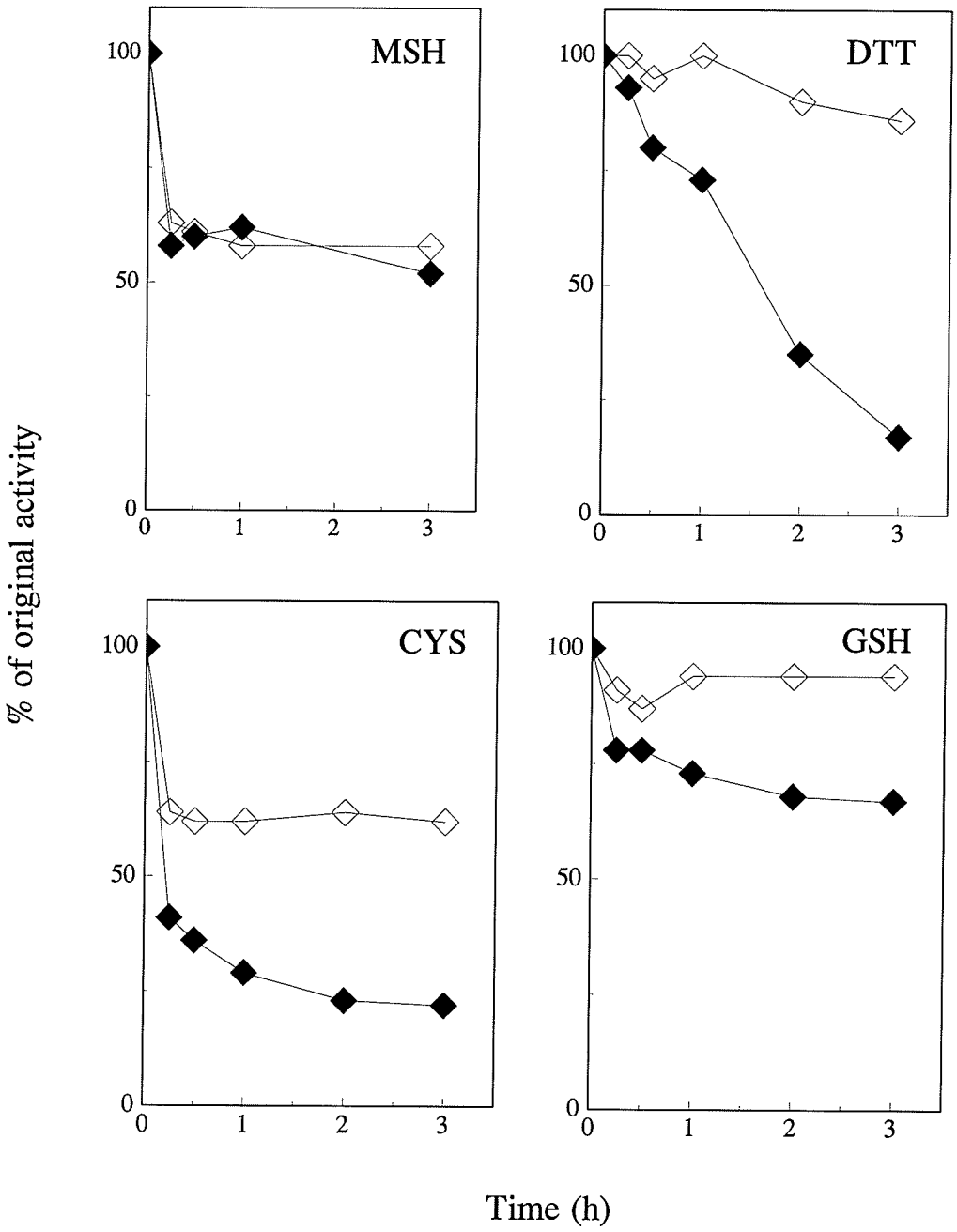


Figure 3.1.10. Comparison of the effects of various sulfhydryl compounds on mutant R260A (◆) and wild type HPII (◇) activities. Each enzyme and 5 mM of the sulfhydryl reagent was incubated at 37℃ in SM buffer and aliquots were taken at various intervals and assayed. Sulfhydryl reagents: MSH, 2-mercaptoethanol; DTT, dithiothreitol; CYS, L-cysteine; GSH, reduced glutathione.

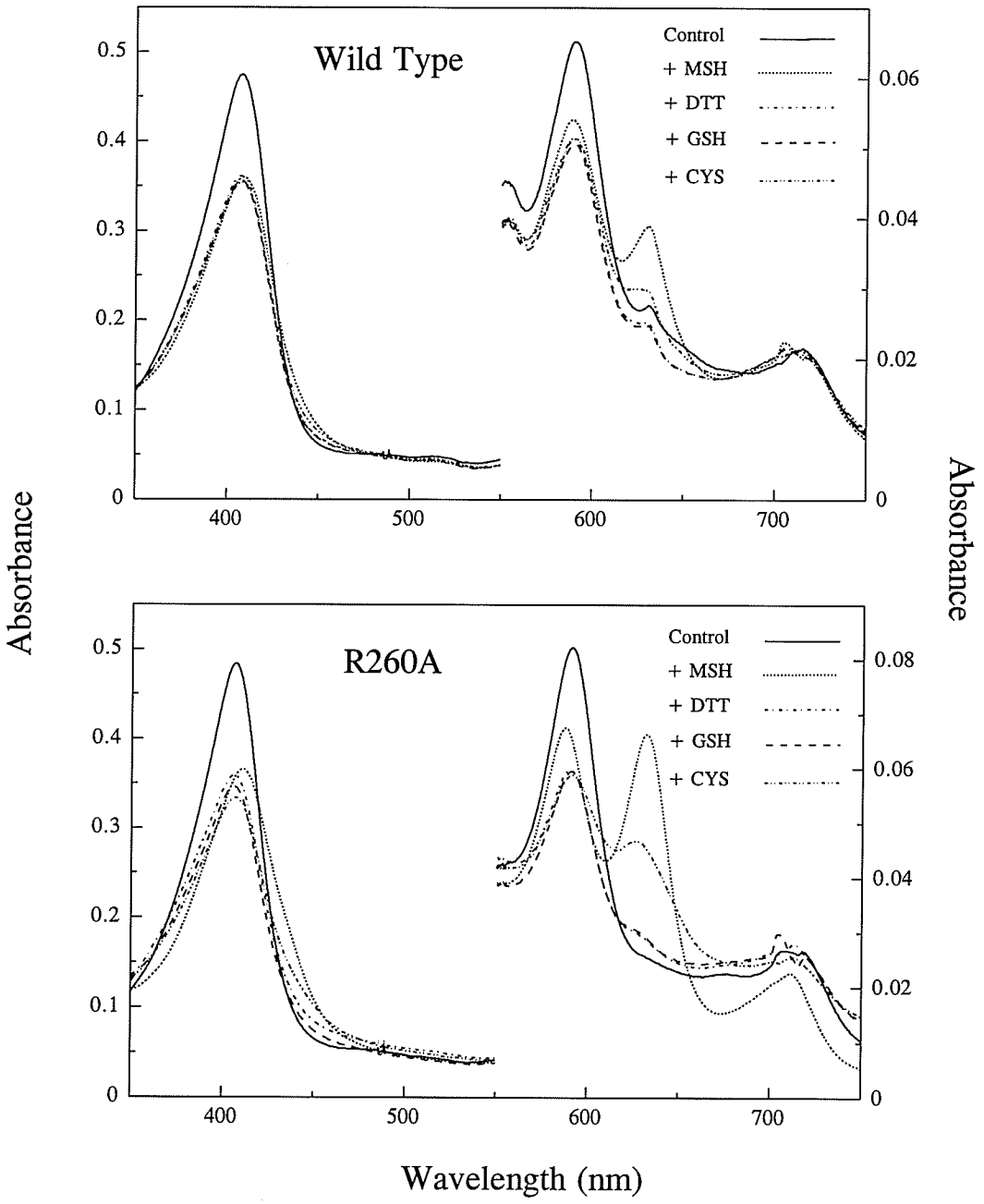


Figure 3.1.11. Visible spectra of wild type HPII and mutant R260A in the presence and absence of various sulfhydryl compounds. Sulfhydryl compounds added at 5 mM concentrations: MSH, 2-mercaptoethanol; DTT, dithiothreitol; CYS, L-cysteine; GSH, reduced glutathione. Spectra were taken after 3 h incubation of each enzyme with the inhibitors at room temperature. The left axis for the range from 350 to 550 nm while the right axis is for the range from 550 to 750 nm.

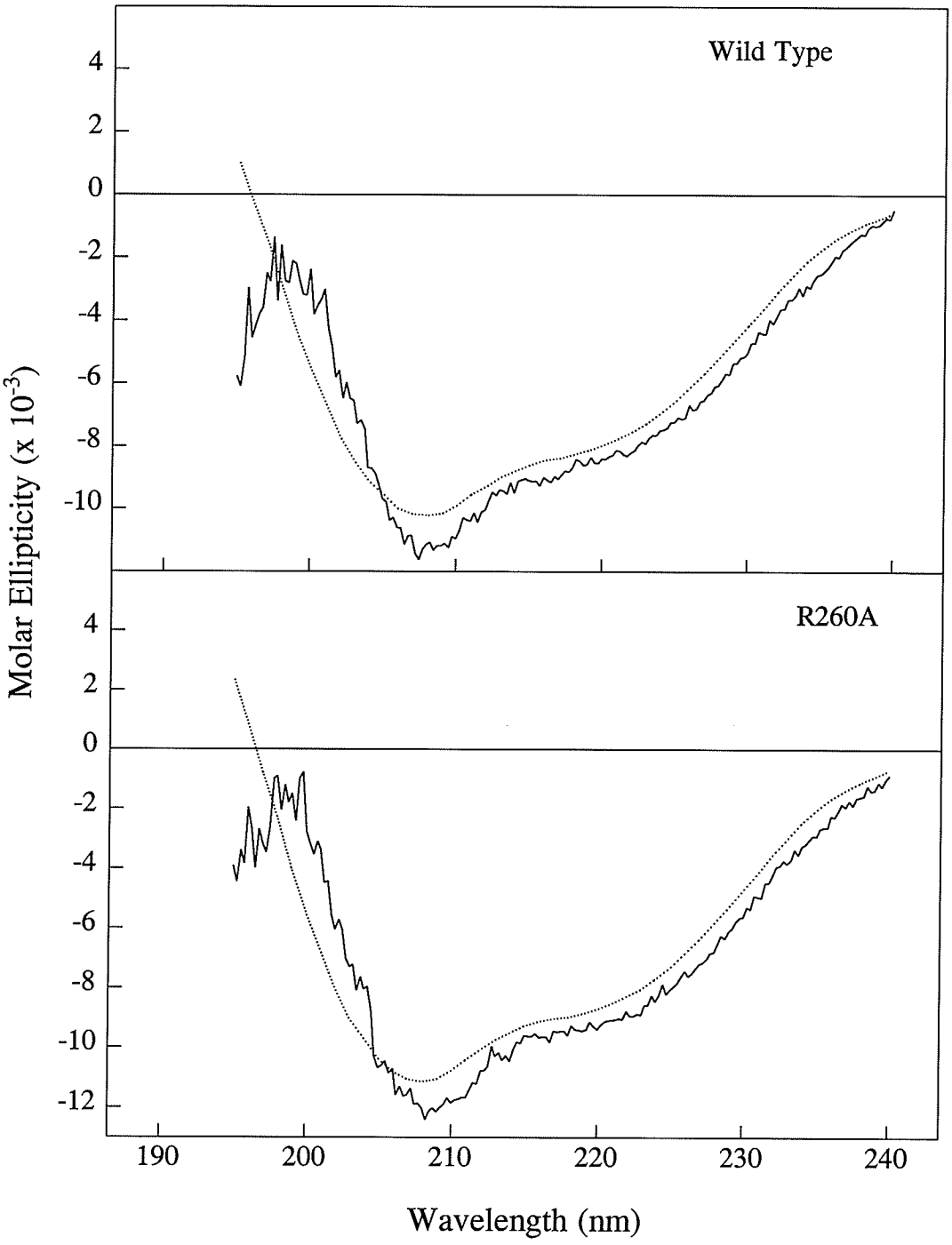


Figure 3.1.12. Circular dichroism spectroscopy analysis of mutant R260A and wild type HPII. Observed (——) and calculated (·····) spectra.

Table 3.1.5. Secondary structure analysis of the R260A enzyme and wild type HPII by circular dichroism spectroscopy.

Mutant	% secondary structure		
	Unordered	**β-sheet**	**α-helix**
R260A	51.1	29.1	19.7
Wild Type	51.6	25.7	22.7

3.2. Construction and characterization of the cysteine replacement mutants

3.2.1. Introduction

Most catalases have a subunit size of 60 kDa and contain a small number of cysteine residues. For example, plant catalases have 6-8 cysteines per subunit and bovine liver catalase contains 4 cysteines per subunit. Despite its larger size (84.2 kDa), HPII catalase contains only two cysteine residues, at positions 438 and 669. Residue C438 is conserved among catalases from different species, including bovine catalase (von Ossowski *et al.*, 1993). Analysis of the crystal structure of the HPII subunit located C438 on the surface of the enzyme, on the opposite side of the subunit from the entrance to the channel leading to the active site (Bravo *et al.*, 1995). The other cysteine, C669, is not conserved among catalases and is located on the C-terminal domain approximately 63 Å from C438 (Figure 3.2.1). The possibility of intrasubunit or intersubunit disulfide linkages was remote but this needed confirmation. Furthermore, it was not known if the cysteines had any role in catalysis. In order to study their role, both cysteines were changed to serine or alanine as single and double mutants.

3.2.2. Cysteine to serine replacement mutants

The yield of mutant protein containing C669S was similar to the wild type enzyme while the C438S and C438S/C669S proteins were produced at 60% and 70% of the wild type yield at 37°C (Table 3.2.1). Unlike the NADPH-binding site

Figure 3.2.1. Localization of the cysteines of HPII.

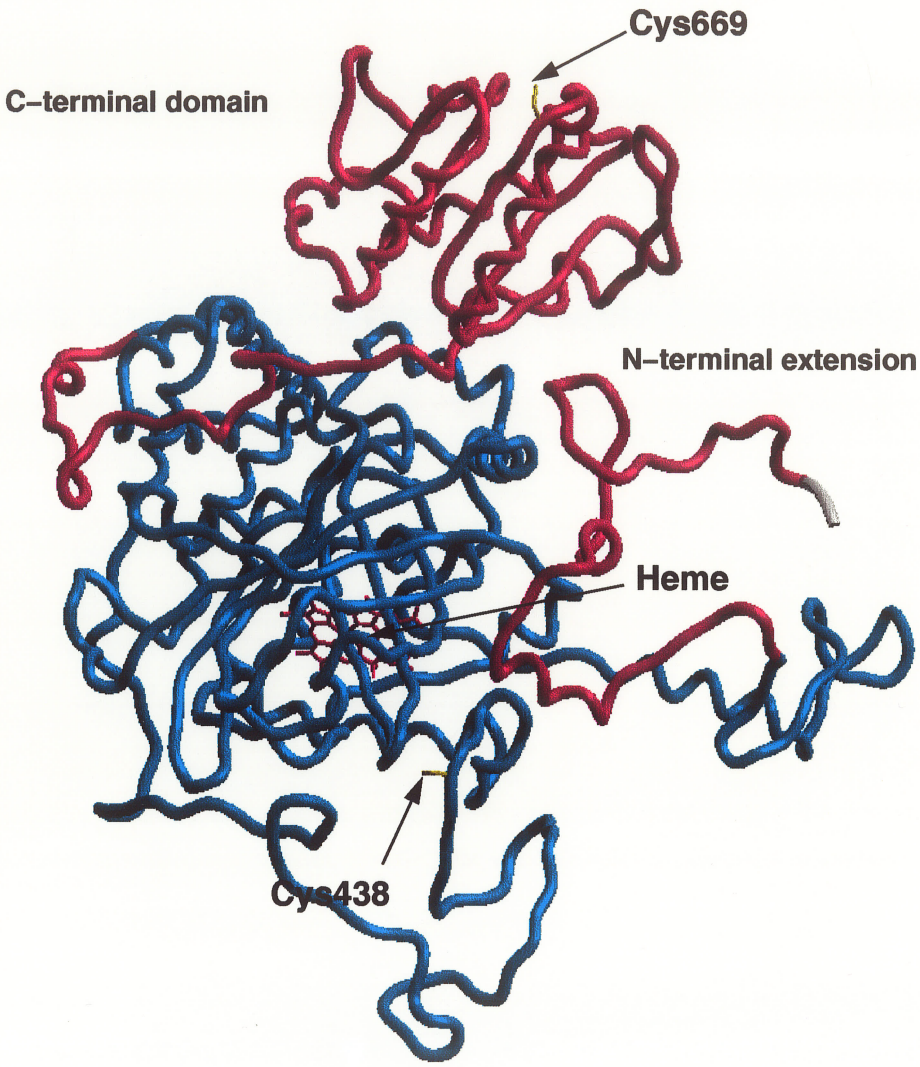

Cys669
C–terminal domain
N–terminal extension
Heme
Cys438

Table 3.2.1. Comparison of the effect of the culture growth temperature on the production of the wild type and various mutant proteins of HPII. Cultures of *E. coli* strain UM255 containing plasmids encoding mutant HPII proteins were shaken at 37°C for 16-17 h or at 28°C for 20-22 h in LB medium supplemented with ampicillin.

Mutant	Catalase Activty (units/mg dry cell weight) 37°C	28°C	Ratio of Activities 28°C/37°C
C438S	261 ± 14	276 ± 33	1.1
C669S	453 ± 67	336 ± 24	0.7
C438S/C669S	297 ± 40	307 ± 31	1.0
C438A	233 ± 29	273 ± 48	1.2
C669A	439 ± 49	344 ± 19	0.8
C438A/C669A	246 ± 24	261 ± 30	1.1
Wild Type	425 ± 51	388 ± 59	0.9

mutants, decreasing the growth temperature to 28°C did not increase the yield.

The mutant proteins were purified and analyzed by SDS-PAGE, revealing >95% purity (Figure 3.1.2). Most physical and kinetic properties of the mutant enzymes were similar to the wild type enzyme. The specific activities of the mutants were determined revealing only small changes from the wild type enzyme (Table 3.2.2). Similarly, the kinetic properties (apparent Km, V_{max} and k_{cat}) and Michaelis-Menten-like substrate saturation curves of the mutants were little changed from those of the wild type enzyme (Table 3.2.2 and Figure 3.2.2). The visible spectra of the mutant proteins were similar to the spectrum of the wild type enzyme with a Soret band at 407 nm and a characteristic heme *d* peak at 590 nm (Figure 3.2.3). Finally, the HPLC profiles (Figure 3.2.4) of the extracted hemes from the mutants were similar to the profile of the wild type enzyme, with a predominant heme *d* and a smaller heme *b* peak.

3.2.3. Cysteine to alanine replacement mutants

Because cysteine to serine replacement did not cause major changes in the properties of HPII, the cysteines were replaced with alanine residues to create the single and double mutant proteins, C438A, C669A and C438A/C669A in which the -CH_2-SH groups were replaced with CH_3. The mutant enzymes were purified and characterized. Their expression at 37°C and 28°C (Table 3.2.1), specific activities and kinetic properties, purity (Table 3.2.2 and Figures 3.1.2 and 3.2.2), visible spectra (Figure 3.2.3) and the heme content (Figure 3.2.4) were all similar to the wild

Table 3.2.2. Comparison of the kinetic and physical properties of purified wild type and HPII mutant proteins.

Mutant	Specific Activity (u/mg)	A_{407}/A_{280}	Apparent Km (mM)	Apparent V_{max} (mol/min)	Apparent k_{cat} (sec^{-1})
C438S	11 418 ± 180	0.966	27.1 ± 0.8	14.3 ± 0.6	2.1 ± 0.1 x 10^7
C669S	12 835 ± 126	0.921	31.3 ± 1.0	16.0 ± 2.5	2.3 ± 0.5 x 10^7
C438S/C669S	11 107 ± 404	0.952	28.1 ± 4.3	14.3 ± 2.4	2.0 ± 0.3 x 10^7
C438A	12 082 ± 568	0.924	29.8 ± 6.0	16.4 ± 3.7	2.3 ± 0.5 x 10^7
C669A	14 505 ± 175	0.955	31.2 ± 3.4	20.6 ± 2.7	2.9 ± 0.4 x 10^7
C438A/C669A	12 870 ± 871	0.965	27.8 ± 4.5	16.7 ± 3.0	2.4 ± 0.4 x 10^7
Wild Type	14 322 ± 690	0.912	27.8 ± 5.4	17.8 ± 2.9	2.5 ± 0.4 x 10^7

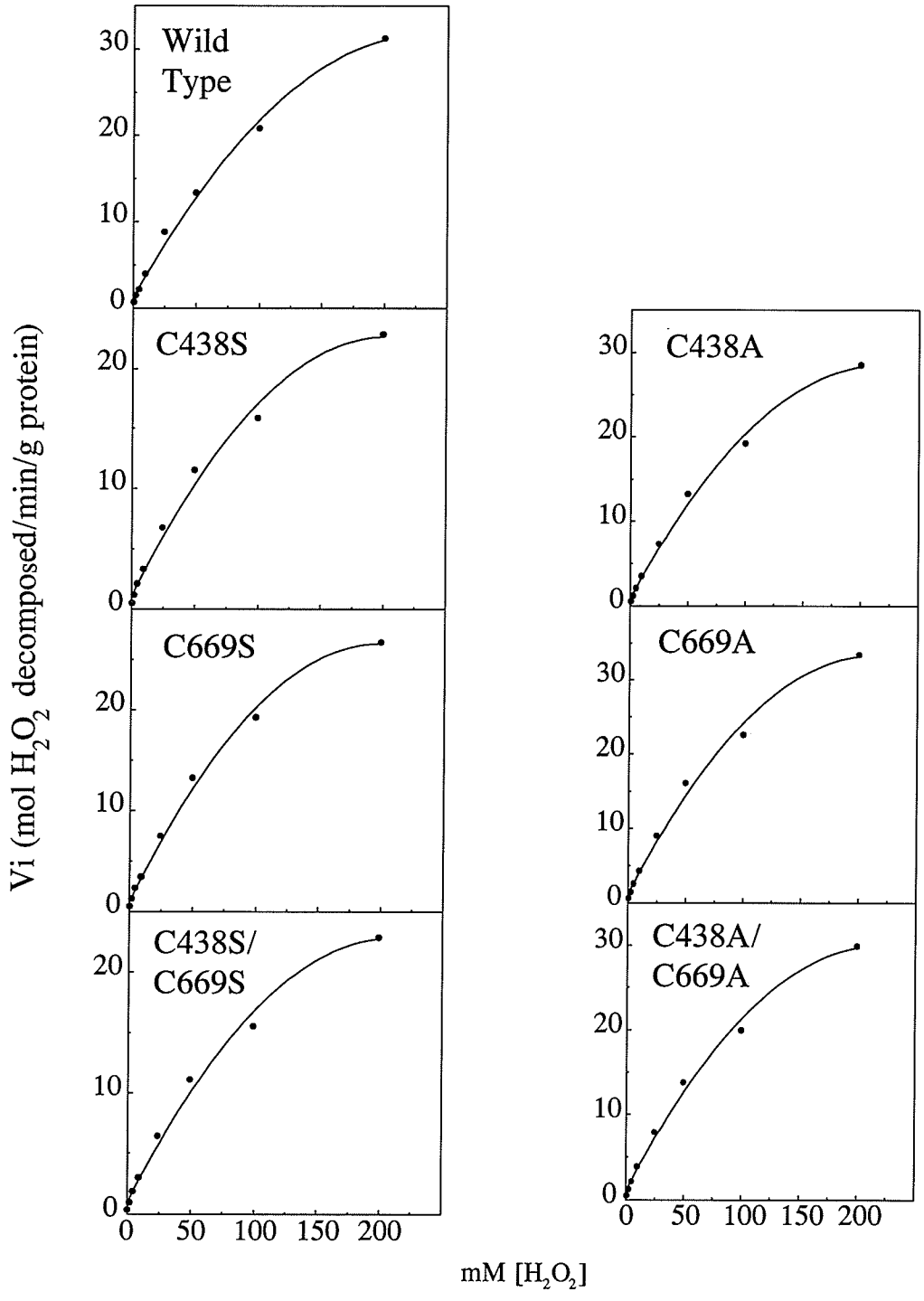


Figure 3.2.2. Comparison of the effect of hydrogen peroxide concentrations on the initial velocities (Vi) of various mutants and wild type HPII catalases.

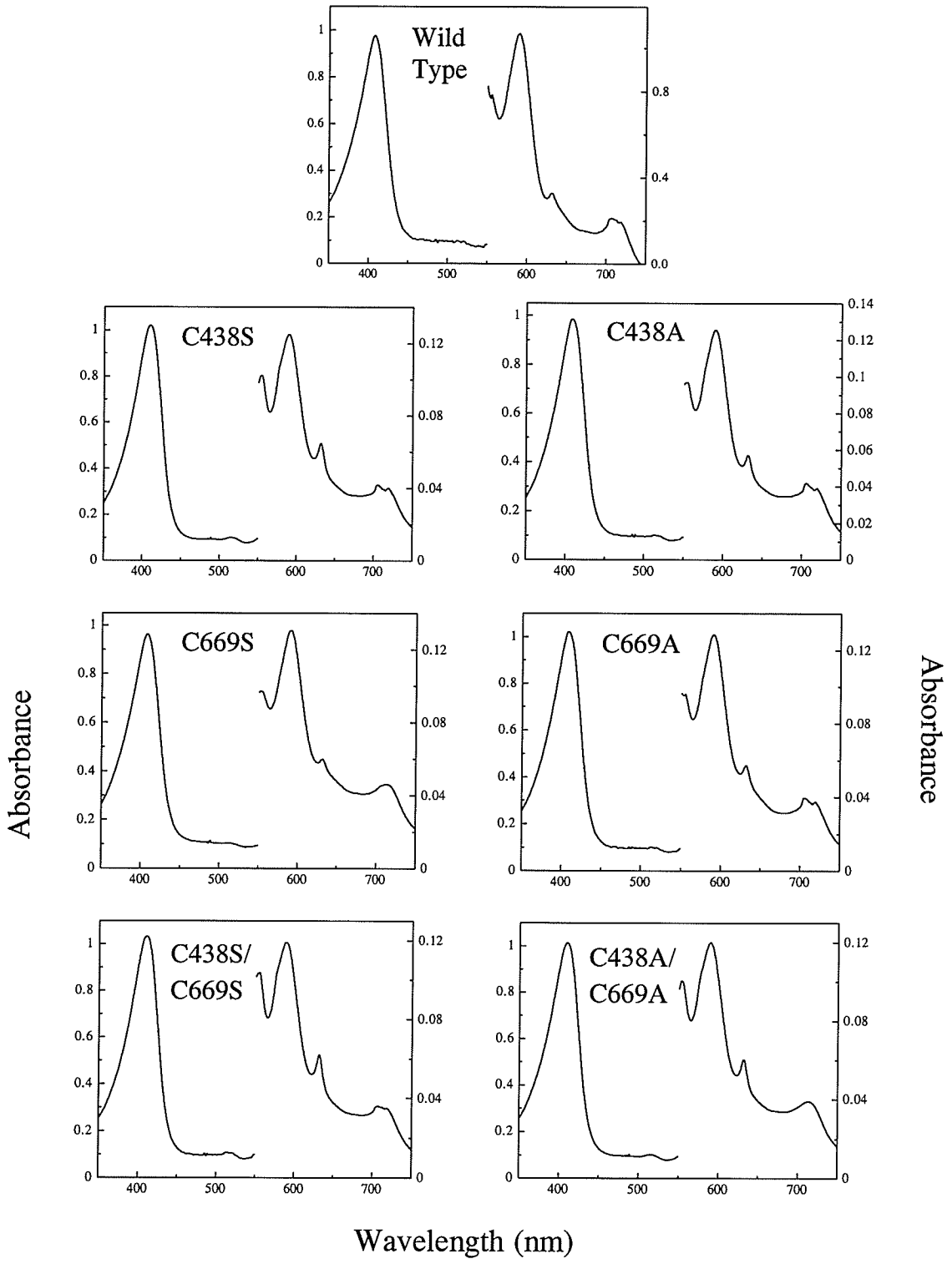


Figure 3.2.3. Visible spectra of wild type HPII and various mutants. The left axis is for the range from 350 to 550 nm while the right axis is for the range from 550 to 750 nm.

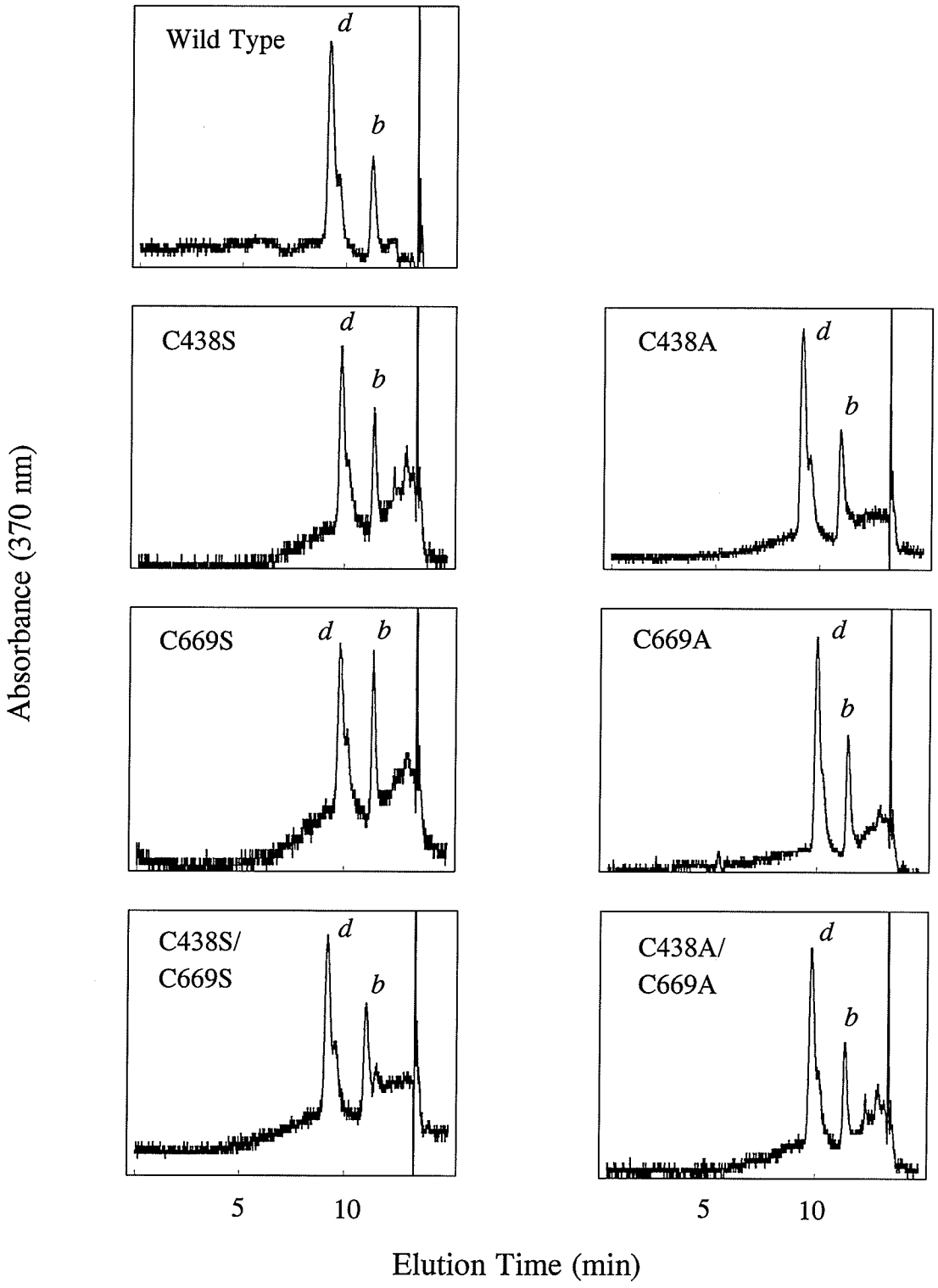


Figure 3.2.4. Elution profiles from C18 reverse phase HPLC chromatography of heme extracted from various HPII mutants. The letters '*b*' and '*d*' denote the locations of heme *b* and heme *d* respectively.

type enzyme and serine replacement mutants.

3.2.4. Quantitation of the sulfhydryl groups on HPII catalase

In order to verify that the cysteines had been replaced by either serine or alanine, the mutant enzymes were titrated with DTNB reagent using wild type HPII as a control. Surprisingly, only one of the two cysteines on HPII was reactive regardless of whether the assay was carried out with native or denatured enzyme, or enzyme that was purified from an anaerobically grown culture (Table 3.2.3). Analysis of the mutant enzymes indicated that the unreactive cysteine was C438, suggesting that this cysteine was modified resulting in a blocked -SH group. For example, the C669S and C669A enzymes did not react with DTNB whereas the C438S and C438A enzymes did. Various possibilities were investigated to determine the nature of the block. An intrasubunit disulfide bond was obviously not possible because only one of the cysteines was blocked and the residues were too far apart. An intersubunit disulfide bond was also eliminated when no difference in subunit mobility on denaturing SDS-PAGE was found with or without 2-mercaptoethanol in the sample buffer. A simple disulfide bond with another mercaptan was ruled out when treatment with 2-mercaptoethanol or sodium borohydride did not generate a free sulfhydryl group. Further studies included treating the enzyme with 0.25 M NaOH followed by neutralizing with HCl. This resulted in generation of a reactive -SH group at C438 (Table-3.2.3), eliminating the possibility of a cysteic acid which is not alkali labile. Treatment of the enzyme with methylamine or hydroxylamine failed to

Table 3.2.3. Quantitation of free sulfhydryl groups of the cysteine replacement mutants of HPII with 5,5'-dithiobis-(2-nitrobenzoic acid).

Mutant	(-SH/subunit) Native	NaOH-treated
Wild Type	1.04 ± 0.08	1.92 ± 0.20
C438S	1.09 ± 0.09	0.97 ± 0.04
C669S	<0.01	1.20 ± 0.07
C669S	<0.01 (boiled)	not determined
C438S/C669S	<0.01	0.08 ± 0.03
C438A	1.04 ± 0.01	0.90 ± 0.10
C669A	<0.01	1.17 ± 0.03
C438A/C669A	<0.01	0.09 ± 0.08
Wild Type ($-O_2$)	0.96 ± 0.07	not determined

generate a free -SH group or a hydroxamate derivative eliminating the possibilities of thiol ester and carbamoyl derivatives.

The nature of modification on C438 was further investigated by matrix assisted laser desorption/ionization mass spectroscopy (MALDI/MS). Both wild type and mutant HPII were digested with CNBr and the mixtures were analyzed by MALDI/MS. The expected mass for the C438-containing fragment is 8524 Da for unmodified C438 and 8508 Da for C438S. A total of 13 CNBr peptides would have been generated but the closest ones in mass to the 8500 Da fragment were 12070 Da and 4835 Da resulting in clearly separated fragments. MALDI/MS analysis showed that the C438 containing fragments from the wild type HPII and C669S mutant had molecular masses of 8567 $\pm$ 2 Da, 43 Da difference from the expected size (Table 3.2.4). By contrast, the S438 containing fragment from the C438S enzyme had a mass of 8505 $\pm$ 1, very close to the expected mass of 8508 Da. The possibilities for a cysteine modification with an approximate mass of 43 include acetylation to form a thiol ester (-S-$COCH_3$, Δmass = 42 Da), carbamoylation (-S-$CONH_2$, Δmass = 43 Da), hemithioacetal formation with acetaldehyde [-S-$CH(OH)CH_3$, Δmass = 44 Da) and oxidation to cysteic acid (-SO_3H, Δmass = 48 Da). As described above all of these possibilities except hemithioacetal modification have been eliminated, but more work is needed for confirmation.

Table 3.2.4. Observed mass of the CNBr peptides containing Cys438 or Ser438 determined by MALDI/MS. For calculated mass, it was assumed that cysteine and serine were unmodified.

	Mass (Da)		
Mutant	**Calculated**	**Observed**	**Difference**
C438S	8508	8505 ± 1	-3
C669S	8524	8506 ± 2	+42
C438S/C669S	8508	8505 ± 1	-3
Wild Type	8524	8567 ± 2	+43

3.3. Construction and characterization of C-terminus truncation mutants

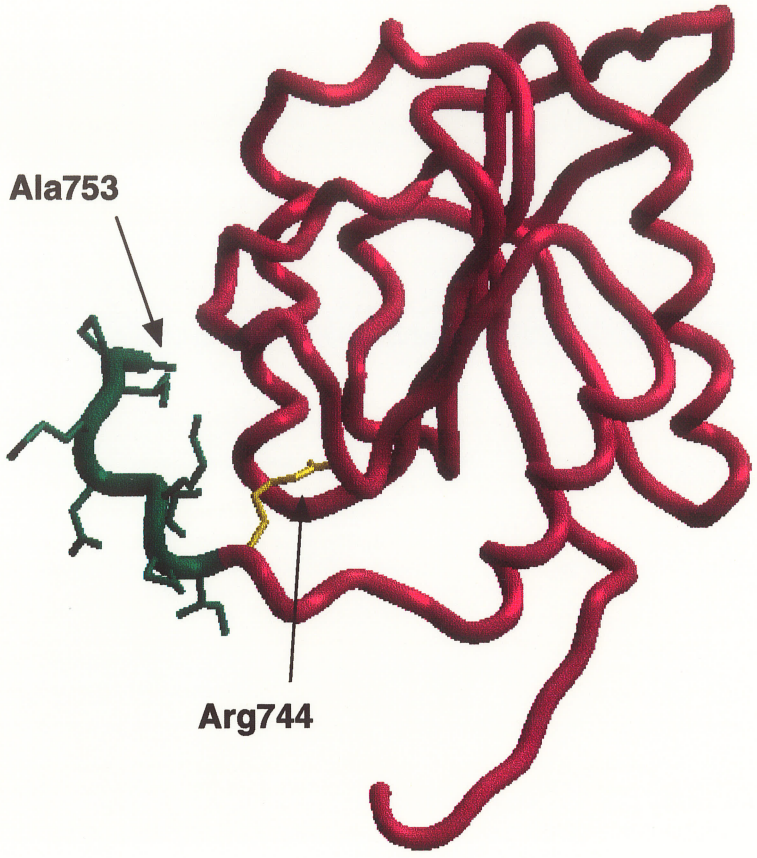
3.3.1. Introduction

HPII catalase contains additional amino acid sequences at both the N- and the C-termini that are absent in typical small subunit catalases, such as bovine liver catalase (Fita & Rossmann, 1985) and *Proteus mirabilis* catalase (Gouet *et al.*, 1995). The 175 additional amino acids at the C-terminus of HPII form a flavodoxin-like domain (Figure 3.1.1) which is also found in *Penicillium vitale* catalase. At present, the role of this extended C-terminus is not known, but it has been speculated that it may contribute to the enhanced stability of the enzyme under harsh chemical and physical conditions as well as interfering with compound II formation and eliminating the need for NADPH binding (Loewen, 1996). In order to study the role of this extended region and determine the minimum required amino acid sequence of HPII a number of C-terminus truncation mutations were constructed and characterized.

3.3.2. Characterization of the mutant proteins

Initially, HPII was truncated progressively from the C-terminus by inserting stop codons in the gene in place of the codons for K750, K747, P746, I745, R744 and W742. At 37°C, the truncated enzymes up to P746 accumulated normal amounts of protein. However, the P746Stop enzyme was produced at 50% of the wild type level, the I745Stop mutant was produced at 15% and the R744Stop mutant did not accumulate protein. Lowering the growth temperature to 28°C increased the yield for

Figure 3.3.1. The C-terminus of HPII. The protein backbone of the C-terminal domain of the HPII subunit from residue 575 to 744 is colored red. The protein backbone from residue 745 to 753 that can be removed is colored green. The side chains of the terminal 10 residues are shown and the location of the side chain of Arg744 projecting into the β-barrel where it forms ionic bonds is highlighted.


Ala753
Arg744

mutants I745Stop and P746Stop closer to the wild type level (Table 3.3.1) and resulted in detectable activity (5.6% and 1.5% respectively) for the R744Stop and W742Stop mutants. These results indicate that the last nine residues of HPII up to R744 were dispensable at least in part for formation of an active enzyme. The structure of this region is shown in Figure 3.3.1.

Once R744 was identified as a critical residue, the role of the arginine side group was investigated. R744 was changed first to lysine to test the importance of its positive charge although modelling indicated that the lysine side chain could not fulfil the same charge role as arginine, and then to alanine. The mutagenesis of R744 was carried out alone and in combination with a stop codon at I745 to generate four mutants: R744A, R744A/I745Stop, R744K and R744K/I745Stop. Except for R744A which accumulated twice as much, this group of mutants all produced about the same amount of protein as the R744Stop mutant confirming that the complete arginine side chain was required for efficient folding of HPII.

In order to determine if the whole flavodoxin-like domain was essential, the V603Stop mutant was constructed. Analysis of protein extracts revealed that this mutant did not accumulate any HPII-like protein even at 28°C. It was therefore possible to conclude that the entire C-terminus of 150 amino acids is required for folding of the HPII subunit into an active structure.

The purifications of all ten mutants were carried out and their purities were analyzed by SDS-PAGE (Figure 3.3.2). The mutants truncated at I745 or longer were produced in wild type quantities and were more than 95% pure. Furthermore,

Table 3.3.1. Comparison of the effect of the culture growth temperature on the production of the wild type and various mutant proteins of HPII. Cultures of *E. coli* strain UM255 containing plasmids encoding mutant HPII proteins were shaken at 37°C for 16-17 h or at 28°C for 20-22 h in LB medium supplemented with ampicillin.

Mutant	Catalase Activity (units/mg dry cell weight) 37°C	28°C	Ratio of activities 28°C/37°C
V603Stop	<0.1	<0.1	N/A
W742Stop	<0.1	6.5 ± 1.3	N/A
R744Stop	<0.1	24 ± 4	N/A
R744A	<1.0	54 ± 7	N/A
R744A/I745Stop	<0.1	24 ± 4	N/A
R744K	<0.1	21 ± 2	N/A
R744K/I745Stop	<0.1	29 ± 5	N/A
I745Stop	59 ± 5	288 ± 30	4.9
P746Stop	226 ± 24	353 ± 24	1.6
K747Stop	367 ± 26	344 ± 33	0.9
K750Stop	436 ± 33	345 ± 28	0.8
Wild Type	425 ± 51	388 ± 59	0.9

N/A: not applicable.

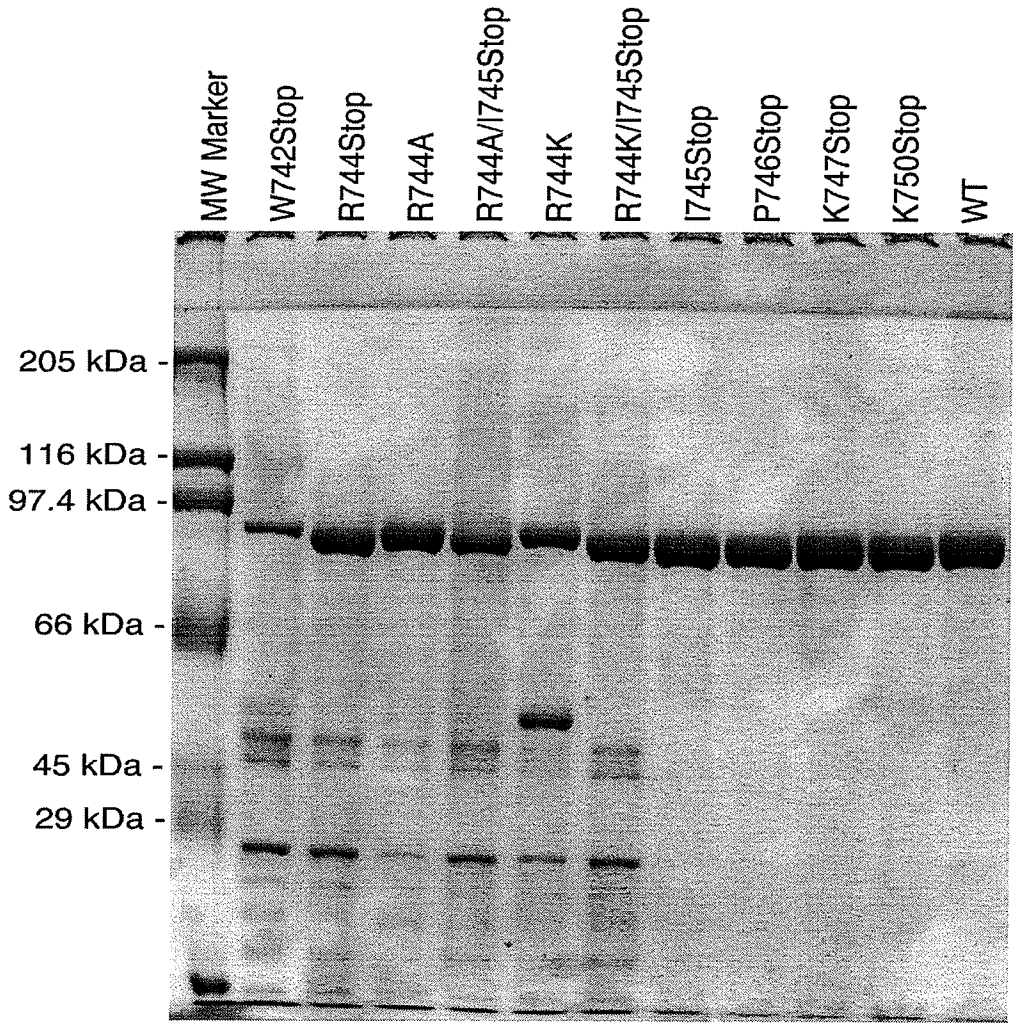


Figure 3.3.2. SDS-polyacrylamide gel electrophoresis analysis of various mutants of HPII following purification. Samples of approximately 10 μg were electrophoresed in an 8% gel and stained with Coomassie Brillant Blue.

the physical and kinetic properties of the I745Stop and longer mutants resembled those of the wild type enzyme, having similar specific activities and apparent Km, V_{max} and k_{cat} values (Table 3.3.2). The profiles of the saturation curves (Figure 3.3.3), the visible spectra (Figure 3.3.4) and the HPLC heme chromatography (Figure 3.3.5) of these longer mutants were also similar to those of the wild type enzyme. On the other hand, the purities of the R744Stop and shorter mutants were significantly lower because their lower yields made purification more difficult and repeated attempts to improve the purity were unsuccessful. The kinetic properties of all mutants were determined (Table 3.3.2) revealing lower specific activities for the less pure enzymes as expected. If the specific activities were corrected using the A_{407}/A_{280} ratio as a measure of purity, the less pure proteins appeared to be as active as the wild type enzyme (data not shown). Kinetic experiments carried out with the low purity mutants revealed similar Vi vs. [S] curves (Figure 3.3.3), similar apparent Km values but lower V_{max} and k_{cat} values, presumably as a result of their low purity. The visible spectra of the mutant proteins were also similar to the wild type enzyme with all mutants having a Soret band as well as a heme *d* peak at 590 nm (Figure 3.3.4). In addition, the HPLC profiles of the extracted hemes from these mutants were similar to the wild type enzyme, having both heme *d* and heme *b* peaks (Figure 3.3.5).

Table 3.3.2. Comparison of the kinetic and physical properties of purified wild type and HPII mutant proteins.

Mutant	Specific Activity (u/mg)	A_{407}/A_{280}	Apparent Km (mM)	Apparent V_{max} (mol/min)	Apparent k_{cat} (sec^{-1})
W742Stop	2 624 ± 9	0.185	36.7 ± 3.4	3.5 ± 0.3	4.9 ± 0.4 x 10^6
R744Stop	8 600 ± 116	0.543	30.6 ± 2.8	11.1 ± 0.9	1.6 ± 0.2 x 10^7
R744A	8 786 ± 288	0.597	28.6 ± 0.8	11.1 ± 0.2	1.6 ± 0.1 x 10^7
R744A/I745Stop	6 929 ± 326	0.475	29.5 ± 0.9	8.9 ± 0.4	1.3 ± 0.1 x 10^7
R744K	5 832 ± 378	0.361	32.2 ± 3.4	9.0 ± 0.5	1.3 ± 0.1 x 10^7
R744K/I745Stop	9 284 ± 347	0.579	40.0 ± 5.5	15.3 ± 2.3	2.2 ± 0.4 x 10^7
I745Stop	13 753 ± 566	0.832	26.7 ± 1.1	18.8 ± 2.9	2.7 ± 0.5 x 10^7
P746Stop	15 022 ± 891	0.850	34.8 ± 4.7	25.5 ± 5.6	3.8 ± 0.5 x 10^7
K747Stop	15349 ± 921	0.882	33.4 ± 1.8	25.9 ± 3.3	3.6 ± 0.4 x 10^7
K750Stop	15 067 ± 1 168	0.877	34.0 ± 7.9	25.7 ± 9.3	3.6 ± 1.3 x 10^7
Wild Type	14 322 ± 690	0.912	27.8 ± 5.4	17.8 ± 2.9	2.5 ± 0.4 x 10^7

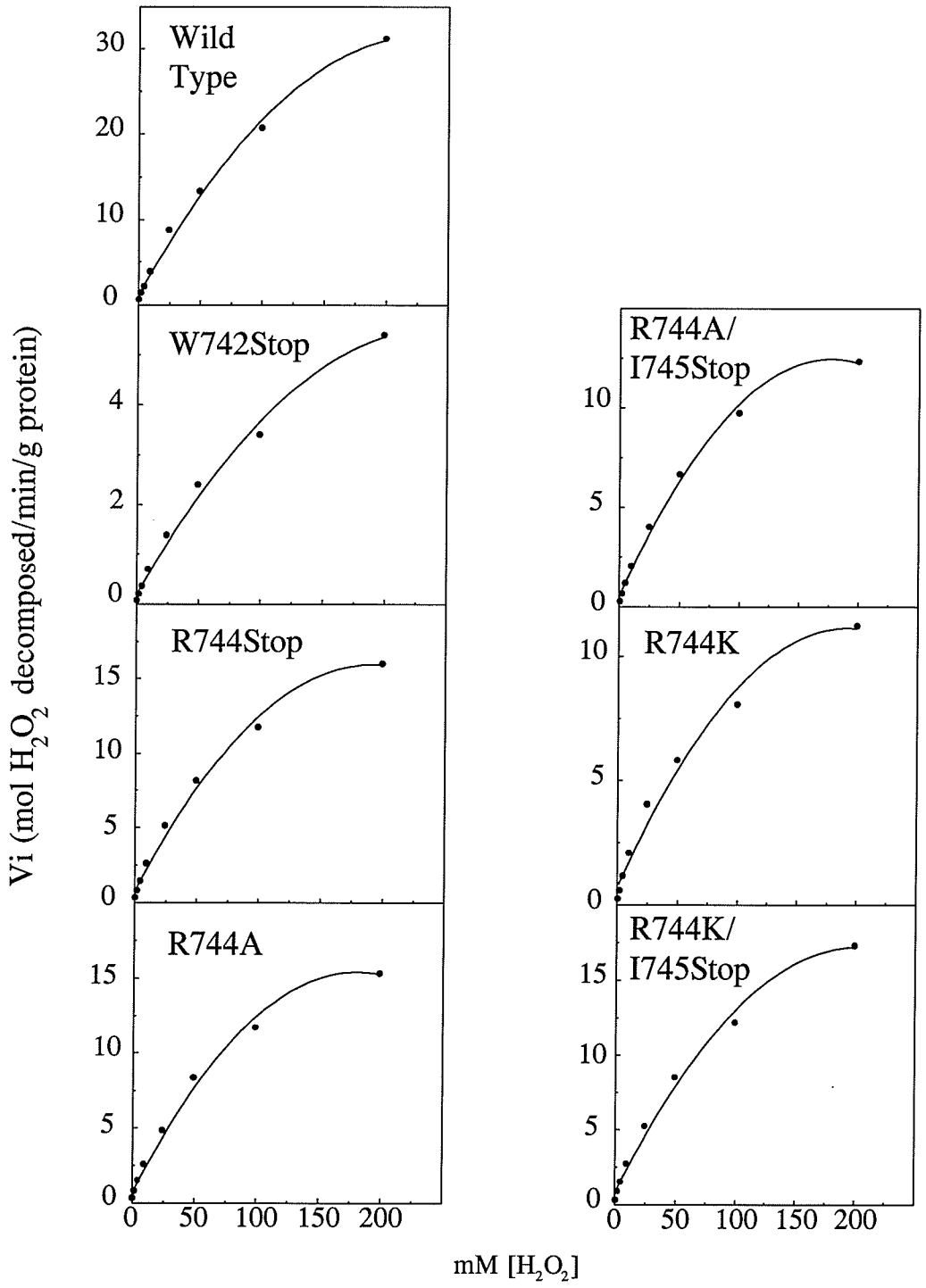


Figure 3.3.3. Comparison of the effect of hydrogen peroxide concentrations on the initial velocities (Vi) of wild type HPII and various mutants.

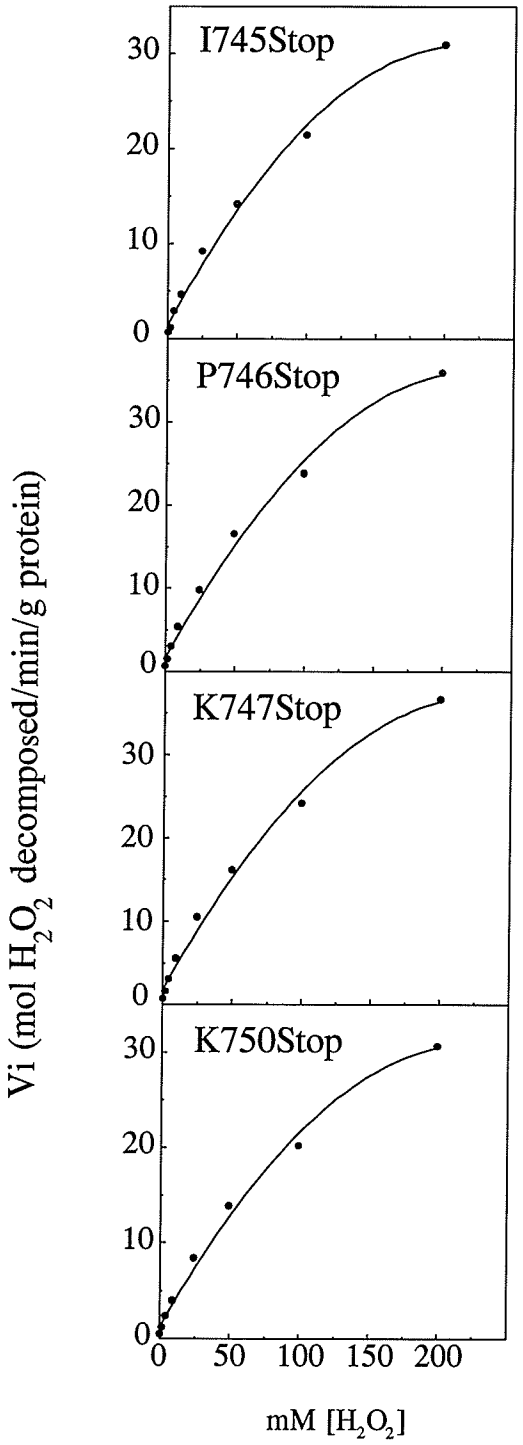


Figure 3.3.3. Continued.

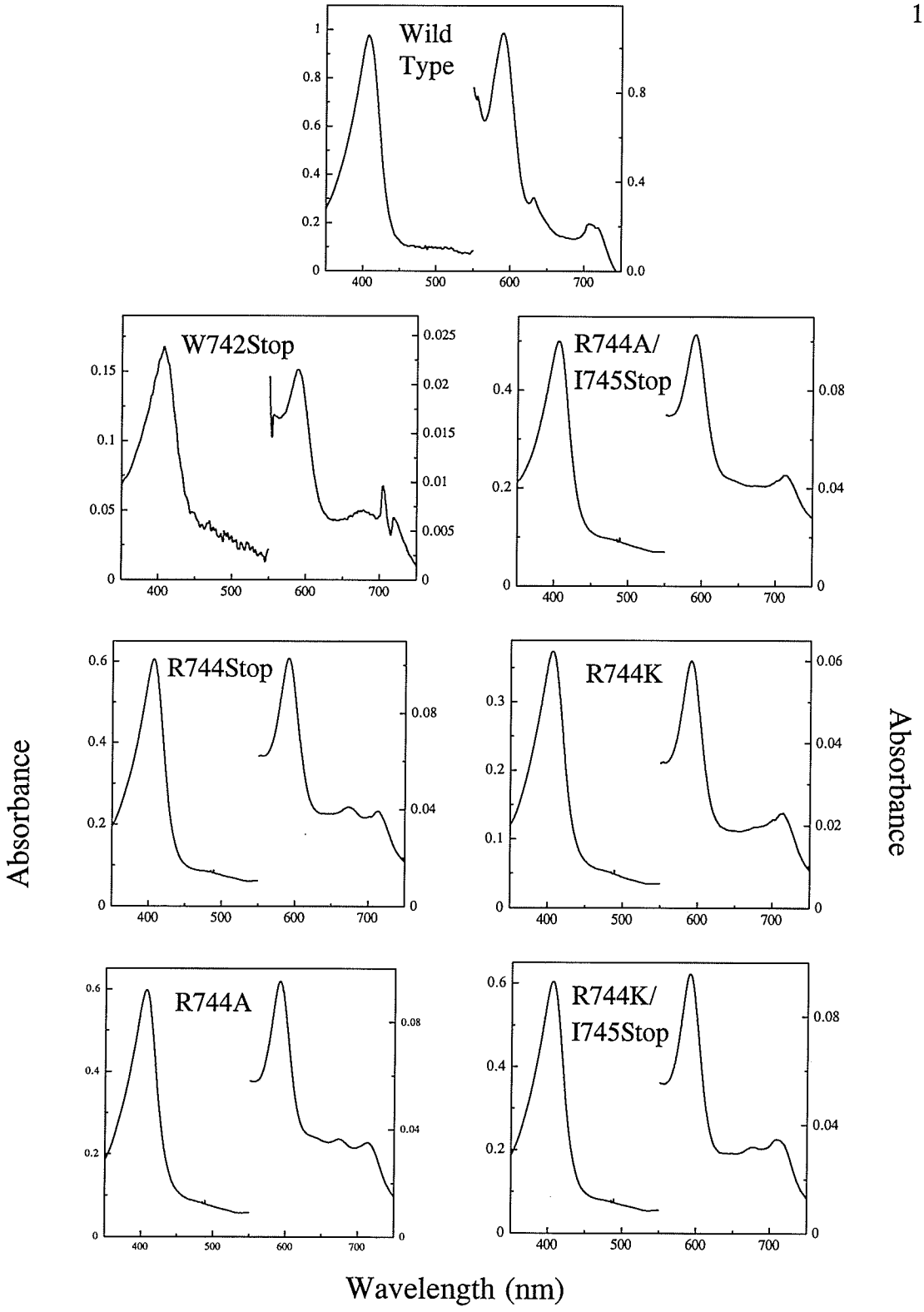


Figure 3.3.4. Visible spectra of wild type HPII and various mutants. The left axis is for the range from 350 to 550 nm while the right axis is for the range from 550 to 750 nm.

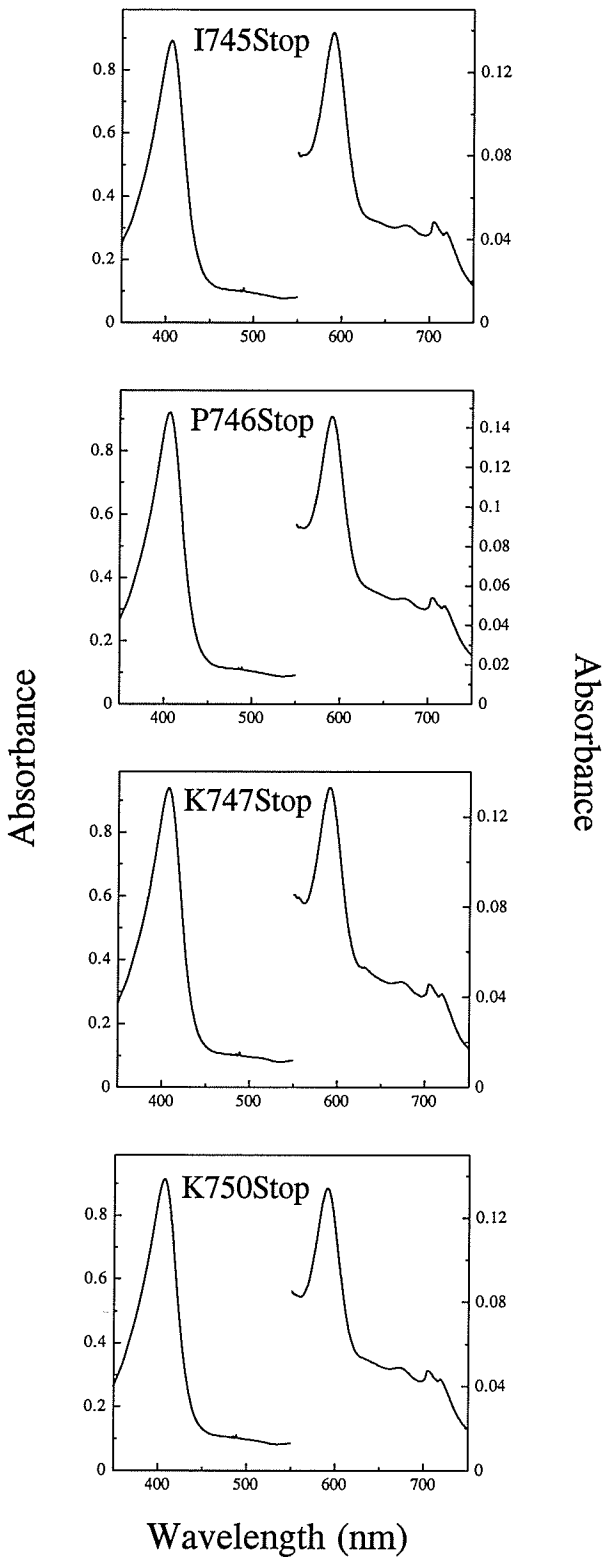


Figure 3.3.4. Continued.

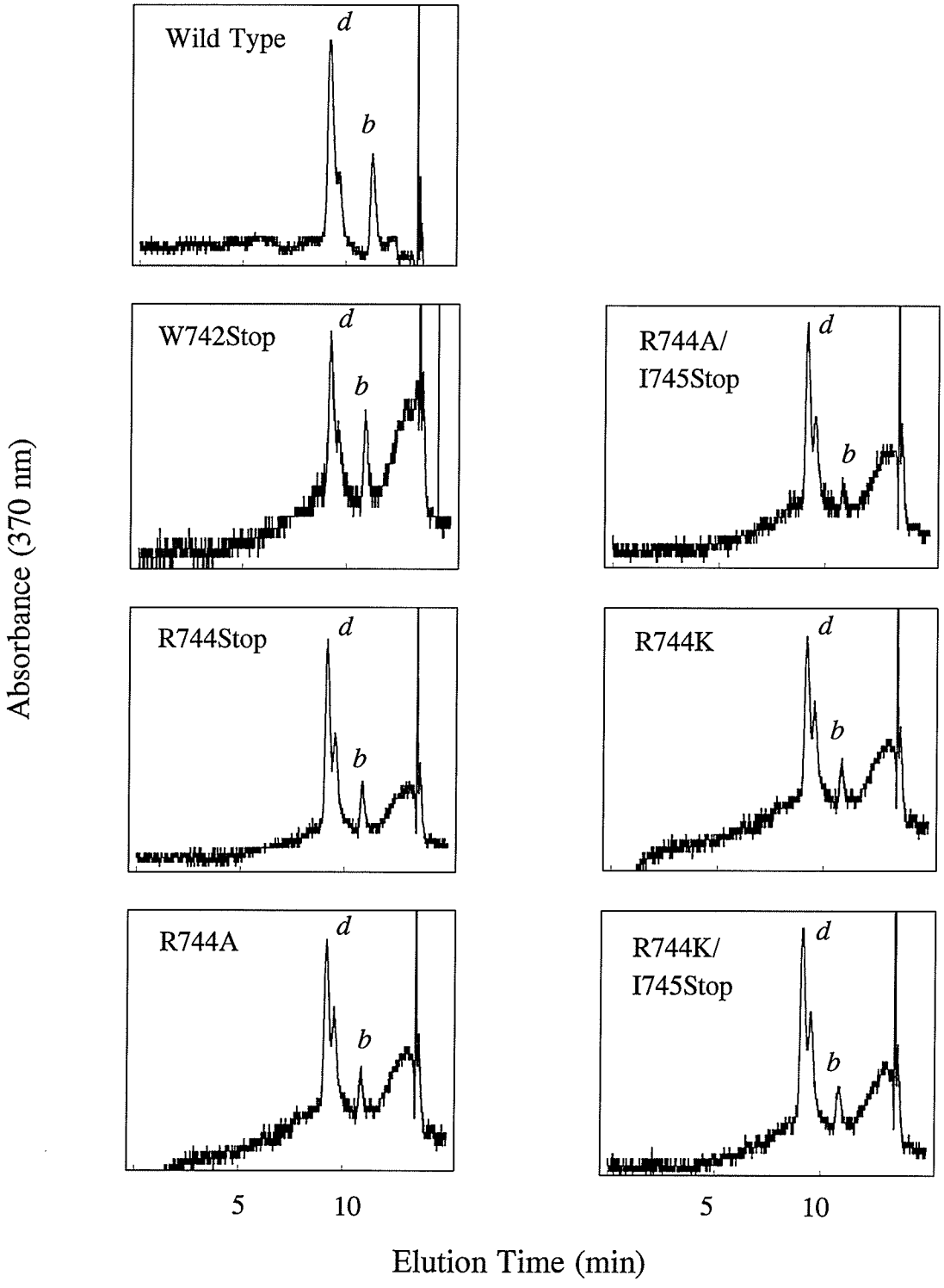


Figure 3.3.5. Elution profiles from C18 reverse phase HPLC chromatography of heme extracted from various HPII mutants. The letters '*b*' and '*d*' denote the locations of heme *b* and heme *d* respectively.

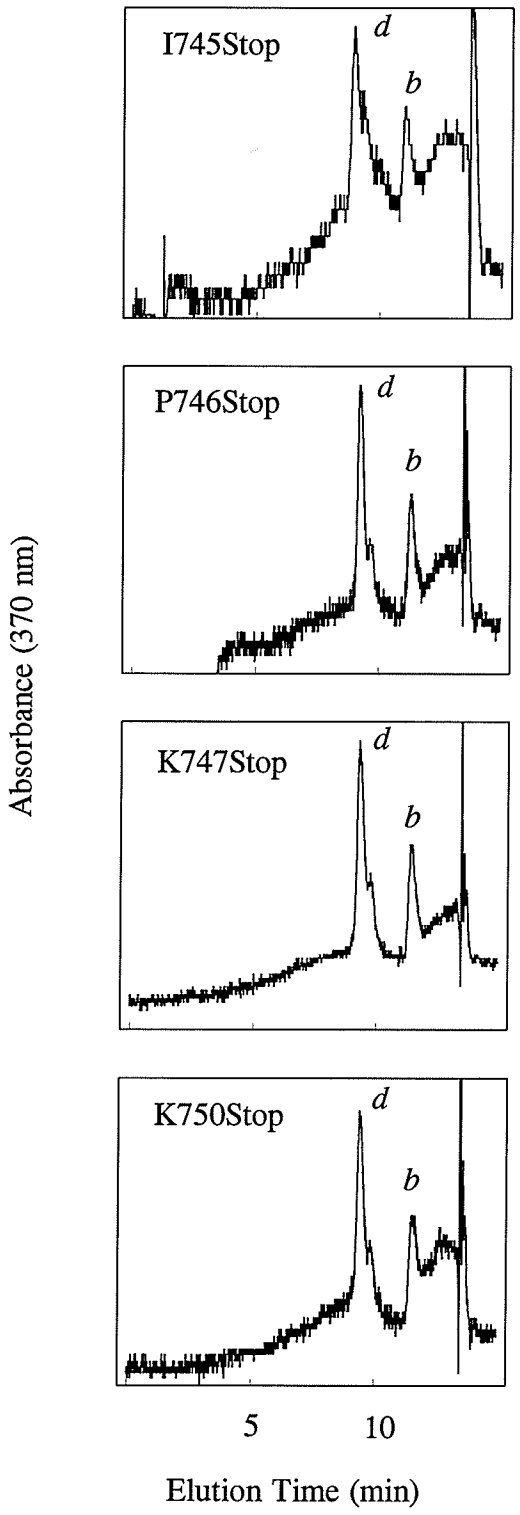


Figure 3.3.5. Continued.

3.4. Construction and characterization of the mutants that might affect heme orientation

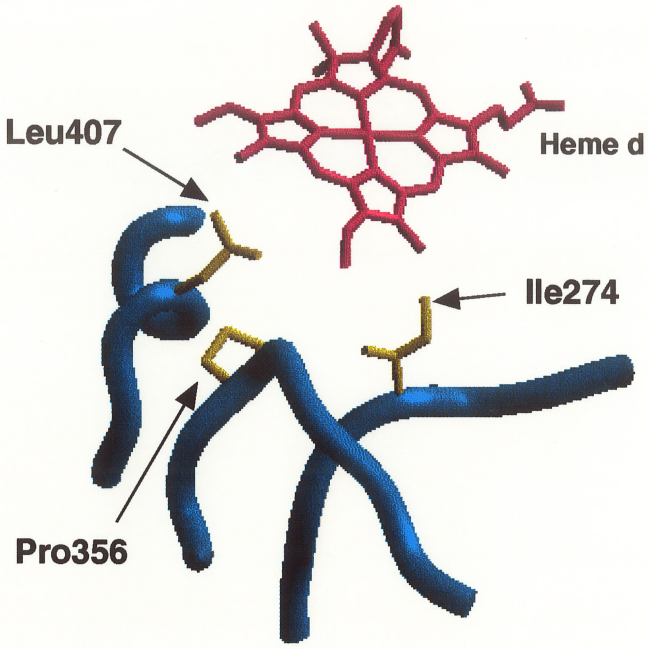
3.4.1. Introduction

In most catalases the hydrophobic residues surrounding the heme group are well conserved as is the orientation of the heme. HPII differs from most catalases in having heme *d* in place of heme *b* (Chiu *et al.*, 1989) in an inverted or flipped conformation (Bravo *et al.*, 1995). The heme *d* structure in HPII and *Penicillium vitale* enzyme has been determined to be *cis*-hydroxychlorin γ-spirolactone (Murshudov *et al.*, 1996). The inversion of the heme is presumably controlled by the heme contact residues including I41, V209, P291 and L342 in the *P. vitale* enzyme and I274, P356 and L407 in HPII (Figure 3.4.1) which differ significantly from their bovine counterparts M60, S216, L298 and M349. In order to investigate the importance of these residues in determining the orientation of the heme, a number of mutants of HPII were constructed. Specifically HPII residues were changed to the bovine enzyme counterparts including single, double and triple mutants: I274S, P356L, L407M, I274S/P356L, I274S/L407M, P356L/L407M, I274S/P356L/L407.

3.4.2. Characterization of the mutant proteins

The I274S double and triple mutants produced no detectable enzyme at 37°C and barely detectable activity at 28°C. Furthermore, HPII-like protein was not visible in the crude extracts of these mutants separated by SDS-PAGE. Therefore, no further

Figure 3.4.1. Organization of the heme-binding residues of HPII. The protein backbone of the relevant sections is colored blue and and the residue side chains are highlighted.


Leu407
Heme d
Ile274
Pro356

attempts were made to purify protein from these mutants. On the other hand, the I274S, P356L and P356L/L407M enzymes were produced in small but detectable amounts (2%, 14% and 3% of wild type levels respectively) quantities at 37°C and higher (14%, 50% and 23% of wild type levels respectively) at 28°C (Table 3.4.1). The L407M mutant accumulated in near wild type levels at both 37°C and 28°C. These mutant proteins were purified and visualized by SDS-PAGE revealing purities similar to the wild type protein. Only the I274S enzyme contained a small amount of contaminating protein (Figure 3.4.2). The purified P356L and L407M and P356L/L407M enzymes had specific activities and kinetic properties (apparent Km, V_{max} and k_{cat}) similar to the wild type enzyme (Table 3.4.2) while the I274S enzyme had a lower specific activity and much lower V_{max} and k_{cat} values despite its Km remaining at the wild type levels. Whether these results were a result of abnormal heme contacts or some other changes in protein conformation could not be determined.

All four mutant enzymes had substrate saturation curves similar to the wild type enzyme (Figure 3.4.3). Moreover, the visible spectra of all four purified mutant enzymes were similar to the wild type enzyme including a Soret band at 407 nm and a heme *d* peak at 590 nm (Figure 3.4.4). There are minor variations in the spectra between 630 nm to 700 nm that cannot be readily explained because the HPLC chromatograms of the extracted hemes from all four mutant proteins contained normal heme *d* and heme *b* peaks (Figure 3.4.5).

Table 3.4.1. Comparison of the effect of the culture growth temperature on the production of the wild type and various mutant proteins of HPII. Cultures of *E. coli* strain UM255 containing plasmids encoding mutant HPII proteins were shaken at 37 °C for 16-17 h or at 28 °C for 20-22 h in LB medium supplemented with ampicillin.

Mutant	Catalase Activity (units/mg dry cell weight) 37°C	28°C	Ratio of activities 28°C/37°C
I274S	10 ± 2	55 ± 10	5.5
P356L	60 ± 3	203 ± 20	3.4
L407M	363 ± 45	329 ± 40	0.9
I274S/P356L	<0.1	1.5 ± 0.5	N/A
I274S/L407M	<0.1	3.0 ± 1.0	N/A
P356L/L407M	13 ± 2	91 ± 9	7.0
I274S/P356L/L407M	<0.1	<0.3	N/A
Wild Type	425 ± 51	388 ± 59	0.9

N/A: not applicable.

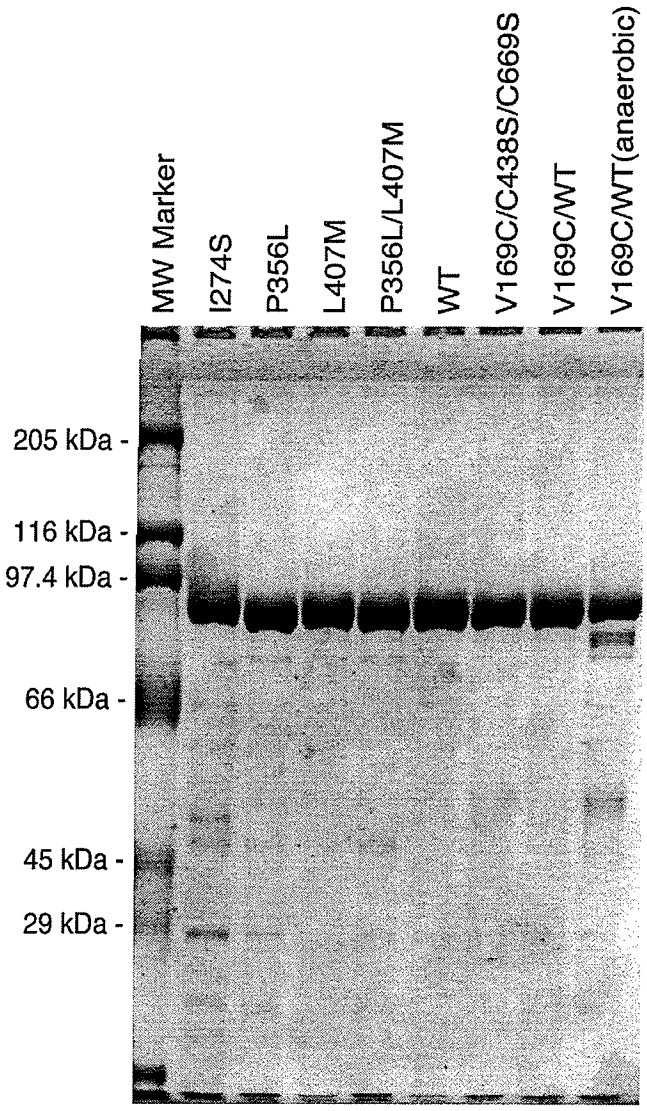


Figure 3.4.2. SDS-polyacrylamide gel electrophoresis analysis of various mutants of HPII following purification. Samples of approximately 10 μg were electrophoresed in an 8% gel and stained with Coomassie Brillant Blue.

Table 3.4.2. Comparison of the kinetic and physical properties of purified wild type and HPII mutant proteins.

Mutant	Specific Activity (u/mg)	A_{407}/A_{280}	Apparent Km (mM)	Apparent V_{max} (mol/min)	Apparent k_{cat} (sec^{-1})
I274S	3 416 ± 238	0.561	25.2 ± 3.4	3.9 ± 0.6	5.5 ± 0.8 x 10^6
P356L	13 220 ± 849	0.788	28.1 ± 7.9	18.5 ± 5.5	2.6 ± 0.8 x 10^7
L407M	12 679 ± 308	0.812	29.1 ± 4.6	17.0 ± 3.1	2.4 ± 0.4 x 10^7
P356L/L407M	12 437 ± 786	0.786	29.6 ± 5.3	16.6 ± 3.0	2.4 ± 0.4 x 10^7
Wild Type	14 322 ± 690	0.912	27.8 ± 5.4	17.8 ± 2.9	2.5 ± 0.4 x 10^7

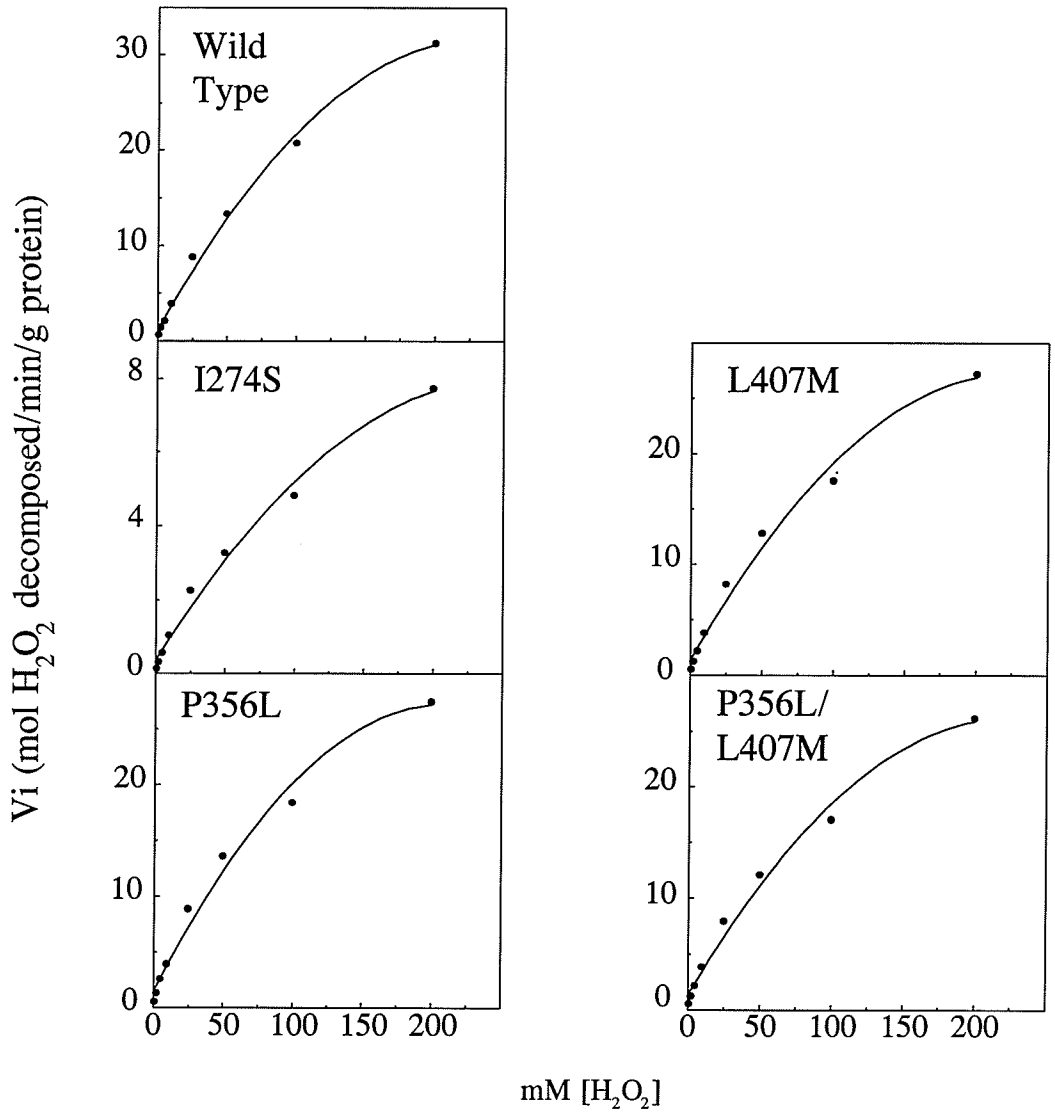


Figure 3.4.3. Comparison of the effect of hydrogen peroxide concentrations on the initial velocities (Vi) of the various mutants and wild type HPII.

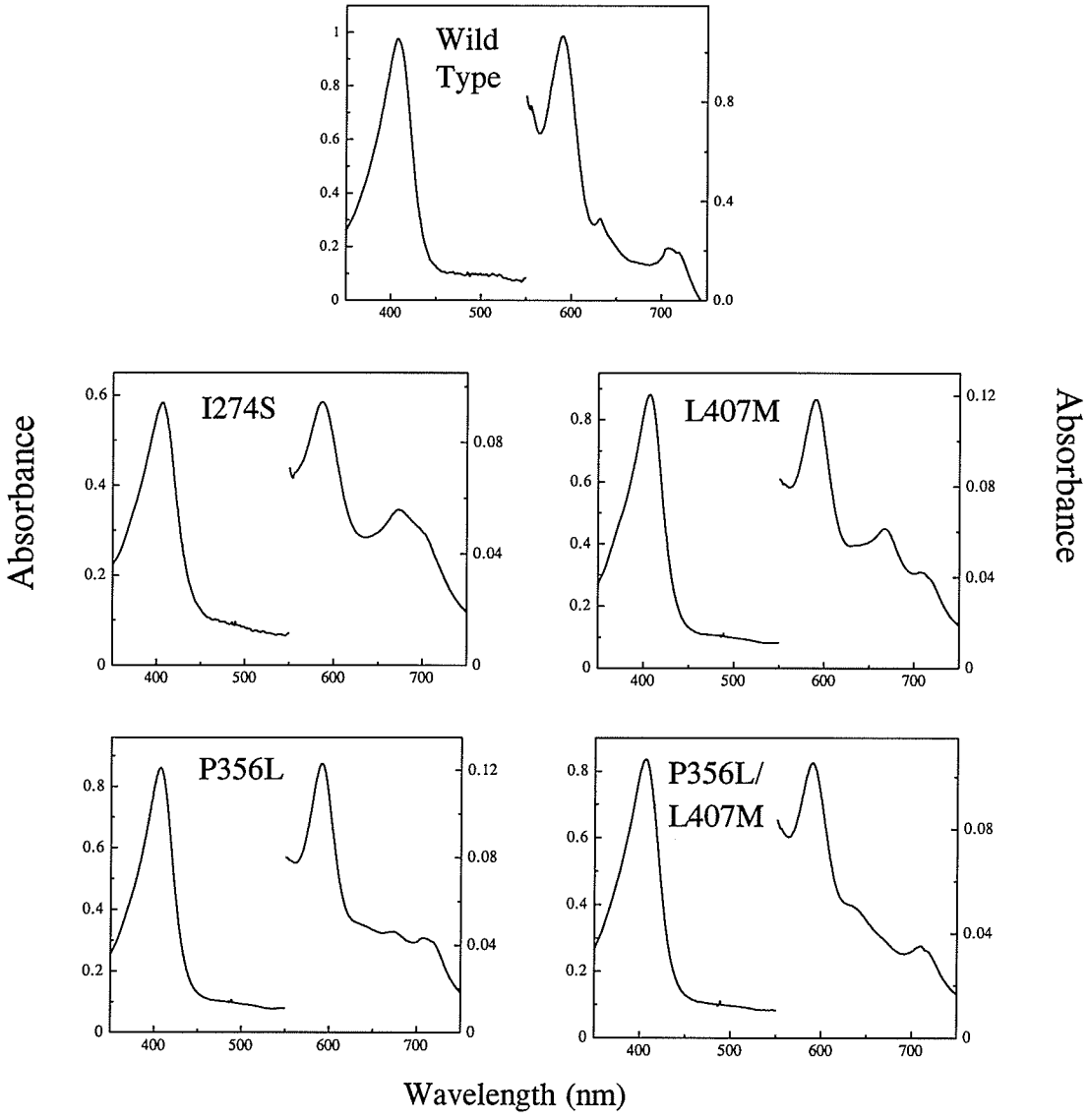


Figure 3.4.4. Visible spectra of wild type HPII and various mutants. The left axis is for the range from 350 to 550 nm while the right axis is for the range from 550 to 750 nm.

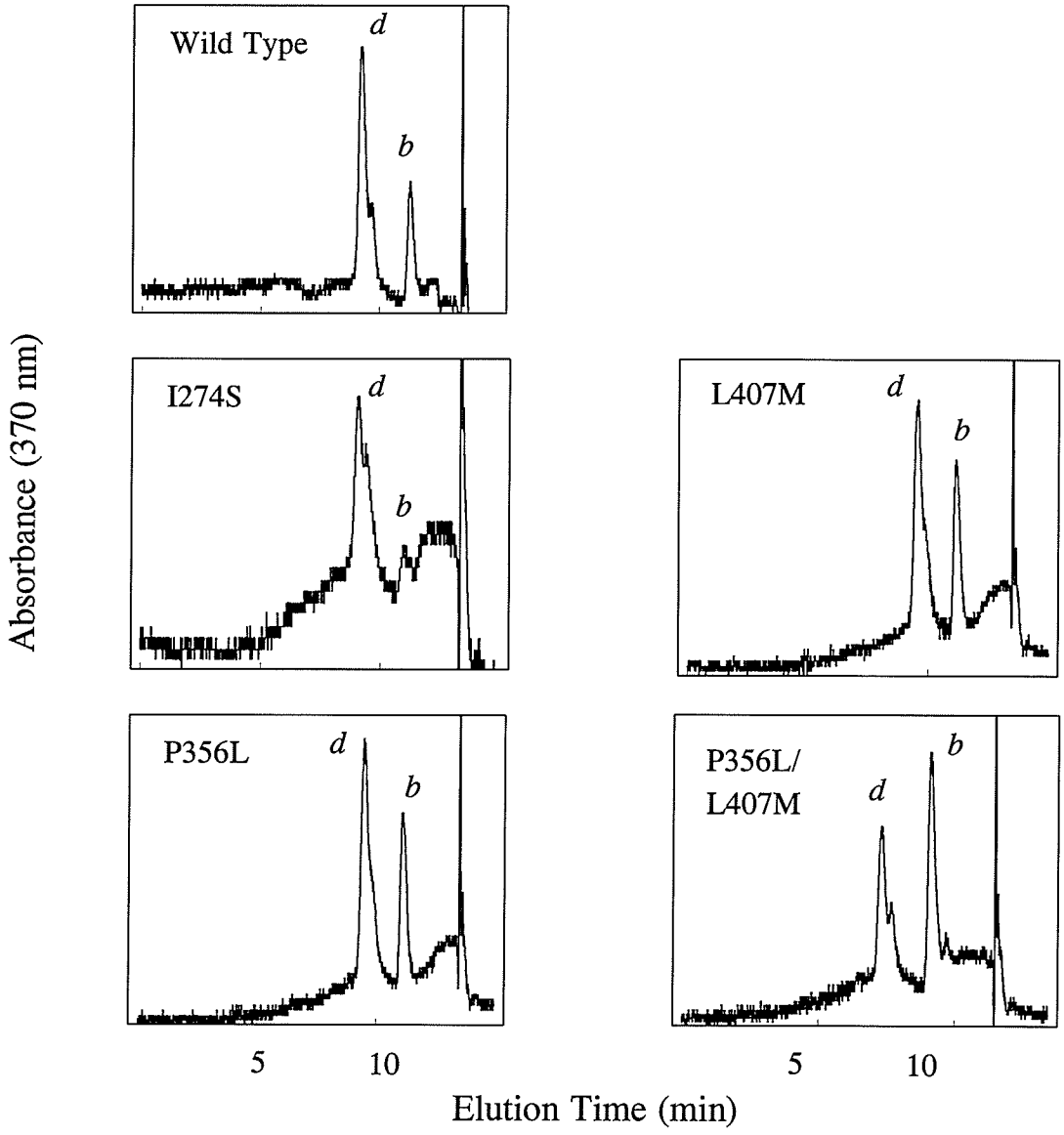


Figure 3.4.5. Elution profiles from C18 reverse phase HPLC chromatography of heme extracted from various HPII mutants. The letters '*b*' and '*d*' denote the locations of heme *b* and heme *d* respectively.

3.5. Construction and characterization of a mutant blocking the channel to the active site

3.5.1. Introduction

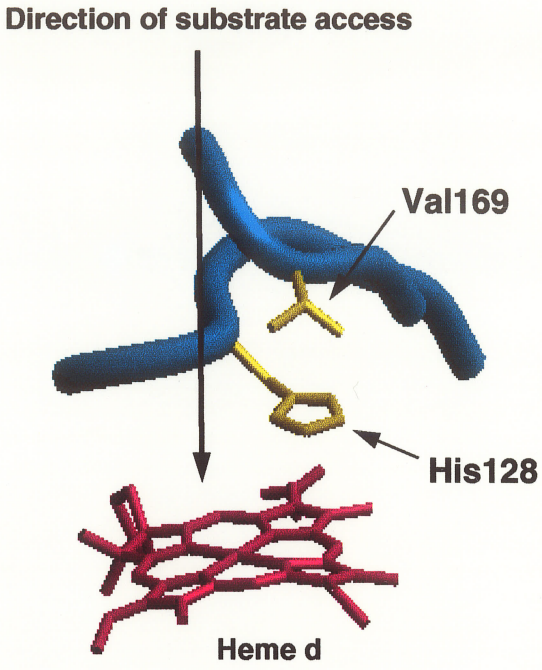
The core domain of HPII catalase is well conserved among catalases. This region includes a channel that leads to the active site that is 30 Å long and 15 Å wide situated adjacent to a β-barrel in both the bovine enzyme and HPII (Murthy *et al.*, 1981). The residues lining the channel are largely conserved with polar residues near the entrance and hydrophobic residues towards the interior. Similarly, the fifth heme ligand Y415 and active site residues H128, S167 and N201 of HPII are conserved. Consequently, the overall structure of the channel and active sites of the enzymes are very similar.

In order to study the importance of the residues in the channel leading to the active site, mutagenesis of a number of residues was proposed. The first residue chosen was V169 situated deep in the channel, close to the active site (Figure 3.5.1). It was changed to cysteine so that it would be possible to subsequently modify the -SH and increase the bulkiness of the residue to study the effect of progressive channel blockage. The V169C mutation was also combined with the C438S/C669S double mutation to remove any background influence of the other cysteines.

3.5.2. Characterization of the V169C enzymes

Cells carrying a plasmid encoding the V169C enzyme, regardless of whether

Figure 3.5.1. Orientation of Val169 relative to His128 in the active site of HPII. The protein backbone is colored blue and heme *d* is shown in red. The residue side chains are highlighted.


Direction of substrate access
Val169
His128
Heme d

as a single mutant or in combination with the C438S/C669S mutations, exhibited only 1-2 u/mg dry cell weight catalase activity at both 28°C and 37°C. Analysis of crude extracts by SDS-PAGE revealed normal or wild type level of the protein suggesting that the mutant protein was largely inactive. Both V169C and V169C/C438S/C669S were purified and characterized revealing similar properties and A_{407}/A_{280} ratios to the wild type enzyme (Table 3.5.1 and Figure 3.4.2). The specific activities were 1000 times lower than for the wild type enzyme and even purification from an anaerobically grown culture caused only a 20-fold increase in its specific activity, still only 1-2 % of the wild type enzyme. Surprisingly, both V169C proteins were brown in color, not green, suggesting the presence of heme *b* and a lack of heme *b* to heme *d* conversion. The spectral analysis confirmed this conclusion, revealing a typical heme *b* peak at 630 nm and the absence of the heme *d* peak at 590 nm (Figure 3.5.2), as did HPLC analysis of the extracted heme, which revealed exclusively heme *b* (Figure 3.5.3). The visible spectrum and the HPLC chromatogram of anaerobically grown V169C also revealed only heme *b*.

3.5.2.a. Titration of the free sulfhydryl groups in the V169C enzymes

Analysis of free sulfhydryl group in the V169C and V169C/C438S/C669S enzymes revealed that the cysteine at position 169 was unreactive suggesting modification of the SH. (Table 3.5.2). Treatment of the mutants with NaOH resulted in the production of approximately half of the expected amount of free -SH. Whether the partial reactivity was the result of only partial regeneration of the -SH or to steric

Table 3.5.1. Comparison of the specific activities and the A_{407}/A_{280} ratio of the purified V169C enzymes and wild type HPII.

Mutant	**Specific activity (u/mg protein)**	**A_{407}/A_{280}**
V169C/C438S/C669S	16 ± 1	1.014
V169C/Wild Type	10 ± 1	0.961
V169C/Wild Type (anaerobic)	197 ± 9	0.646
Wild Type	14 322 ± 690	0.912

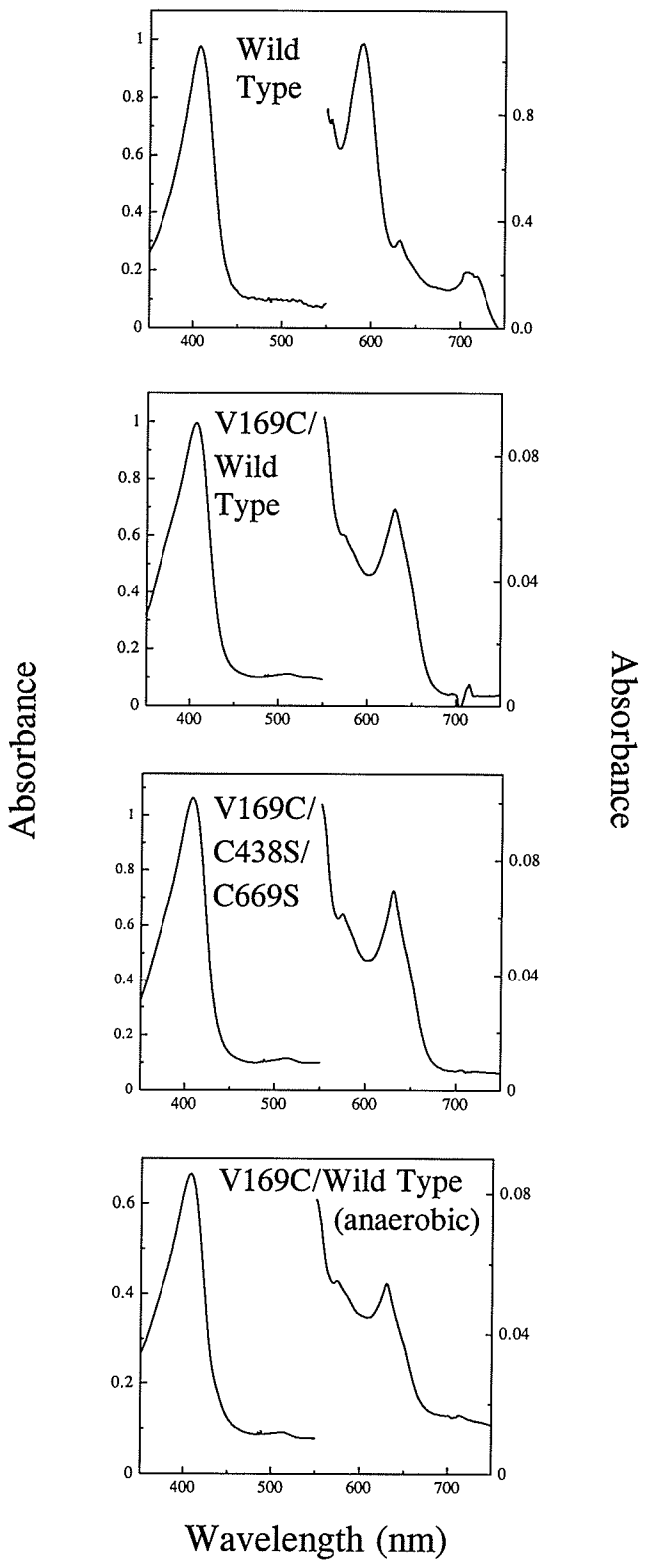


Figure 3.5.2. Visible spectra of wild type HPII and V169C mutants. The left axis is for the range from 350 to 550 nm while the right axis is for the range from 550 to 750 nm.

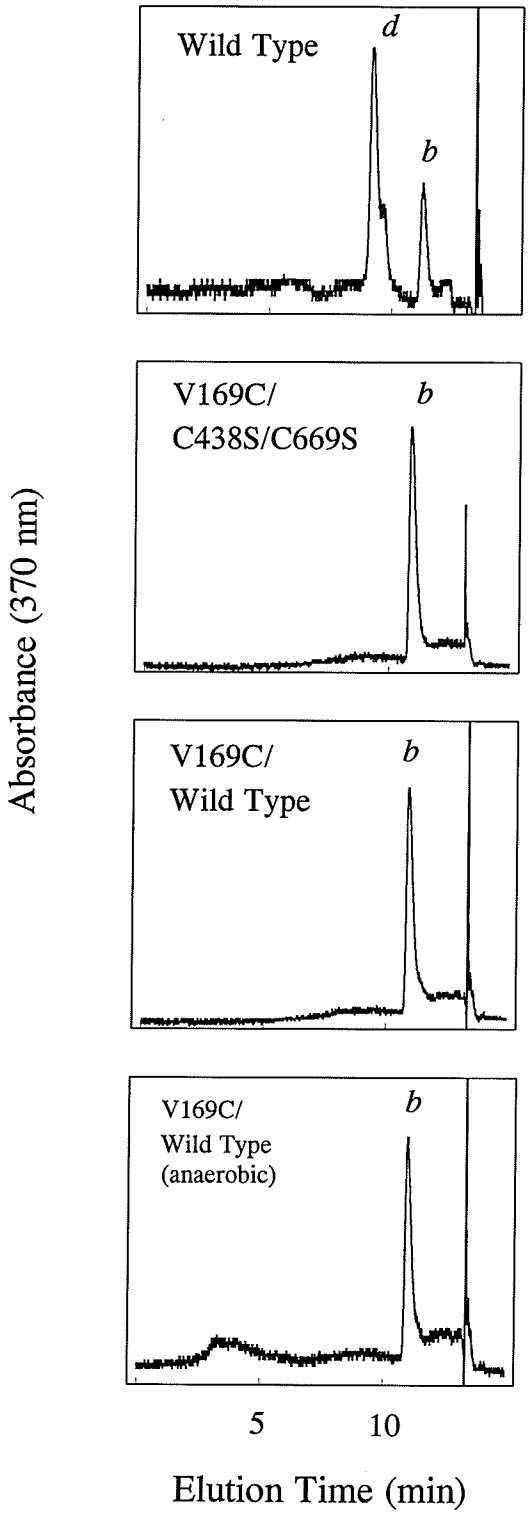


Figure 3.5.3. Elution profiles from C18 reverse phase HPLC chromatography of heme extracted from V169C enzymes and wild type HPII. The letters '*b*' and '*d*' denote the locations of heme *b* and heme *d* respectively.

Table 3.5.2. Quantitation of free sulfhydryl groups in the V169C enzymes using 5,5'-dithiobis-(2-nitrobenzoic acid).

Mutant	(-SH/subunit) Native	NaOH-treated
V169C/C438S/C669S	0.0	0.50 ± 0.08
V169C/WT	1.14 ± 0.20	2.64 ± 0.11
V169C/WT ($-O_2$)	1.02 ± 0.07	2.68 ± 0.13
Wild Type	1.04 ± 0.08	1.92 ± 0.20

inaccessibility to DTNB deep in the heme channel was not determined. The fact that NaOH did generate free -SH indicates that the modification did not involve either a disulfide bond or oxidation to cysteic acid.

3.5.2.b. Circular dichroism spectroscopy analysis of the V169C enzymes

Circular dichroism spectroscopy analysis was performed on both V169C enzymes (Figure 3.5.4) revealing a significant decrease in the β-sheet structure content from 25% to 11-13%. Even the enzyme from the anaerobic cells contained only 17% β-sheet (Table 3.5.4). The loss in the β-sheet content was accompanied by increases in both α-helical and unordered structure of 5 to 8%. These data suggested significant changes in the internal structure of the enzymes as a result of the V169C mutation.

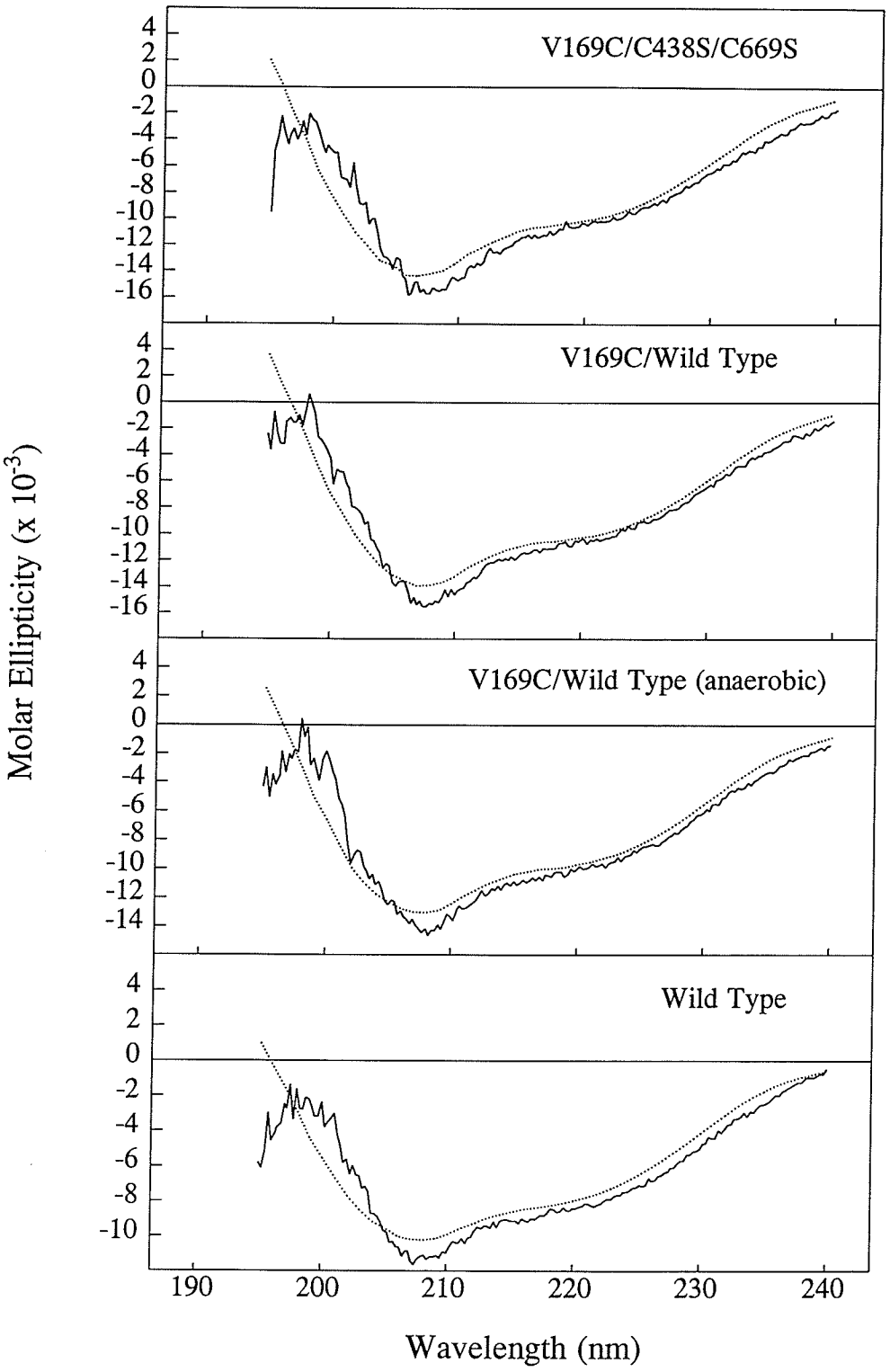


Figure 3.5.4. Comparison of the circular dichroism spectra of wild type HPII and the V169C enzymes. Observed (——) and calculated (·····) spectra.

Table 3.5.3. Secondary structure analysis of V169C enzymes and wild type HPII by circular dichroism spectroscopy.

Mutant	% secondary structure		
	Unordered	β-sheet	α-helix
V169C/C438S/C669S	61.8	11.1	27.2
V169C/Wild Type	57.9	13.6	28.5
V169C/Wild Type (anaerobic)	57.2	16.8	26.0
Wild Type	51.6	25.7	22.7

4. DISCUSSION

4.1. Temperature-dependent production of the mutant proteins

Many of the HPII mutants that were produced in small quantities during growth at 37°C could be increased in amount of activity by growth at 28°C. For example, mutants in the putative NADPH-binding site, truncation mutants and mutants involved in heme-binding all fell into this category. Other mutants including the C438S/A family did not exhibit a yield increase at 28°C. Generally, the smaller the yield at 37°C, the greater was the increase in yield at 28°C although some mutants, which were not detectable at 37°C, were either not detectable or were barely detectable at 28°C. It cannot be absolutely certain what causes the lack of protein production in some mutants but disruption of the normal folding pathway is the most likely explanation. As a result of this disruption in folding, the excess of abnormal protein in the cell may induce, or signal the initiation of proteolytic degradation (Fergus *et al.*, 1992). Lowering the growth temperature to 28°C would slow down the protease activity which should allow more time for correct folding to occur consistent with what is observed in a number of mutants. Chaperones are involved in the folding process presumably by preventing the formation of structures that would be irreversibly inactive. The mutations that interfere with the formation of correctly folded structures may result in folding intermediates being bound to chaperones for longer periods of time. It is during this extended period of folding that proteases have a chance to cleave the protein resulting in the low yields. In the case of proteins

with apparently serious folding problems, like the double and triple mutants of I274S, and C-terminal truncation beyond R744, the temperature did not provide the chaperones with sufficient time to promote folding before proteolysis occurred.

There was also a relationship between the yield and the extent of purification. Generally, proteins produced in lower yields were more difficult to purify and the standard purification protocol resulted in protein with significant contaminating protein.

4.2. Failure of binding of NADPH to HPII

The attempts to modify the putative NADPH-binding site in HPII by incorporating the corresponding residues from the bovine enzyme was initiated before the crystal structure of HPII was available. A comparison of the structures of the two enzymes in this region reveals a number of differences that are now known to preclude NADPH-binding even in the presence of the modifications. By comparing the structures of the bovine enzyme and HPII, one can see major differences, such as the counterparts of the bovine NADPH-binding residues being oriented in opposite directions in HPII and in locations that do not leave enough space for NADPH-binding. Considering these major structural differences between both enzymes, introduction of the four bovine NADPH-binding residues into HPII would not be expected to form a structure capable of NADPH-binding.

4.3. High activity of mutant R260A

The mutant R260A was initially constructed as part of the study of the putative NADPH-binding site and the mutation was a significant distance from the active site. This made it difficult to rationalize why the mutation caused such a significant enhancement in specific activity. At present, HPII exhibits one of the highest turnover rates of any enzyme and certainly the highest rate of all catalases. It is therefore surprising to find an even higher catalytic rate in the mutant where R260 is replaced with alanine. By comparing various catalase inhibitors of different sizes, it was possible to conclude that the heme channel and the active site must be more accessible in R260A than wild type HPII. The residues lining the heme channel of HPII and the bovine enzyme are superimposible, and the channel itself has a funnel like structure, getting increasingly narrower as the active site is approached (Murthy *et al.*, 1981). The data from Table 3.1.4 are consistent with this conclusion of enhanced accessibility to the active site of R260A mutant. For example, the mutant is more sensitive to azide than the wild type enzyme but equally sensitive to cyanide, a smaller molecule. Similarly, the R260A mutant enzyme is more sensitive to hydroxylamine and its alkyl derivatives than the wild type enzyme, requiring 4-fold less inhibitor for similar levels of inhibition. Also consistent with the concept of steric factors constraining access to the active site is the progression to less effective inhibition in the series, hydroxylamine, methylhydroxylamine and ethylhydroxylamine. The R260A mutant protein is also more sensitive to some thiol reagents than the wild type enzyme. For example, both the mutant and wild type

enzymes are equally sensitive to 2-mercaptoethanol, the smallest of the thiol reagents studied but the larger cysteine causes greater inhibition of the R260A enzyme (80% loss of activity) than the wild type (40% loss of activity). Even larger thiols, dithiothreitol and glutathione, do not affect HPII activity but inhibit the R260A enzyme by 80% and 40% respectively. The pattern of inhibition by these latter reagents suggests a slower access to the active site than for cysteine and 2-mercaptoethanol. These data are all consistent with the channel leading to the active site being larger in the R260A enzyme than in HPII.

The crystal structure of HPII contains 18 α-helixes and 16 β-strands. The residue R260 is located in a loop region between α_5-helix and β_5-strands (Bravo, 1996). The secondary structure analysis of the R260A enzyme revealed a small increase in the β-sheet content compared to HPII. Most of the β-sheets are located in the core region that contains the β-barrel surrounding the active site and small increases in the β-sheet content in this region may relax the barrel enlarging the heme channel and allowing easier access to the active site. The structural changes in R260A HPII will be further substantiated by X-ray crystallography.

4.4. Mechanism of hydroxylamine inhibition

Cyanide and azide are well characterized as reversible inhibitors of catalases (Beyer & Fridovich, 1988) and their reactions with HPII are consistent with these earlier results. Hydroxylamine is not well characterized as a catalase inhibitor. It has been reported to react with compound I of catalase, forming nitric oxide (Demaster *et*

al., 1989; Marcocci *et al.*, 1994), and nitric oxide can potentially react with heme to cause inhibition of the enzyme (Doyle *et al.*, 1981). Consistent with this hypothesis, it has recently been shown that nitric oxide causes rapid but reversible inhibition of bovine catalase (Brown, 1995). Hydroxylamine was also reported to irreversibly inhibit the non-heme, Mn-catalase from *Lactobacillus plantarum* while methylhydroxylamine was 100-times less effective (Kono & Fridovich, 1983). By contrast HPII was inhibited reversibly by hydroxylamine, but methylhydroxylamine was 10,000 times less effective. Nitric oxide was not studied as an inhibitor of HPII.

4.5. Inhibition of HPII by thiol compounds

The effect of thiol reagents on catalase has been reported (Takeda *et al.*, 1980). The porcine catalase suffers almost complete, irreversible inactivation when treated with 2-mercaptoethanol, and partial, reversible inhibition by dithiothreitol and glutathione. An active oxygen species resulting from the reaction of dithiothreitol and glutathione with oxygen was proposed to be the inhibitory species, but no explanation was proposed for the effect of 2-mercaptoethanol. In support of this explanation is the fact that the reaction of reducing agents with dissolved oxygen produces superoxide anion which inhibits bovine catalase (Kono & Fridovich, 1982; Schimizu *et al.*, 1984) by converting it to inactive compounds I and III.

The sulfhydryl reagents 2-mercaptoethanol and cysteine cause a partial inhibition of HPII while the larger reducing agents dithiothreitol and glutathione do not affect the enzyme activity and cause only a transient change in the absorption

spectrum via a superoxide-mediated reaction. The reaction of HPII with 2-mercaptoethanol poses a puzzle. Ethanol is a substrate for catalases and can therefore traverse the channel to the active site making it a reasonable assumption that 2-mercaptoethanol can do the same. However, because ethanol does not affect catalase activity, it can be concluded that it is the sulfhydryl part of 2-mercaptoethanol that is causing the inhibition. While an adduct with the iron or other part of the heme seems most likely, an irreversible reaction with another component of the active site cannot be eliminated.

4.6. Modification on C438

Of the two cysteines in HPII, C438 is conserved among catalases which may lead to conjecture about its catalytic and structural roles. The fact that C438 is modified is further evidence that the free sulfhydryl group does not have a role in the catalytic mechanism. Furthermore, even the modified cysteine is largely dispensable because its replacement with serine or alanine by site-directed mutagenesis caused only a small reduction in specific activity. The possibility remains that a reduced cysteine at this location is detrimental to the enzyme although this seems unlikely because the bovine enzyme apparently contains an unmodified cysteine at the equivalent location. The modification may simply be another example of how HPII has been modified to achieve maximum stability under more stressful conditions. A free -SH would be more reactive and its blockage would probably prevent other unwanted reactions.

Two classes of cysteine modifications other than the common disulfide linkage have been reported. One involves oxidation to a cysteine-sulfinic acid and cysteic acid such as in *Streptococcus* NADH peroxidase where the oxidation occurs during the catalytic process (Pool & Claiborne, 1989). The second involves a thiol-ester linkage in α-macroglobulin (Motoshima *et al.*, 1988) and between ubiqutin and ubiqutin-conjugating enzyme (Sullivan & Vierstra, 1993). Therefore, the hemithioacetal structure proposed for the group blocking the sulfhydryl group of C438 is a novel modification and further work is required to confirm the structure. A thiamine pyrophosphate mediated two carbon acetaldehyde transfer from pyruvate to the sulfhydryl group of HPII is a possible source of modification.

The mechanism giving rise to and the role of the modification of C438 is still unclear. Unlike the postulated catalytic role of oxidized cysteine in NADH peroxidase, there does not seem to be a mechanistic role for the blocked cysteine of HPII. The residue is on the surface of the enzyme on the opposite side of the subunit from the entrance to the channel leading to the active site. Therefore, the H_2O_2 should be able to enter the channel without contacting the cysteine, and the distance precludes any influence of the putative hemithioacetal on the active site itself. The modified cysteine enhances the activity of the enzyme by 25% but its removal in the C438S/A mutant of HPII resulted in no other observable structural or spectral changes. One hypothesis that will be investigated is that it is a means of marking the stationary phase proteins to protect them against proteolytic degradation.

4.7. Importance of the C-terminal domain

The C-terminal domain is essential for the correct folding and accumulation of HPII. Truncation beyond R744 where the residue side chains begin to form an integral part of the domain results in no enzyme accumulating in growing cells. The side chain of R744 extends into the interior of the C-terminal domain (Figure 3.3.1) where it forms several hydrogen bonds that would stabilize the folding. In addition the backbone N-H of R744 forms a stabilizing H-bond with an adjacent residue providing a further role for the residue. The preceding residues P746 and I745 have lesser roles in domain stabilization because truncations at these locations result in either 50% or 15% of wild type accumulation. The effect of the truncations is best explained as interfering with folding because any protein that did accumulate had a specific activity similar to wild type HPII.

The role of this C-terminal domain remains obscure. There has been some speculation that it confers stability on the enzyme under extreme physical and chemical conditions or abolishing the need for NADPH-binding (Loewen, 1996), but the importance of the domain in folding suggests a different or auxiliary role. For example, the region on the main domain of HPII that interacts with the C-terminal domain contains a number of hydrophobic residues that would be exposed in the absence of the C-terminus. Improper folding or removal of the C-terminal domain may expose these residues to the medium causing disruption and precipitation or proteolytic degradation of the protein.

4.8. I274: a possible key heme contact residue

The object of changing a number of key heme interaction residues to their bovine counterparts was to see if the heme could be "flipped" to the opposite orientation. Isoleucine 274 of HPII was found to play an important role because the I274S mutation reduced accumulation of the enzyme and caused a 50% reduction in the specific activity. Whether this was a result of interference with heme insertion into the subunit or interference with protein folding could not be determined.

The P356L/L407M enzyme exhibited similar specific activity to the wild type indicating that these two residues have little influence on heme binding or that the change of side chains did not affect heme-binding. Incorporation of other mutations with the I274S resulted in no protein being produced revealing that the original object of inserting the bovine heme contact residues into HPII was not enough to change the orientation of the heme into bovine orientation. In fact the changes prevented folding and accumulation of the protein. In the case of rat liver catalase it was suggested that attachment of heme to the apoenzyme takes place following the synthesis of the monomer (de Duve, 1974). If the same mechanisms applied to HPII, the mutated heme contact residues may interfere with the binding of heme into the apoenzyme subunits suggesting that the heme pocket of HPII has evolved to accommodate the heme in only one orientation.

4.9. Blocking the heme channel of HPII

The V169C mutation clearly blocked the access of substrates to the active site.

Because H_2O_2 is required for heme conversion, the blockage presumably also prevented the formation of heme *d*. The sulfhydryl groups in C169 could be oxidized by the incoming H_2O_2 although the final product has not been identified. In contrast to the alkali-labile C438, residue C169 was not fully reactive with DTNB even after treatment with NaOH suggesting that partial oxidation to cysteic acid or some other form may have occurred. Whether or not the inserted C169 is modified may eventually be determined by MALDI/MS. Secondary structure analysis showed that the V169C mutation caused a sharp decrease in the β-sheet content suggesting a distortion of the β-barrel surrounding the active site. X-ray crystallography analysis of the structure will be required to characterize the exact changes.

4.10. Future directions

Work can now proceed in a number of different directions. A protocol possibly involving the construction of protease deficient hosts should be developed to allow the production of the less stable mutant proteins. In the absence of proteases it may be possible that larger amounts of these enzymes will fold correctly and accumulate in the cell. The nature of the modification on C438 remains to be determined and this will probably require the purification of large quantities of the C438-containing CNBr fragment for NMR and IR analysis. The role of the C-terminal domain requires further study and a first approach may involve the construction of a mutant lacking the domain entirely. Similarly, the role of the N-terminal extension could be studied by first removing it to see what effect this has on

the enzyme properties. Finally, a more detailed study of the R260A enzyme, possibly including X-ray structure analysis, is required to determine the reason for the enhanced activity.

5. REFERENCES

Abril N. & C. Pueyo. 1990. Mutagenesis in *Escherichia coli* lacking catalase. *Environ. Molec. Mutagen.* **15:** 184-189.

Aebi H., C.H. Schneider, H. Gang & U. Wiesmann. 1964. Separation of catalase and other red cell enzymes from hemoglobin by gel filtration. *Experientia* **20:** 103-104.

Aebi H., E. Bossi, M. Cantz, S. Matsubara & H. Suter. 1967. V. Berliner symposium über struktur und funktion der erythrocyten. Humboldt Universität Berlin, DDR.

Afanas'ev I.B. 1989. *Superoxide Ion: Chemistry and Biological implications,* Vol. 1, CRC Press, Florida.

Ahern H. 1993. How bacteria cope with oxidatively damaged DNA- *Escherichia coli* has several ways of recognizing and repairing damage to particular nucleotide bases. **59:** 119-122.

Allen R.G. & V.S. Venkitraj. 1992. Oxidants and antioxidants in development and differentiation. *J. Nutr.* **122 (3S):** 631-635.

Allgood G.S. & J.J. Perry. 1986. Characterization of a manganese-containing catalase from the obligate thermophile *Thermoleophilum album. J. Bacteriol.* **168:** 563-567.

Almagor M., S. Kahane & S. Yatziv. 1984. Role of superoxide anion in host cell injury induced by *Mycoplasma pneumonia* infection. A study in normal and trisomy 21 cells. *J. Clin. Invest.* **73:** 842-847.

Almarrson O., A. Sinha, E. Gopinath & T.C. Bruice. 1993. Mechanism of one-electron oxidation of NAD(P)H and function of NADPH bound to catalase. *J. Am. Chem. Soc.* **115:** 7093-7102.

Ames B.N. 1983. Dietary carcinogens and anticarcinogens. *Science* 221: 1256-1264.

Andrea U., J.Singh & K. Siegler-Skylakakis. 1985. Pyruvate and related α-keto acids protect mammalian cells in culture against hydrogen peroxide-induced cytotoxicity. *Toxicol. Lett.* **28:** 93-98.

Andrews S.C., P.H. Harrison & J.R. Guest. 1989. Cloning, sequencing, and mapping of the bacterioferritin gene (*bfr*) of *Escherichia coli* K12. *J. Bacteriol.* **171:** 3940-3947.

Archibald F.S. & M.N. Duong. 1986. Superoxide dismutase and oxygen toxicity defences in the genus *Neisseria*. *Infec. Immun.* **51:** 631-641.

Ausubel F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith & K. Struhl. 1989. *Current Protocols in Molecular Biology*, Green Publishing Associates and Wiley-Interscience, New York.

Barth M., C. Marschall, A. Muffler, D. Fischer & R. Hengge-Aronis. 1995. Role for the histone-like protein H-NS in growth phase-dependent and osmotic regulation of σ^S and many σ^S-dependent genes in *Escherichia coli*. *J. Bacteriol.* **177:** 3456-3464.

Battistoni A. & G. Rotilio. 1995. Isolation of an active and heat-stable monomeric form of Cu, Zn superperoxide dismutase from the periplasmic space of *Escherichia coli*. *FEBS Lett.* **374:** 199-202.

Benov L., L.Y. Chang, B. Day & I. Fridovich. 1995. Copper, zinc superoxide dismutase in *Escherichia coli*: periplasmic localization. *Arc. Biochem. Biophys.* **319:** 508-511.

Beyer W.F. & I. Fridovich. 1988. Catalases: with and without heme. In *Oxygen Radicals in Biology and Medicine,* M.G. Simic, K.A. Taylor, J.F. Ward & C. von Sonntag (eds.), Plenum Press, New York.

Bochreinger J., D. Fischer, G. Mosler & R. Henge-Aronis. 1995. UDP-glucose is a potential intracellular signal molecule in the control of expression of σ^S and σ^S-dependent genes in *Escherichia coli*. *J. Bacteriol.* **177:** 413-422.

Bol D.K.& P.E. Yasbin. 1991. The isolation. cloning and identification of a vegetative catalase-peroxidase gene from *Bacillus subtilis*. *Gene* **109:** 34-37.

Bravo J. 1996. Estructura de la catalase HPII de *Escherichia coli*. PhD Thesis. Universitat Politècnica de Catalunya, Spain.

Bravo, J., N. Verdaguer, J. Tormo, C. Betzel, J. Switala, P.C. Loewen & I. Fita. 1995. Crystal structure of catalase HPII from *Escherichia coli*. *Structure* **3:** 491-502.

Brawn K., I. Fridovich. 1981. Strand scission by enzymatically generated oxygen radicals. *Arch. Biochem. Biophys.* **206:** 414-419.

Britton L. & I. Fridovich. 1977. Intracellular location of the superoxide dismutases of *Escherichia coli*- a re-evaluation. *J. Bacteriol.* **131:** 815-820.

Brodie A.E. & D.J. Reed. 1987. Reversible oxidation of glyceraldehyde-3-phosphate dehydrogenase thiols in human lung carcinoma cells by hydrogen peroxide. *Biochem. Biophys. Res. Com.* **148:** 120-125.

Brot N., L. Weissbach, J. Werth & H. Weissbach. 1981. Enzymatic reduction of protein-bound methionine sulfoxide. *Proc. Natl. Acad. Sci. U.S.A.* **78:** 2155-2158.

Brown G.C. 1995. Reversible binding and inhibition of catalase by nitric oxide. *Eur. J. Biochem.* **232:** 188-191.

Brown O.R. & F. Yein. 1978. Dihydroxyacid dehydratase: The site of hyperbaric oxygen poisoning in branch chain amino acid biosynthesis. *Biochem. Biophys. Res. Commun.* **85:** 1219-1221

Brown-Peterson N.J. & M.L. Salin. 1993. Purification of a catalase peroxidase from *Halobacterium halobium* characterization of some unique properties of the halophilic enzyme. *J. Bacteriol.* **175:** 4197-4202.

Buzy A., V. Bracchi, R. Sterjiades, J. Chroboezek, P. Thibault, J. Gagnon, H.M. Jouve & G. Hudry-Clergeon. 1995. Complete amino acid sequence of *Proteus mirabilis* PR catalase. Occurrence of a methionine sulfone in the close proximity of the active site. *J. Prot. Chem.* **14:** 59-72.

Carlsson J. & V.S. Carpenter. 1980. The *recA* gene product is more important than catalase and superoxide dismutase in protecting *Escherichia coli* against oxygen toxicity. *J. Bacteriol.* **142:** 319-321.

Cathcart R., E. Schwiers, R.L. Saul & B.N. Ames. 1984. Thymine glycol and thymidine glycol in human and rat urine: a possible assay for oxidative damage. *Proc. Natl. Acad. Sci. U.S.A.* **81:** 5633-5637.

Cerutti P.A. 1985. Prooxidant states and tumour production. *Science* **227:** 375-381.

Chan P.C.& B.H.J. Bielski. 1980. Glyceraldehyde-3-phosphate dehydrogenase catalyzed chain oxidation of reduced nicotinamide adenine dinucleotide by perhydroxyl radicals. *J. Biol. Chem.* **255:** 874-876.

Chan E. & B. Weiss. 1987. Endonuclease IV of *Escherichia coli* is induced by paraquat. *Proc. Natl. Acad. Sci., U.S.A.* **84:** 3189-3193.

Chance B. 1954. Enzyme mechanisms in living cells. In *A Symposium on the Mechanism of Enzyme Action*. pp. 399-460, W.D. McElroy & B. Glass (eds.), The John Hopkins Press, Baltimore.

Chance B. 1952. The state of catalase in the respiring cell. *Science* **116:** 202-203.

Chance B., H. Sies & A. Boveris. 1979. Hydroperoxide metabolism in mammalian organs. *Physiol. Rev.* **59:** 527-605.

Chang J.Y. & W.A. Schroeder. 1972. Reaction of 3-amino-1:2:4-triazole with bovine liver catalase and human eyrthrocyte catalase. *Arch. Biochem. Biophys.* **148:** 505-508.

Chiu J.T., P.C. Loewen, J. Switala, R.B. Gennis & R. Timkovich. 1989. Proposed structure for the prosthetic group of the catalase HPII from *Escherichia coli*. *J. Am. Chem. Soc.* **111:** 7046-7050.

Christman M., R. Morgan, F. Jacobson & B.N. Ames. 1985. Positive control of a regulon for defence against oxidative stress and some heat shock proteins in *Salmonella typhimurium*. *Cell* **41:** 753-762.

Christman M., R. Morgan & B.N. Ames. 1989. OxyR, a positive regulator of hydrogen peroxide inducible genes in *Escherichia coli* and *Salmonella typhimurium*, is homologous to a new family of bacterial regulatory proteins. *Proc. Natl. Acad. Sci., U.S.A.* **86:** 3484-3488.

Chung C.T., S.L. Niemela & R.H. Miller. 1989. One-step preparation of competent *Escherichia coli*: Transformation and storage of bacterial cells in the same solution. *Proc. Natl. Acad. Sci. U.S.A.* **86:** 2172-2175.

Claiborne A. & I. Fridovich. 1979. Purification of the *o*-dianisidine peroxidase from *Escherichia coli* B. *J. Biol. Chem.* **254:** 4245-4252.

Claiborne A., D.P. Malinowski & I. Fridovich. 1979. Purification and characterization of hydroperoxidase II of *Escherichia coli* B. *J. Biol. Chem.* **254:** 11664-11668.

Clare D.A., J. Blum & I. Fridovich. 1984. A hybrid superoxide dismutase containing both functional iron and manganase. *J. Biol. Chem.* **259:** 5932-5936.

Clayton R.K. 1959. Purified catalase from *Rhodopseudomonas spheroides*. *Biochim. Biophys. Acta* **36:** 40-47.

Costilow R.N. & B.B. Jr. Keele. 1972. Superoxide dismutase in *Bacillus popilliae*. *J. Bacteriol.* **111:** 628-630.

Cross C.E., B. Halliwell, E.T. Borish, W.A. Pryor, B.N. Ames, R.L. Saul, J.M. McCord & D. Harman. 1987. Oxygen radicals and human disease. *Ann. Intern. Med.* **107:** 526-545.

Dahl T.A., W.R. MIdden & P.C. Hartman. 1988. Pure exogenous singlet oxygen: Nonmutagenicity in bacteria. *Mutat. Res.* **201:** 127-136.

Darr D. & I. Fridovich. 1985. Inhibition of catalase by 3, 3'-diamino-benzidine. *Biochem. J.* **226:** 781-787.

Davies K.J., M.E. Delsignore & S.W. Lin. 1987. Protein damage by oxygen radicals: II. Modification of amino acids. *J. Biol. Chem.* **262:** 9902-9907.

Day W. 1984. *Genesis on Planet Earth*. 2nd edit. Yale University Press, New Haven.

de Duve C. & P. Boudhuin. 1966. Peroxisomes (microbodies and related particles). *Physiol. Rev.* **46:** 323-357.

de Duve C. 1974. Intracellular localization, biosynthesis and functions of rat liver catalase. In *Alcohol and Aldehyde Metabolizing Systems*. pp. 161-168, R.G. Thurman, T Yonetani, J.R. Williamson and B. Chance (eds.), Academic Press, New York.

DeMaster E.G., L. Raij, S. I. Archer & E.K. Weir. 1989. Hydroxylamine is a vasorelaxant and a possible intermediate in the oxidative convertion of L-arginine to nitric oxide. *Biochem. Biophys. Res. Commun.* **163:** 527-533.

Demple B., J. Halbrook & S. Linn. 1983. *Escherichia coli xthA* mutants are supersensitive to hydrogen peroxide. *J. Bacteriol.* **153:** 11079-11082.

Demple B. & S. Linn. 1982. 5,6-saturated thymine lesions in DNA: production by ultraviolet light of hydrogen peroxide. *Nucl. Acids Res.* **10:** 3781-3789.

Demple B. & J. Halbrook. 1983. Inducible repair of oxidative DNA damage in *Escherichia coli*. *Nature, London* **304:** 466-468.

Desiseroth A. & A.L. Dounce. 1970. Catalase: physical and chemical properties, mechanism of catalysis and physiological role. *Physiol. Rev.* **50:** 319-375.

Dickerson R.E. H.B. Gray, M.Y. Darensboug &D.J. Darensbourg. 1984. *Chemical Principles*, 4th edit., The Benjamin/Cummings, California.

Diguiseppi J & I. Fridovich. 1984. The toxicity of the molecular oxygen. *CRC Crit. Rev. Toxicol.* **12:** 315-342.

Dingman D.W. & D.P. Stahly. 1984. Protection of *Bacillus larvae* from oxygen toxicity with emphasis on the role of catalase. *Appl. Environ. Microbiol.* **47:** 1228-1237.

Doyle M.P., R.A. Pickering, T.M. DeWeert, J.W. Hoekatra & D. Peter. 1981. Kinetics and mechanism of the oxidation of human deoxyhemoglobin by nitrites. *J. Biol. Chem.* **256:** 12393-12398.

Ellman G.L. 1959. Tissue sulfhydryl groups. *Arch. Biochem. Biophys.* **82:** 70-77.

Esaki M. & T. Asahi. 1982. Purification and properties of catalase from sweet potato root microbodies. *Plant Cell Physiol.* **23:** 315-322.

Farr, S.B. & T. Kogoma. 1991. Oxidative stress responses in *Escherichia coli* and *Salmonella typhimurium. Microbiol. Rev.* **55:** 561-585.

Fenton H.J.H. 1894. Oxidation of tartaric acid in presence of iron. *J. Chem. Soc., Lon.* **65:** 899-910.

Fergus J.D. & R.J. Mayer. 1992. Intracellular protein degradation. In *Focus*, D. Rickwood (ed.), IRL Press. New York, U.S.A.

Filho C.M., M.E. Hoffman & R. Meneghini. 1984. Cell killing and DNA damage by hydrogen peroxide are meditated by intracellular iron. *Biochem. J.* **218:** 273-275.

Fita I. & M.G. Rossmann. 1985. The active center of catalase. *J. Mol. Biol.* **185:** 21-37.

Forkl H., J. Vandekerckhove, G. Drews & M.H. Tadros. 1993. Molecular cloning, sequence analysis and expression of the gene for catalase-peroxidase (*opeA*) from the photosynthetic bacterium *Rhodobacter capsulatus* B10. *Eur. J. Biochem.* **214:** 251-258.

Fridovich I. 1976. Oxygen radicals. hydrogen peroxide, and oxygen toxicity. In *Free Radicals in Biology*. pp. 239-277, W.A. Pryor (ed.), Academic Press, New York.

Fridovich I. 1978. The biology of oxygen radicals. *Science* **201:** 875-880.

Fridovich I. 1986. Biological effects of the superoxide radical. *Arch. Biochem. Biophys.* **247:** 1-11.

Fridovich S.H. & N.E. Porter. 1981. Oxidation of arachidonic acid in micelles by superoxide and hydrogen peroxide. *J. Biol. Chem.* **256:** 260-265.

Gentry D.R., V.J. Hemandez, L.H. Nuygen, D.B. Jensen & M. Cashel. 1993. Synthesis of the stationary phase sigma factor σ^S is positively regulated by ppGpp. *J. Bacteriol.* **175:** 7982-7989.

Goldberg I. & A. Hochman. 1989a. Purification and characterization of a novel catalase from the bacterium *Klebsiella pnuemoniae*. *Biochim. Biophys. Acta* **991:** 330-336.

Goldberg I. & A. Hochman. 1989b. Three different types of catalases in *Klebsiella pnuemoniae*. *Arch. Bichem. Biophys.* **268:** 124-128.

Gottstein A. 1893. Über die zerlegung des wasserstoffsuperoxyds durch die zellen, mit bemerkungen über eine makroskopische reaction für bacterien. *Virchows Arch. Path. Anat.* **133:** 295-307.

Gouet P. 1993. Determination de la structure 3-D de la catalase de la bacterie *Proteus mirabilis* par diffraction aux rayons X structure avec et sans NADPH. PhD thesis, University of Paris, XI Orsay, France.

Gouet P., H-M. Jouve & O. Didelberg. 1995. Crystal structure of *Proteus mirabilis* catalase with and without bound NADPH. *J. Mol. Biol.* **249:** 933-954.

Greenberg J.T. & B. Demple. 1988. Overproduction of peroxide-scavenging enzymes in *Escherichia coli* supresses spontaneous mutagenesis and sensitivity to redox-cycling agents in *oxyR*-mutants. *EMBO J.* **7:** 2611-2617.

Greenberg J.T. & B. Demple. 1989. A global response induced in *Escherichia coli* by redox-cycling agents overlaps with that induced by peroxide stress. *J. Bacteriol.* **171:** 3933-3939.

Gutteridge J.M.C. 1986. Iron promoters of the Fenton reaction and lipid peroxidation can be released from hemoglobin by peroxidation. *FEBS Lett.* **201:** 291-295.

Haber F. & J. Weiss. 1934. The catalytic decomposition of hydrogen peroxide by iron salts. *Proc. Roy. Soc. Ser. A* **147:** 332-351.

Hagensee M.E. & R.E. Moses. 1986. Repair response of *Escherichia coli* to hydrogen peroxide. *J. Bacteriol.* **168:** 1059-1065.

Hagensee M.E., S.K. Bryan & R.E. Moses. 1987. DNA polymerase III requirement for repair of DNA damage caused by methylmethane sulfonate and hydrogen peroxide. *J. Bacteriol.* **169:** 4608-4613.

Hagensee M.E. & R.E. Moses. 1989. Multiple pathways for repair of hydrogen peroxide-induced DNA damage in *Escherichia coli*. *J. Bacteriol.* **171:** 991-995.

Halliwell B., D.A. Rowley & J.M.C Gutteridge. 1983. Transition metal catalysis and oxygen radical reactions. In *Life Chemistry Reports Supplement 2 (Oxidative damage and related enzymes-EMBO Workshop, 1983)* pp. 8-14.

Halliwell B. & J.M.C. Gutteridge. 1986. Oxygen free radicals and iron in relation to biology and medicine: Some problems and concepts. *Arch. Biochem. Biophys.* **246:** 501-514.

Halliwell B. & J.M.C. Gutteridge. 1989. *Free Radicals in Biology and Medicine*. 2nd ed. Oxford University Press, New York.

Hassan H.M. & I. Fridovich. 1977. Enzymatic defences against the toxicity of oxygen and streptonigrin in *Escherichia coli*. *J. Bacteriol.* **129:** 1574-1583.

Hassan H.M. & I. Fridovich. 1979. Paraquat and *Escherichia coli*. *J. Biol. Chem.* **254:** 10846-10852.

Heimberger A. & A. Eisenstark. 1988. Compartmentalization of catalases in *Escherichia coli*. *Biochem. Biophys. Res. Comm.* **154:** 392-397.

Hennessey J.P. Jr. & W.C. Jr. Johnson. 1982. Experimental errors and their effect on analyzing circular dichroism spectra of proteins. *Anal. Biochem.* **125:** 177-188.

Herbert D. & J. Pinsent. 1948. Crystalline bacterial catalase. *Biochem. J.* **43:** 193-204.

Heym B., Y. Zhang, S. Poulet, D. Young & S.T. Cole. 1993. Characterization of the *katG* gene encoding a catalase-peroxidase required for the isoniazid susceptibility of *Mycobacterium tuberculaosis*. *J. Bacteriol.* **175:** 4255-4359.

Hicks D.B. 1995. Purification of three catalase isozymes from facultatively alkaliphilic *Bacillus firmus* OF4. *Biochim. Biophys. Acta* **1225:** 347-355.

Hillar A.P., P. Nicholls. 1992. A mechanism for NADPH inhibition of catalase compound II formation. *FEBS Lett.* **314:** 179-182.

Hillar A.P., P. Nicholls, J. Switala & P.C. Loewen. 1994. NADPH binding and control of catalase compound II formation: comparison of bovine, yeast and *Escherichia coli* enzymes. *Biochem. J.* **300:** 531-539.

Hochman A. & A. Shemesh. 1987. Purification and characterization of a catalase-peroxidase from the photosynthetic bacterium *Rhodopseudomonas capsulata*. *J. Biol. Chem.* **262:** 6871-6876.

Hochman A. & I. Goldberg. 1991. Purification and characterization of a catalase-peroxidase from a typical catalase from the bacterium *Klebsiella pneumonia*. *Biochim. Biophy. Acta* **1077:** 299-307.

Hollstein M., P. Brooks, S. Linn & B.N. Ames. 1984. Hydroxymethyluracil DNA glycosylase in mammalian cells. *Proc. Natl. Acad. Sci., U.S.A.* **81:** 4003-4007.

Huisman G.W. & R. Kolter. 1994. Sensing starvation: a homoserine-lactone-dependent signalling pathway in *Escherichia coli*. *Science* **265:** 537-539.

Imlay J.A. & I. Fridovich. 1991. DNA damage by hydrogen peroxide through the Fenton reaction *in vivo* and *in vitro*. *Science* **240:** 640-642.

Imlay J.A. & S. Linn. 1987. Mutagenesis and stress responses induced in *Escherichia coli* by hydrogen peroxide. *J. Bacteriol.* **169:** 2967-2976.

Issajew W. 1904. Über die hefekatalase. *Hoppe-Seyler's Zeitschrift für Physiologisches Chemie* **42:** 102-116.

Ivanova A., C. Miller, G.Glinsky & A. Eisenmark. 1994. Role of Rpo (katF) in oxyR-dependent regulation of hydroperoxidase I in *Escherchia coli*. *Molec. Microbiol.* **12:** 571-578.

Jacobson F., R. Morgan, M.F. Christman & B.N. Ames. 1989. An alkyl hydroperoxidase reduction from *Salmonella typhimurium* involved in the defence of DNA against oxidative damage. *J. Biol. Chem.* **264:** 1488-1496.

Jouva H.M., C. Lasauiere & J. Relmont. 1983. Properties of a catalase from a peroxidase-resistant mutant of *Proteus mirabilis*. *Can. J. Biochem. Cell Biol.* **62:** 935-944.

Jouve H.M., J. Gaillard & J. Pelmont. 1984. Characterization and spectral properties of *Proteus mirabilis* PR catalase. *Can J. Biochem. Cell Biol.* **62:** 935-944.

Jouve H.M., J. Pelmont & J. Gaillard. 1986. Interaction between pyridine adenine dinucleotides and bovine liver catalase: A chromatographic and spectral study. *Arch. Biochem. Biophys.* **248:** 71-79.

Jouve H.M., F. Beaumont, I. Leger, J. Foray & J. Pelmont. 1989. Tightly bound NADPH in *Proteus mirabilis* PR catalase. *Biochem. Cell Biol.* **67:** 271-277.

Keele B.B., J.M. Jr. McCord & I. Fridovich. 1970. Superoxide dismutase from *Escherichia coli* B. A new manganese containing enzyme. *J. Biol. Chem.* **245:** 6176-6181.

Keyse S.M. & R.M. Tyrrell. 1989. Heme oxygenase is the major 32 kDa stress protein induced in human skin fibroblasts by DNA radiation, hydrogen peroxide, and sodium arsenite. *Proc. Natl. Acad. Sci. U.S.A.* **86:** 99-103.

Kikuchi-Torii K., S. Hayashi., H. Nakamoto & S. Nakamura. 1982. Properies of *Aspergillus niger* catalase. *J. Biochem.* **92:** 1449-1456.

Kim H.I., K. Kim & S.G. Rhee. 1989. Induction of an antioxidant protein of *Saccharomyces cerevisiae* by O_2, Fe^{3+}, or 2-mercaptoethanol. *Proc. Natl. Acad. Sci. U.S.A.* **86:** 6018-8022.

Kirkmann H.N. & G.F. Gaetani. 1984. Catalase: a tetrameric enzyme with four tightly bound molecules of NADPH. *Proc. Natl. Acad. Sci. U.S.A.* **81:** 4343-4348.

Kirkmann H.N., S. Galiano & G.F. Gaetani. 1987. The function of catalase-bound NADPH. *J. Biol. Chem.* **262:** 660-666.

Klotz M.G., Y.C. Kim. J. Katsuwon & A.J. Anderson. 1995. Cloning, characterization and phenotypic expression in *Escherichia coli* of *catF*, which encodes the catalytic subunit of catalase isozyme CatF of *Pseudomonas syringae*. *Appl. Microbiol. Biotechnol.* **43:** 656-666.

Knauf H.J., R.F. Vogel & W.P. Hammes. 1992. Cloning, sequence and properties and immunological detection of a bromoperoxidase-catalase of *Lactobacillus sake* LTH677. *App. Environ. Microbiol.* **58:** 832-839.

Kono Y. & I. Fridovich. 1982. Superoxide radical inhibits catalase. *J. Biol. Chem.* **257:** 5751-5754.

Kono Y. & I. Fridovich. 1983. Isolation and characterization of the pseudocatalase of *Lactobacillus plantarum*. *J. Biol. Chem.* **258:** 6015-6019.

Kunkel T.A., J.D.Roberts & R.A. Zakour. 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol.* **154:** 367-382.

Lange R. & R. Hengge-Aronis. 1991a. Identification of a central regulator of stationary phase gene expression in *Escherichia coli*. *Mol. Microbiol.* **5:** 49-59.

Lange R. & R. Henge-Aronis. 1991b. Growth phase regulated expression of *bolA* and morphology of stationary phase *Escherichia coli* cells is controlled by the novel sigma factor σ^S *J. Bacteriol.* **173:** 4474-4481.

Lange R., D. Fischer & R. Hengge-Aronis. 1995. Identification of transcriptional start sites and the role of ppGpp in the expression of *rpoS*, the structural gene for the σ^S subunit of RNA polymerase in *Escherichia coli*. *J. Bacteriol.* **177:** 4676-4680.

Lardinois O.M. 1995. Reactions of bovine liver catalase with superoxide radicals and hydrogen peroxide. *Free Rad. Res.* **22:** 251-274.

Layne E. 1957. Spectrophotometric and turbidometric methods for measuring proteins. *Methods Enzymol.* **3:** 447-454.

Lazarow P.B. & Y. Fujiki. 1985. Biogenesis of peroxisomes. *Ann. Rev. Cell Biol.* **1:** 489-530.

Lee M.H. & J.W. Park. 1995. Lipid peroxidation products mediate damage of superoxide dismutase. *Biochem. Mol. Biol. Int.* **35:** 1093-1102.

Levin D.E., M. Hollstein, M.F. Christman, E.A. Schwiers & B.N. Ames. 1982. A new *Salmonella* tester strain (TA102) with A:T base pairs at the site of mutation detects oxidative mutagens. *Proc. Natl. Acad. Sci. U.S.A.* **79:** 7445-7449.

Levitz S.M. & R.D. Diamond. 1984. Killing of *Aspergillus fumigatus* spores and *Candida albicans* yeast phase by the iron-hydrogen peroxide-halide system. *Infect. Immun.* **43:** 1100-1102.

Lipmann F. & L. C. Turtle. 1945. A specific micromethod for the determination of acyl phosphates. *J. Biol. Chem.* **159:** 21-28.

Loew O. 1901. Catalase, A new enzyme of general occurrence with special reference to the tobacco plant. *U.S. Dept. Agr. Rep.* **#68**. p.47.

Loewen P.C. 1984. Isolation of catalase-deficient *Escherichia coli* mutants and genetic mapping of *katE*, a locus that affects catalase activity. *J. Bacteriol.* **157:** 622-626.

Loewen P.C. 1992. Regulation of bacterial catalase synthesis. In *Molecular Biology of Free Radical Scavenging Systems*. pp. 97-115, J.G. Scandalios (ed.), Cold Spring Harbor Laboratory Press

Loewen P.C. 1996. Bacterial catalases. (in press)

Loewen P.C. & B.L. Triggs. 1984. Genetic mapping of *katF*, a locus that with *katE*, affects the synthesis of a second catalase species in *Escherichia coli*. *J. Bacteriol.* **160:** 668-675.

Loewen P.C., J. Switala & B.L. Triggs-Raine. 1985a. Catalases HPI and HPII in *Escherichia coli* are induced independently. *Arch. Biochem. Biophys.* **243:** 144-149.

Loewen P.C., B.L. Triggs, C.S. George & B.E. Hrabarchuk. 1985b. Genetic mapping of *katG*, a locus that affects the synthesis of the bifunctional catalase-peroxidase hydroperoxidase I in *Escherichia coli*. *J. Bacteriol.* **162:** 661-667.

Loewen P.C. & G.V. Stauffer. 1990. Nucleotide sequence of *katG* of *Salmonella typhimurium* LT2 and characterization of its product, hydroperoxidase I. *Mol. Gen. Genet.* **224:** 147-151.

Loewen P.C. & J. Switala. 1986. Purification and characterization of catalase HPII in *Escherichia coli* K12. *Biochem. Cell Biol.* **64:** 638-646.

Loewen P.C. & J. Switala. 1987. Multiple catalases in *Bacillus subtilis*. *J. Bacteriol.* **169:** 3601-3607.

Loewen P.C. & J. Switala. 1988. Purification and characterization of spore-specific catalase-2 from *Bacillus subtilis*. *Biochem. Cell Biol.* **66:** 707-714

Loewen P.C., J. Switala, I. von Ossowski, A. Hillar, A. Christie, B. Tattrie & P. Nicholls. 1993. Catalase HPII of *Escherichia coli* catalyzes the conversion of protoheme to *cis*-heme *d*. *Biochemistry* **32:** 10159-10164.

Loewen P.C. & R. Hengge-Aronis. 1994. The role of the sigma factor σ^S (KatF) in bacterial global regulation. *Ann. Rev. Microbiol.* **48:** 53-80.

Loprasert S., S. Negoro & H. Okada. 1989. Cloning, nucleotide sequence, and expression in *Escherichia coli* of the *Bacillus stearothermophilus* peroxidase gene (*perA*). *J. Bacteriol.* **171:** 4871-4875.

Maeda Y., A. Trautwein, U. Gonser, K. Yoshida, K. Kikuchi-Tori, T. Homma & Y. Ogura. 1973. Mössbauer effect in bacterial catalase. *Biochim. Biophys. Acta.* **303:** 230-236.

Marcinkeviciene J.A., R.S. Magliozza & J.S. Blanchard. 1995. Purification and characterization of the *Mycobacterium smegmatis* catalase-peroxidase involved in isoniazid activation. *J. Biol. Chem.* **270:** 22290-22395.

Marcocci L., J.J. Maguire, M.T. Droy-Lefaix & L. Packer. 1994. The nitric oxide scavenging properties of *Ginko biloba* extract EGb 761. *Biochem. Biophys. Res. Commun.* **201:** 748-755.

Margoliash E. & A. Novogrodsky. 1958. A study of the inhibition of catalase by 3-amino-1:2:4-triazole. *Biochem. J.* **68:** 468-475.

Margoliash E., A. Novogrodsky & A. Schejter. 1960. Irreversible reaction of 3-amino-1:2:4-triazole and related inhibitors with the protein of catalase. *Biochem. J.* **74:** 339-350.

Marquis R.E. & S.Y. Shin. 1994. Mineralization and responses of bacterial spores in heat and oxidative agents. *FEMS Microbiol. Rev.* **14:** 375-379.

Matin A. 1990. Molecular analysis of the starvation stress in *Escherichia coli*. *FEMS MIcrobiol. Ecol.* **74:** 185-196.

McCann M.P., J.P. Kidwell & A. Maidan. 1991. The putative σ factor KatF has a central role in the development of starvation-mediated general resistance in *Escherichia coli*. *J. Bacteriol.* **173:** 4188-4194.

Mead J.F. 1976. Free radical mechanisms of lipid damage and consequences for cellular membranes. In *Free Radicals in Biology*. pp. 51-68, W.A. Pryor (ed.), Academic Press, New York.

Mead D.A., E.S. Skorupa & B. Kemper. 1985. Single-stranded DNA SP6 promoter plasmids for engineering mutant RNAs and proteins: synthesis of a 'stretched' preparathyroid hormone. *Nucleic Acids Res.* **13:** 1103-1118.

Meir E. & E. Yagil. 1985. Further characterization of the two catalases in *Escherichia coli*. *Curr. Microbiol.* **12:** 315-320.

Melik-Adamyan W.R., V.V. Barynin, A.A. Vagin, V.V. Borisov, B.K. Vainshtein, I. Fita, M.R.N. Murthy & M.G. Rossmann. 1986. Comparison of beef liver and *Penicillium vitalae* catalases. *J. Mol. Biol.* **188:** 63-72.

Metzner H. 1978. *Photosynthetic Oxygen Evolution*. Academic Press, New York.

Morgan R.W., M.F. Christman, F.S. Jacobson, G. Storz & B.N. Ames. 1986. Hydrogen peroxide-inducible proteins in *Salmonella typhimurium* overlap with heat shock and other stress proteins. *Proc. Natl. Acad. Sci. U.S.A.* **83:** 8059-8063.

Motoshima A., M. Sera, T. Funakoshi, S. Shoyi, Y. Kubota & H. Ueli. 1988. Electrophoretic and spectrophoretic analysis of equine α_2-macroglobulin with cleavage of the thiol ester bonds by methylamine. *Arch. Biochem. Biophys.* **26:** 517-524.

Mulvey M.R., J. Switala, A. Borys & P.C. Loewen. 1990. Regulation of transcription of *katE* and *katF* in *Escherichia coli*. *J. Bacteriol.* **172:** 6713-6720.

Mulvey M.R., P.A. Sorby, B.L. Triggs-Raine & P.C. Loewen. 1988. Cloning and physical characterization of *katE* and *katF* required for catalase HPII expression in *Escherichia coli*. *Gene* **73:** 337-345.

Mulvey M.R. & P.C. Loewen. 1989. Nucleotide sequence of *katF* of *Escherichia coli* suggests KatF protein is a novel σ transcription factor. *Nucl. Acids Res.* **17:** 9979-9991.

Murshodov G.N., A.I. Grebenko, V. Barynin, Z. Dauter, K.S. Wilson, B.K. Vainshtein, W. Melik-Adamyan, J. Bravo, J.M. Ferran, J.C. Ferrer, J. Switala, P.C. Loewen & I. Fita. 1996. Structure of the heme *d* of *Penicillium vitale* and *Escherichia coli* catalases. *J. Biol. Chem.* **271:** 8863-8868.

Murshudov G.N., W.R. Melik-Adamyan, A.I. Grebenko, V.V. Barynin, A.A. Vagin, B.K. Vainshtein, Z. Dauter & K.S. Wilson. 1992. Three-dimentional structure of catalase from *Micrococcus lysodeikticus* at 1.5 Å resolution. *FEBS Lett.* **312:** 127-131.

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

Nadler V., I. Goldberg & A. Hochman. 1986. Comparative study of bacterial catalases. *Biochem. Biophys. Acta.* **882:** 234-241.

Nagy J.M., A.E.G. Cass & K.A. Brown. 1995. Progress in the characterization of catalase-peroxidase from *Mycobacterium tuberculosis*. *Biochem. Soc. Trans.* **23:** 152S.

Neubert D., A.B. Wojtczak & A.L. Lehninger. 1962. Purification and enzymatic identity of mitochondrial contraction-factors I and II. *Proc. Nat. Acad. Sci. U.S.A.* **48:** 1651-16

Nicholls P. & G.R. Schonbaum. 1952. Catalases. In *The Enzymes*, Vol. 8, pp. 147-225, 2nd edit., P.D. Boyer, H. Lardy & K. Myrback (eds.), Academic Press, New York.

Pegg M., D. Crane & C. Masters. 1986. Confirmation that catalase is a glycoprotein. *Biochem. Int.* **12:** 831-838.

Peppers R.E. & R.N. Costilow. 1965. Electron transport in *Bacillus popillaea*. *J. Bacteriol.* **89:** 271-276.

Perczel A., K. Park & G.D. Fasman. 1992. Analysis of the circular dichroism spectrum of proteins using the convex constraint algorithm: a practical guide. *Anal. Biochem.* **203:** 83-93.

Pichorner H., G. Jessner & R. Ebermann. 1993. tBOOH acts as a suicide substrate for catalase. *Arch. Biochem. Biophys.* **300:** 258-264.

Poole L.B. & A. Claiborne. 1989. The non-flavin center of the Streptococcal NADH peroxidase. Evidence for a stabilized cysteine-cysteic acid. *J. Biol. Chem.* **264:** 12330-12338.

Radi T.J., J.F. Turrens, L.Y. Chang, K.M. Bush, J.D. Crapo & B.A. Freeman. 1991. Detection of catalase in rat heart mitochondria. *J. Biol. Chem.* **366:** 22028-22034.

Reid T.J., M.R.N. Murty, A. Sicignano, N. Tanaka, W.D.L. Musick & M.G. Rossmann. 1981. Structure and heme environment of beef liver catalase at 2.5 Å resolution. *Proc. Natl. Acad. Sci. U.S.A.* **78**: 4767-4771.

Repine J.E., R.B. Fox. & E.M. Berger. 1981. Hydrogen peroxide kills *Staphylococcus aureus* by reacting with staphylococcal iron to form hydroxyl radical. *J. Biol. Chem.* **256:** 7094-7096.

Richter H.E. & P.C. Loewen. 1982. Catalase synthesis in *Escherichia coli* is not controlled by catabolite repression. *Arch. Biochem. Biophys.* **215:** 72-77.

Roth M. & P.K. Jensen. 1967. Determination of catalase activity by means of the Clark electrode. *Biochim. Biophys. Acta* **139:** 171-173.

Sambrook J., E.F. Fritsch & T. Maniatis. 1989. *Molecular Cloning: A laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Sammartano L.J., R.W. Tuveson & R. Davenport. 1986. Control of sensitivity to inactivation by H_2O_2 and broad-spectrum near-UV radiation by the *Escherichia coli katF* locus. *J. Bacteriol.* **4168:** 13-21.

Sanger F.S., S. Nicklen & A.R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. U.S.A.* **74:** 5463-5467.

Sawyer D.T. & J.S. Valentine. 1981. How super is superoxide? *Acc. Chem. Res.* **14:** 393-400.

Sawyer D.T. 1987. The chemistry and activation of dioxygen species (O_2, O_2^-, HOOH) in biology. In *Oxygen Complexes and Oxygen Activation by Transition Metals*. pp. 131-148, A.E. Martell & D.T. Sawyer (eds.), Plenum Publishing, New York.

Schell M.A. 1993. Molecular biology of the LysR family of transcriptional regulators. *Ann. Rev. Microbiol.* **47:** 597-626.

Schellhorn H.E. & H.M. Hassan. 1988. Response of hydroperoxidase and superoxide dismutase deficient mutants of *Escherichia coli* K-12 to oxidative stress. *Can. J. Microbiol.* **34:** 1171-1176.

Schellhorn H.E. & V.L. Stones. 1992. Regulation of *katF* and *katE* in *Escherichia coli* K12 by weak acids. *J. Bacteriol.* **174:** 4769-4776

Schonbaum G.R. & B. Chance. 1976. Catalase. In *The Enzymes*, Vol. 13, 3rd edit., pp. 363-408, P.D. Boyer (ed.), Academic Press, New York.

Schroeder W.A., J.R. Shelton, J.B. Shelton, B. Robberson & G. Appell. 1969. The amino acid sequence of bovine liver catalase: a preliminary report. *Arch. Biochem. Biophys.* **131:** 653-655.

Sha Z., T.J. Stabel & J.E. Mayfield. 1994. *Brucella abortis* catalase is a periplasmic protein lacking a standard signal sequence. *J. Bacteriol.* **126:** 7375-7377.

Sharma K.D. L.A. Andersson, T.M. Loehr, J. Terner & H.M. Goff. 1989. Comparative spectral analysis of mammalian, fungal and bacterial catalases. *J. Biol. Chem.* **264:** 12772-12779.

Shimizu N., K. Kobayashi & K. Hayashi. 1984. The reaction of superoxide radical with catalase. Mechanism of the inhibition of catalase by superoxide radical. *J. Biol. Chem.* **259:** 4414-4418.

Simic M.G. & K.A. Taylor. 1987. Introduction to peroxidation and antioxidation mechanisms. In *Oxygen Radicals in Biology and Medicine*. pp. 1-10, M.G. Simic, K.A. Taylor, J.F. Ward & C. von Sonntag (eds.), Plenum Press, New York.

Smith J. & A. Schrift. 1979. Phylogenic distribution of glutathione peroxidase. *Comp. Biochem. Physiol.* **638:** 39-44.

Sohal R.S., R.G. Allen, K.J. Farmer, R.K. Newton & P.L. Toy. 1985. Effects of exogenous antioxidants, on the levels of endogenous antioxidants, lipid-soluble fluorescent material and life span in the house fly, *Musca domestica*. *Mech. Ageing Dev.* **31:** 329-336.

Storz G., L.A. Tartaglia & B.N. Ames. 1990. Transcriptional regulator of oxidative stress-inducible genes: Direct activation by oxidation. *Science* **248:** 189-194.

Storz G., F.S. Jacobson, L.A. Tartaglia, R.W. Morgan, L.A. Silveira & B.N. Ames. 1989. An alkyl hydroperoxidase reductase induced by oxidative stress in *Salmonella typhimurium* and *Escherichia coli*: Genetic characterization and cloning of *ahp*. *J. Bacteriol.* **171:** 2049-2055.

Storz G. & L.A. Tartaglia. 1992. OxyR: A regulator of antioxidant genes. *J. Nutr.* **122** (3S): 627-630.

Straight R.C. & J.D. Spikes. 1985. Photosensitized oxidation of biomolecules. In *Singlet Oxygen*. Vol. 4, pp. 91-143, A.A. Friper (ed.) CRC, Boca Raton, FL.

Sullivan M.L & R.D. Vierstra. 1993. Formation of a stable adduct between ubiquitin and *Arabidopsis* ubiquitin-conjugating enzyme, ArUBC1. *J. Biol. Chem.* **268:** 8777-8780.

Sumner J.B. & A.I. Dounce. 1937. Crystalline catalase. *J. Biol. Chem.* **121:** 417-424.

Sund H., K..Weber & E. Mölbert. 1967. Dissoziation der rinderleber-katalase in ihre untereinheiten. *Eur. J. Biochem.* **1:** 400-410.

Takeda A. T. Miyahara, A. Hachimori & T Samejima. 1980. The interaction of thiol compounds with porcine erythrocyte catalase. *J. Biochem.* **87:** 429-439.

Tanford C. & R. Lovrien. 1962. Dissociation of catalase into subunits. *J. Am. Chem. Soc.* **84:** 1892-1896.

Thorup O.A., J.T. Carpenter & P. Howard. 1964. Human erythrocyte catalase: demonstration of heterogeneity and relationship to erythrocyte ageing *in vivo*. *Brit. J. Haematology*. **10:** 542-550.

Tolbert N.E. 1978. Peroxisomal redox enzymes. *Methods Enzymol.* **52:** 493-505.

Triggs-Raine B.L., B.W. Doble, M.R. Mulvey, P.A. Sorbey & P.C. Loewen. 1988. Nucleotide sequence of *katG* encoding catalase HPI of *Escherichia coli*. *J. Bacteriol.* **170:** 4415-4419.

Vainshtein B.K., W.R. Melik-Adamyan, V.V. Barynin. A.A. Vagin & A.I. Grebenko. 1981. Three-dimensional structure of enzyme catalase. *Nature* **293:** 411-412.

Vainshtein B.K., W.R. Melik-Adamyan, V.V. Barynin, A.A. Vagin, A.I. Grebenko, V.V. Borisov, K.S. Bartels, I. Fita & M.G. Rossmann. 1986. Three-dimensional structure of catalase from *Penicillium vitale* at 2.0 Å resolution. *J. Mol. Biol.* **188:** 49-61.

Vieira J. & J. Messing. 1987. Production of single-stranded plasmid DNA. *Methods Enzymol.* **153:** 3-11.

Volkert M.R. & P.C. Loewen, J. Switala, D. Crowly & M. Conly. 1994. The Δ(*argF-lacZ*) 205(U169) deletion greatly enhances resistance to hydrogen peroxide in stationary-phase *Escherichia coli*. *J. Bacteriol.* **176:** 1297-1302.

von Ossowski I. E. 1993. Characterization of *katE* and its product, catalase HPII, from *Escherichia coli* by sequence analysis and site-directed mutagenesis. PhD thesis, University of Manitoba, Winnipeg, Canada.

von Ossowski I., G. Hausner & P.C. Loewen. 1993. Molecular evolutionary analysis based on the amino acid sequence of catalase. *J. Mol. Evol.* **37:** 71-76.

von Ossowski I., M.R. Mulvey, P.A. Leco, A. Borys & P.C. Loewen. 1991. Nucleotide sequence of *Escherichia coli katE*, which encodes catalase HPII. *J. Bacteriol.* **173:** 514-520.

Walkup L.K.B. & T. Kogama. 1989. *Escherichia coli* proteins inducible by oxidative stress mediated by the superoxide radical. *J. Bacteriol.* **171:** 1476-1484.

Wardell J.L. 1974. Preparation of thiols. In The chemistry of the thiol group. pp. 164-258, S. Patoi (ed.), John Wiley & Sons, London.

Wasserman B.P. & H.O. Hultin. 1981. Effect of deglycolysation on the stability of *Aspergillus niger* catalase. *Arch. Biochem. Biophys.* **212:** 385-392.

Weber K., J. R. Pringle & M. Osborn. 1972. Measurement of molecular weights by electrophoresis on SDS-acrylamide gels. *Methods Enzymol.* **26:** 3-27.

Welinder K.G. 1991. Bacterial catalase-peroxidases are gene duplicated members of the plant peroxidase superfamily. *Biochim. Biophys. Acta* **1080:** 215-220.

Wolff S., J.K. Wiencke, V. Afzal, J. Youngblom & F. Cortes. 1989. In *Low Dose Radiation: Biological Basis of Risk Assesment*. pp. 446-454, K.F. Baverstock & J.W. Stather (eds.), Taylor and Francis, London.

Wood P.M. 1988. The potential diagram of oxygen at pH 7. *Biochem. J.* **253:** 287-289.

Xiang F. & R.C. Beavis. 1994. A method to increase contaminant tolerance in protein matrix-assisted laser desorption by the fabrication of thin protein-doped polycrystalline films. *Rapid Commun. Mass Spectrom.* **8:** 199-204.

Yamoto I., Y. Fukumori & T. Yamanaka. 1990. Purification and characterization of catalase from a facultative alkaliphilic *Bacillus*. *J. Biochem.* **108:** 583-587.

Yanisch-Perron C., J. Vietra & J. Messing. 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequence of the M13mp18 and pUC19 vectors. *Gene* **33:** 103-119.

Yonei S., R. Yokota & Y. Sato. 1987. The distinct role of catalase and DNA repair systems in protection against hydrogen peroxide in *Escherichia coli*. *Biochem. Biophys. Res. Commun.* **143:** 638-644.

Yost F.J. & I. Fridovich. 1973. An iron-containing superoxide dismutase from *Escherichia coli*. *J. Biol. Chem.* **248:** 4905-4908.

Yusifov E.F., A.I. Grebenko, V.V. Barynin, G.N. Murshundou, A.A. Vagin, W.R. Melik-Adamyan & B.K. Vainshtein. 1989. Three-dimensional structure of catalase from *Micrococcus lysodeikticus* at a resolution of 3.0 Å. *Sov. Phys. Crystallogr.* **34:** 870-874.

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