The Role of Key Residues in the Lateral Channel of the *E. coli* Catalase HPII

By

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A Thesis submitted to

the Faculty of Graduate Studies

In Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

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ABSTRACT

The heme-containing active site of catalases is buried in the core of the protein. The flow of substrate and product to and from the site takes place through a network of channels that extend from the molecular surface to the core of the protein. Three channels have been identified in catalases, two of which have been characterized: the main channel and the lateral channel. The main channel with length of 55 Å in catalase HPII approaches the distal side of the heme perpendicular to its plane. The lateral channel with length of 30 Å extends from molecular surface to a cavity where it bifurcates before reaching the active site in the plane of the heme. This study investigates the role of the lateral channel by changing key residues in the heme pocket and the lateral channel using site directed mutagenesis. The targeted residues were isoleucine 274 in the entrance to the lateral channel, phenylalanine 206 and phenylalanine 214, close to the heme, and glutamate 270 about 15 Å up the lateral channel. The conversion of Ile274 to a residue with a smaller side chain, including Ala, Gly and Val, did not increase the protein's activity despite increasing the size of the lateral channel opening. However, each variant exhibited a different heme profile suggesting that the heme environment and possibly the ability of the proteins to bind heme were affected by the mutations. The conversion of Ile274 to a larger residue, Phe, interfered with protein folding and very little protein accumulated. Changes to Glu270 had very little effect on enzyme activity suggesting that increasing the diameter of the lateral channel at the location of E270 is not a determinant of the protein's activity. Phe214 and Phe206 variants all exhibited reduced activities and absorption spectra suggestive of different heme species and contents suggesting that F206 and F214 have a role in determining the heme integrity in HPII.

ACKNOWLEDGEMENT

I want to thank my Lord God and Savior Jesus Christ, for He has blessed me and my family so much that I was able to complete the work of this Master's thesis.

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Away from the academic world, I would like to thank my wife Anna Liza Louis. Her support, understanding, bearing and sacrifices were essential and indispensable for me to complete this work. In my thinking I do believe that both of us contributed equally to this work, each in his/her own way.

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LIST OF ABBREVIATIONS

A	Absorbance
Å	Angestromes
Amp^r	Ampicillin resistant
ATP	Adenosine triphosphate
BLC	Bovine liver catalase
bp	Base pair(s)
CCA	convex constrain algorithm
CPX	Catalases peroxidases
CpdI	Intermediate compound I
Da	Dalton
DEAE	Diethylaminoethyl
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acetic
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
mA	Milli ampers
MALDI/MS	Matrix assisted laser desorption/ionization mass spectroscopy
MSH	2-Mercaptoethanol
MW	Molecular weight
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
NMR	Nuclear magnetic resonance
PAGE	Polyacrylamide gel electrophoresis
PVC	*Penicillium vitale* catalase
ROS	Reactive oxygen species
RT	Room temperature
SDS	Sodium dodecyl sulfate
T_m	Melting temperature
TAE	Tris-Acetate-EDTA
SOD	Superoxide dismutase
V_{max}	Maximum velocity
Vi	Initial velocity
WT	Wild type
w/v	Weight per volume

1. INTRODUCTION

1.1. Oxygen and living organisms.

Life on Earth has existed for at least 3.5 billion years, and during most of that time it consisted mainly of microorganisms. Primitive organisms capable of metabolism (ability to accumulate and modify nutrients for energy) and reproduction (ability to generate more organisms like themselves) that first appeared were most likely "*thermophilic anaerobes*" and may have depended on RNA for both enzymatic and genetic activities (Schwartz and Dayhoff 1978).

Life as we know it is both complex and simple: complex because of myriad of organisms, yet simple because of the small variety of atoms and molecules that life on earth requires (Doolittle and Sapienza 1980). Over the long history of life on earth, photosynthesis has dominated over respiration resulting in a rich store of energy and oxygen. Originally oxygen (O_2) was only a trace element in the earth's atmosphere when prokaryotic life forms emerged from the primordial soup (Chandra and Shethna 1977). It became a significant element when certain bacteria began splitting water to create energy, releasing molecular oxygen, as a bi-product in the photosynthetic process (Xiong *et al*, 2000). Doubtless this new gas was lethal for many of these bacteria, but some of them adapted to use oxygen to oxidize food sources generating energy, in a process known as *"aerobic respiration"*. With O_2 available as a terminal electron acceptor in the electron transport, aerobic organisms developed the ability to derive much more energy from the oxidation of organic compounds than anaerobic organisms (Miller *et al*, 1971).

The world is rich in examples of how more complex organisms have evolved from less complex ones by the process of *"natural selection"* (Darwin, 1859). Efficient

aerobic metabolism developed in many bacteria, and exactly the same aerobic mechanisms operate in the mitochondria of eukaryotes, supporting conjecture that both mitochondria and chloroplasts evolved from aerobic bacteria that had been endocytosed by primitive eukaryotic cells (Schwartz and Dayhoff, 1978).

Living cells require energy to carry out the biotic activities necessary for life. The molecule supplying energy to many reactions is ATP (adenosine triphosphate) and the major source of ATP for all cells is the oxidation of glucose, via the process of cell respiration. The initial steps of cell respiration, called glycolysis, occur in the cells of all organisms (with a few bacterial exceptions) (Fenchel *et al*, 1977) and result in glucose being converted to pyruvic acid. The requirement for oxygen arises subsequent to glycolysis whereby pyruvate is metabolized to CO_2 by the Krebs cycle and electron transport chain. The oxidation of pyruvate involves a series of reactions which transfer electrons from pyruvate and other intermediates to the reduced electron carriers NAD^+ and FAD. Subsequent oxidation of NADH and $FADH_2$ in the electron transport chain powers the movement of hydrogen ions across the cell membrane establishing a concentration and, therefore, a pH gradient. This process is usually coupled to the production of unstable reactive oxygen species.

1.2. Reactive oxygen species

A free radical is a molecule with one or more unpaired electrons in its outer orbital. In most molecules, the electrons in each orbital are paired and have opposite spin. The presence of an unpaired electron makes a molecule less stable and consequently more reactive (Bayer *et al*, 1988). Molecular oxygen and many of its derivatives contain

unpaired electrons making them naturally reactive giving rise to the term "reactive oxygen species" or ROS.

ROS most often refers to compounds, such as singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), and hydroperoxides (ROOH) (Gutteridge and Halliwell, 1989). All these compounds have the potential to cause free-radical reactions (Pryor, 1986) resulting in the non-reduced target molecule often being converted to a radical, which may further react with another molecule. In eukaryotes, ROS are generated in several ways including the electron transport chain from antimicrobial or antiviral responses and from detoxification reactions carried out by the cytochrome system (Raven, 1970). Environmental agents such as ultraviolet light, ionizing radiation, redox chemicals and cigarette smoke also readily generate ROS (Demple and Linn 1982). Usually, ROS do not cause much damage because organisms have one or more defense mechanisms against them, but there are times when ROS accumulate and cause cellular damage. This is known as "*oxidative stress*" (Freemann and Crapo 1982).

1

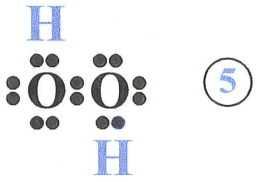
Oxygen

O_2

3

Hydroxyl radical

[• OH]



5

Hydrogen peroxide

H_2O_2

2

Superoxide anion

$\bullet O_2^-$

4

Hydroxyl anion

$[OH^-]$

6

Hypochlorite ion

$[OCl^-]$

1.3. Oxidative stress.

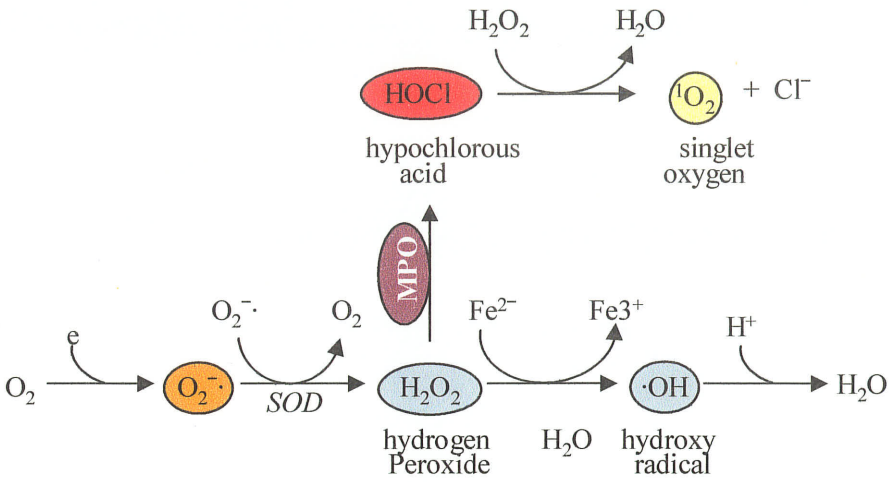
Cellular oxidative stress occurs when cells come in contact with reactive oxygen species (ROS). Whether damage occurs depends on the concentration of ROS and the effectiveness of cellular defenses. In prokaryotes, ROS include extracellular hydrogen peroxide (H_2O_2), that can penetrate the cell membrane to access the cellular compartment, and the intracellular products of oxidases and dehydrogenases (Loewen *et al*, 1997). In eukaryotes, mitochondria are the main source of endogenous ROS including hydrogen peroxide. In combination with reduced trace metals such as iron or copper, hydrogen peroxide is transformed into the highly reactive hydroxyl radical, which causes damage to virtually all macromolecules including proteins (Fenton, 1894).

The oxygen rich environments common to most aerobic organisms produce a variety of chemical modifications in proteins, DNA, lipids, carbohydrates and even amino acids (Freeman *et al*, 1986). Commonly carbonyls and carbonyl adducts, such as 4-hydroxy-2-nonenal (HNE), are formed and deamination, racemization and isomerization reactions occur (Brot *et al*, 1981). These chemical modifications result in protein cleavage, aggregation and loss of catalytic and structural function by distorting the proteins secondary and tertiary structure (Davies and Delsignore 1987). If the oxidatively modified proteins are not repaired, they must be removed, usually by proteolysis.

1.4. Toxicity of Hydrogen peroxide

Hydrogen peroxide (H_2O_2) plays a very important role in ROS toxicity. It is more stable than $O_2^{\cdot-}$, and can diffuse through the plasma membrane. If it is not scavenged by catalase or glutathione peroxidase, H_2O_2 can promote radical reactions far from its origin

(Gutteridge and Halliwell 1989, Parke and Sapota 1996). The most reactive and potentially harmful radical $OH^{\cdot}$ (Halliwell and Gutteridge 1989) is generated from $O_2^{-\cdot}$ and H_2O_2 via the Haber-Weiss reaction or the Fenton reaction in which the electron donor $O_2^{-\cdot}$ is replaced by Fe^{2+} or Cu^{2+} (Fenton, 1894).

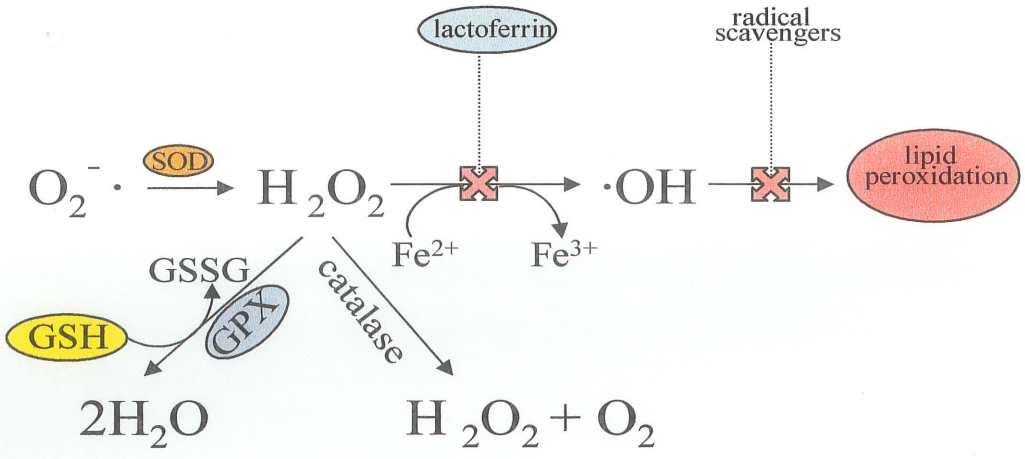


1.5. Oxidative stress and human diseases

Oxidative stress is thought to contribute to the general decline in cellular functions. Although the entire role free radicals play in disease is not totally understood, it is believed that they either cause or accelerate a wide variety of chronic diseases (Rowley and Halliwell, 1983 a&b). These diseases may include Alzheimer's disease, amyotrophic lateral sclerosis (Evans, 1993), Parkinson's disease, atherosclerosis (Halliwell and Cross 1994), ischemia/reperfusion neuronal injuries, degenerative disease of the human temporomandibular-joint, cataract formation, muscular degeneration (Kroemer *et al*, 1998 and Spencer *et al*, 1994), degenerative retinal damage, rheumatoid arthritis, multiple sclerosis, muscular dystrophy, human cancers (Cerutti, 1985) as well as the aging process itself (Halliwell, 1989, Cross *et al*, 1994).

1.6. Antioxidant response.

To help cope with oxygen radicals, living organisms, regardless of complexity, have evolved antioxidant systems (Farr and Kogoma, 1991). The systems consist of specific enzymes, vitamins, minerals and amino acids which have the job of combining with toxic free radicals to neutralize them and prevent cellular damage (Bravo *et al*, 1997, Heffner and Repine 1989). The antioxidant system consists of two major groups of molecules: 1) antioxidant enzymes (Hassan and Schrum, 1994) and, 2) antioxidant nutrients such as vitamins and minerals (Lundberg *et al*, 1999). Antioxidant enzymes include superoxide dismutase (SOD), catalase, and glutathione peroxidase (Fridovich, 1983); anti-oxidant nutrients consist of coenzyme Q, vitamins A, C and E (Burton and Ingold 1989), beta-carotene and bioflavanoids (Halliwell, 1996). The influence or damage of ROS is kept to a minimum by an organism's natural supply of antioxidants (Hassan and Fridovich, 1979). For example transition metals can be inactivated by chelating proteins (e.g., ferritin), while ROS can be removed enzymatically by SOD, peroxidases and catalases or non-enzymatically by antioxidants like vitamin E, vitamin C and glutathione (Benzie, 2000).

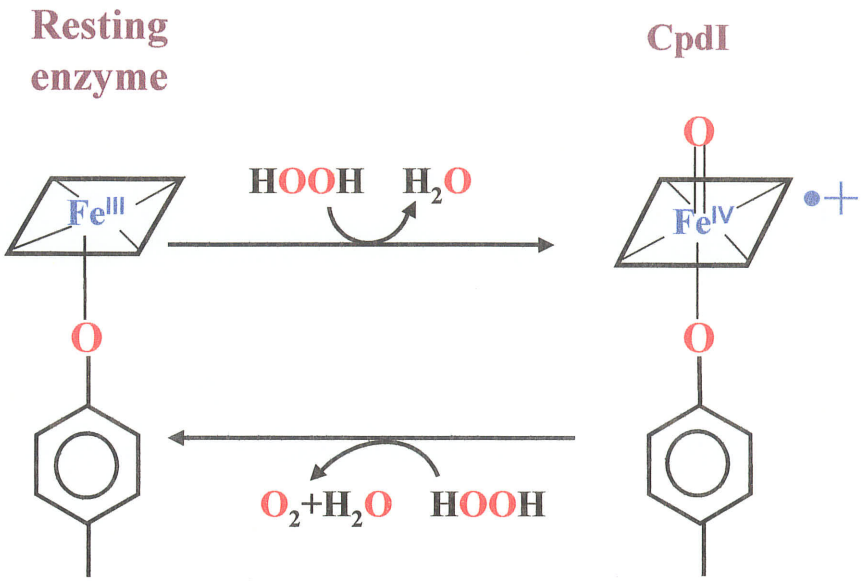


1.7. Catalases

General Characteristics

Catalases have been extensively studied for more than one hundred years since the first report of a catalase in tobacco leaf (Loew, 1901). Bovine liver catalase (BLC) was one of the first proteins to be crystallized (Sumner and Dounce, 1937) and is sometimes referred to as the "standard catalase" (Klotz and Loewen, 2003). New sequences and new crystal structures are published every year providing a data base of more then 250 sequenced genes and 8 crystal structures (Klotz and Loewen, 2003).

In prokaryotes, catalases appear to be "non essential"; that is a bacterial cell deficient in catalase can grow and survive. Catalases are protective enzymes that usually respond to oxidative stress or starvation and sometimes to the virulence system in pathogens. Heme containing catalases are evolutionarily conserved among most aerobic organisms (von Ossowski *et al*, 1993, Klotz *et al*, 1997, Klotz and Loewen, 2003), and decompose two molecules of H_2O_2 into two molecules of water and one molecule of oxygen (Chance *et al*, 1952a, Maehly and Chance, 1954). The reaction takes place in two steps where the first step involves the oxidation of the heme ferric atom by one molecule of H_2O_2 to form the intermediate compound I (CpdI) ($Por^{+\cdot}Fe^{4+}=0$) and one molecule of H_2O (Chance *et al*, 1952b). The second step involves H_2O_2 as an electron donor reducing CpdI back to the resting state releasing the second molecule of H_2O and one molecule of oxygen (Fita and Rossmann 1985a).



Heme containing catalases are relatively large tetrameric proteins in which the active site of each subunit is deeply buried in the core of the protein. This necessitates the flow of substrate and products through channels to access and exit the active site. The high turn over rate of the enzyme suggests that movement through the channels is highly efficient, very likely this mechanism involves not only the active site heme but also a number of residues in the heme vicinity and the channels. In addition to the heme-containing mono-functional catalases, catalase activity is also found in the bifunctional dimeric catalase-peroxidases and the hexameric Mn-catalases (Chelikani *et al*, 2004).

Evolution of Catalases

Monofunctional heme catalases:

The phylogenetic analysis of over 250 mono-functional heme catalase sequences, from a variety of prokaryotes and eukaryotes suggests a common ancestor. The enzymes can be divided into 3 clades (Klotz *et al*, 1997, Klotz and Loewen, 2003) based on phylogenetic analysis; clade 1 and 3 contain small subunit (~60 kDa) catalases and clade 2 contains the large subunit (~80 kDa) enzymes. Generally, clade 1and 2 enzymes seem to be more closely related than they are to clade 3 enzymes in that the heme is flipped 180° in clade 3 and most, but not all, clade 3 enzymes bind NADPH whereas clade 1and 2 enzymes do not. The large subunit, clade 2, catalases have extended N- and C-termini that seem to enhance the stability of the enzymes to both temperature (T_m = 83°C for loss of activity) and proteolysis degradation (Switala *et al*, 1999*)*. Recent studies showed that HPII, a large subunit catalase, with truncated N- and C-termini retained catalytic activity but with less stable structure. This observation suggests that the large subunit catalase may have been the common ancestor for clade 1 and 3 enzymes (Chelikani *et al*, 2004), corroborating the conclusion of the recent phylogenetic analysis (Klotz and Loewen 2003) that suggested that clade 1 and 3 (small subunit) catalases evolved from clade 2 after gene duplication event followed by loss of the sequences. The absence of clade 3 catalases from old taxonomic species suggests that proteins from this clade were the latest to develop (Klotz and Loewen, 2003).

Bacterial catalases are present in all three clades. Some bacterial species even contain representatives from the 3 clades suggesting that catalase gene duplication events happened first in bacteria and were passed on to higher organisms. The fact that all

plant/algal catalases group in clade 1, and the presence of more than one gene in plant genomes, suggest gene duplication events subsequent to clade 1 segregation. Indeed, the presence of multiple catalases may have arisen from the need for organ specific enzymes in plants. Animal catalases fall entirely in clade 3 with higher animals containing only one catalase and lower animals containing more than one. Fungal catalases are found in both clade 2 and clade 3 (Klotz *et al*, 1997).

Bi-functional Catalase-Peroxidases (CPX)

Catalase-peroxidases are genetically unrelated to mono-functional catalases (Nicholls *et al*, 2000, Hillar *et al*, 2000) and phylogenetic studies suggest that CPX enzymes evolved later than monofunctional heme catalases. The presence of two separate but sequence related domains suggest that a gene duplication and fusion event was responsible for the structure. Lateral gene transfer contributed to the distribution of this gene among different taxonomic groups including the eukaryotic ancestor cell. The N-terminal domain of the CPX protein shares various similarities with plant ascorbate peroxidase, suggesting a common ancestor (Klotz and Loewen, 2003).

Nonheme catalase (Mn)

Nonheme catalase genes fall into two groups that might have arisen from a common ancestor by gene duplication, and they probably evolved before the bi-functional catalase-peroxidase but after monofunctional heme catalases (Klotz and Loewen 2003). The Mn-catalase gene is not present in eukaryotic organisms and it has been suggested that the gene was not maintained because Mn-catalases have lower catalytic activities than other catalases making them less essential. The gene has been retained in many species of eubacteria especially methanogens (anaerobes) (Klotz *et al*, 1997).

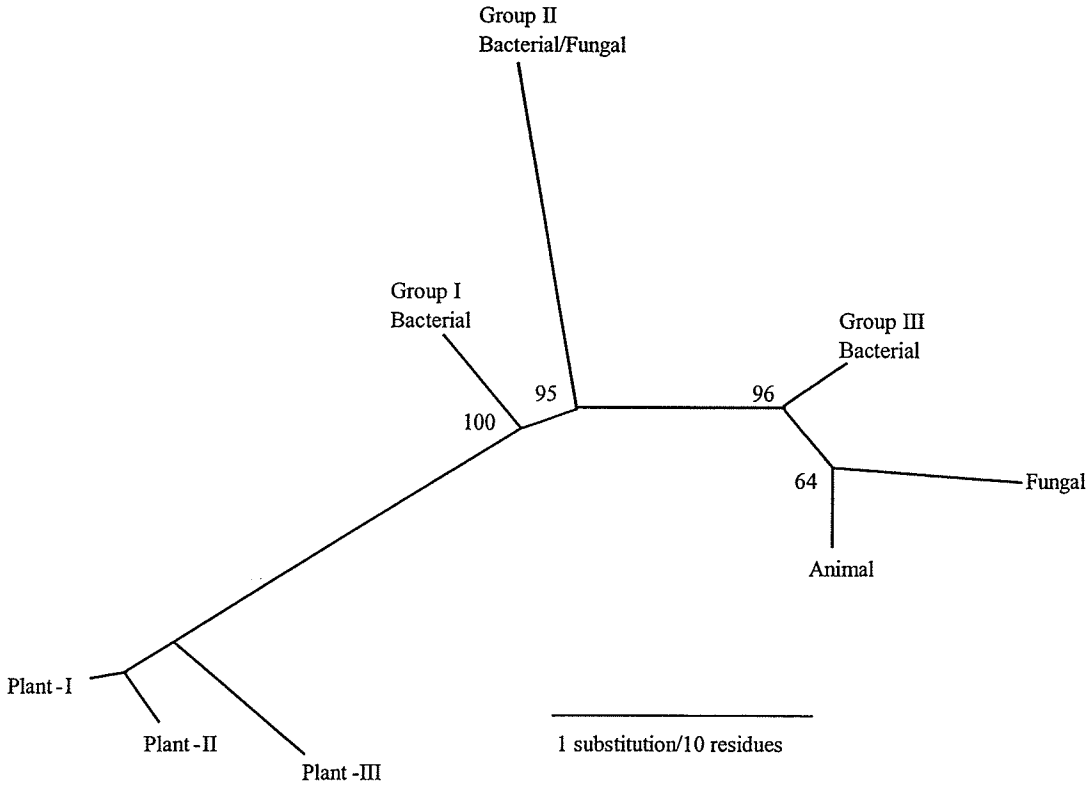


Fig 1.1. Phylogenetic tree showing the 3 clades (groups) of monofunctional heme catalases (Klotz, *et al* 1997).

1.8. *E. coli* Catalases

E. coli was discovered in the human colon in 1885 by The German bacteriologist Theodor Escherich, who showed that certain strains were responsible for infant diarrhea and gastroenteritis, an important public health discovery (Frazer and Curtess 1975). Although the bacteria were initially called *Bacterium coli*, the name was later changed to *Escherichia coli* to honor its discoverer. Soon after its discovery, *E. coli* became a very popular lab organism because scientists could grow it quickly on both simple and complex media. *E. coli* can grow aerobically, using oxygen as a terminal electron acceptor or anaerobically, utilizing fermentative metabolism (Sanderson, 1976). The ability to grow both aerobically and anaerobically classifies the *E. coli* bacteria as a facultative anaerobe (Riley *et al*, 1983).

Although *E. coli* has often been identified as a foodborne pathogen, the vast majority of *E. coli* strains are harmless, including those commonly used in genetics laboratories. *E. coli* is part of the Enterobacteriaceae family, which is informally referred to as the enteric bacteria (Levine and Edelman 1984). Other enteric bacteria include *Salmonella* (a very large family, with many different members), *Klebsiella pneumoniae*, and *Shigella* species, which some consider to be part of the *E. coli* family. Microbiologists have classified *E. coli* into more than 170 serogroups, within each of which there may be one or more serotypes (Evans and Evans 1983).

E. coli has evolved an efficient defense system against hydroxyl radicals including superoxide dismutases and hydroperoxidases (Farr and Kogoma, 1991). *E. coli* produces two inducible catalases or hydroperoxidases called HPI and HPII which have been well studied physiologically and biochemically (Nicholls *et al*, 2000). Early studies

couldn't define clearly the relative importance of the two enzymes because of the overlapping activities, but the isolation of mutants lacking one or the other of the two catalases allowed a differentiation (Loewen, 1984). Not only are the two enzymes structurally and genetically different, the systems controlling their synthesis respond to different stimuli and involve different mechanisms (Obinger *et al*, 1997a).

1.9. Hydroperoxidase I (HPI)

HPI is a bifunctional catalase-peroxidase containing heme b associated in homodimers and homotetramers of 80 kDa subunits (Loewen *et al*, 1985). The subunit of HPI is encoded by the gene *katG,* which has been mapped at 89.2 minutes on the *E. coli* chromosome (Loewen and Triggs 1984). The gene has been cloned and sequenced, revealing an open reading frame of 2181 bp with transcriptional regulatory sequences at -10 and -35 upstream of the transcription start site (Loewen, 1984).

HPI is synthesized constitutively during log phase growth and levels increase about 2 fold as the culture enters stationary phase (Hillar *et al*, 1999). Addition of H_2O_2 increases the levels of HPI, by a mechanism involving OxyR (Storz *et al*, 1990)

1.10. Hydroxyperoxidase II (HPII)

General features

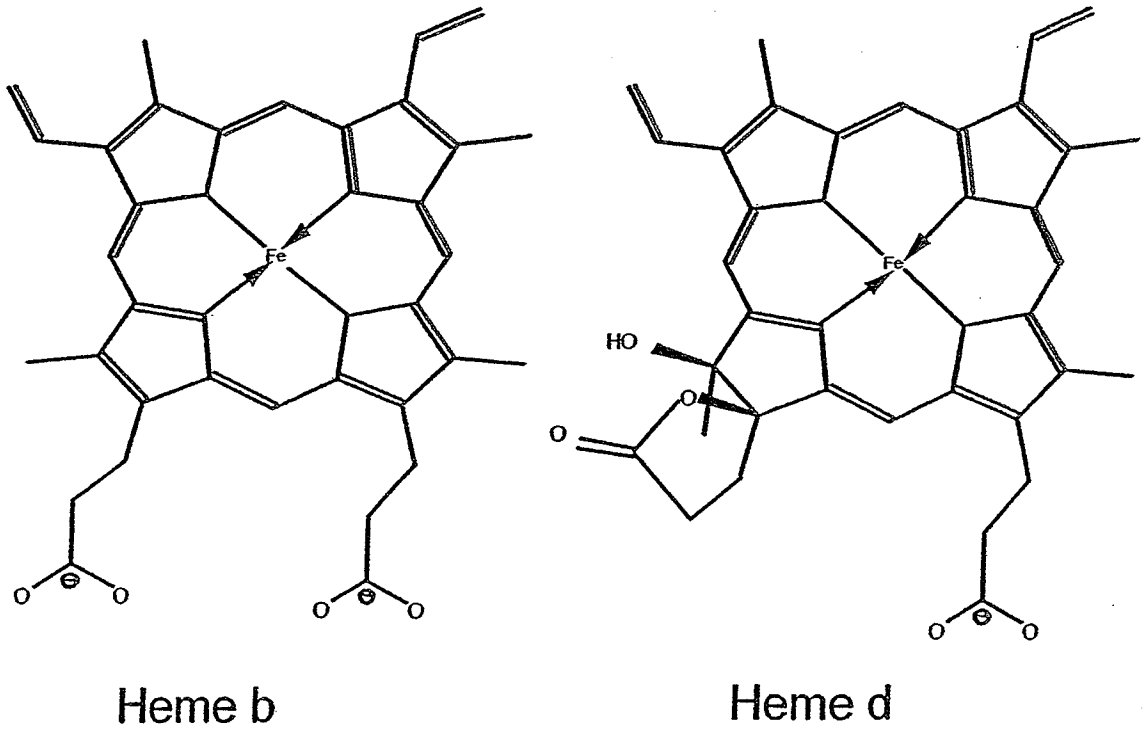
HPII is one of the most well studied catalases, having been first crystallized in 1990 and subsequently, the subject of a structure/function study involving over 100 variants. As a clade 2 monofunctional, heme d containing catalase, it is a homotetramer of 84.2 kDa subunits (Loewen *et al*, 1993b). The larger subunit imparts enhanced

resistance to denaturation in extreme conditions including pH, urea, high temperature and exposure to proteases (Switala *et al*, 1999). HPII is encoded by *katE* gene located at 37.2 minutes on the *E. coli* chromosome. Expression of HPII is constitutive at a low level during log phase growth, and this increases up to 10 fold as the culture enters stationary phase (Loewen and Switala, 1986). RpoS, the stationary phase sigma factor, controls expression of HPII from *katE,* along with the expression from 55 or more stress genes (Loewen *et al*, 1998). The expression of RpoS is in turn controlled by a complex mechanism at the levels of transcription, translation and enzyme stability (Loewen, 1999).

HPII Active site-Environment

The HPII active site in each subunit consists of a heme d prosthetic group surrounded by a number of residues with catalytic and structural roles. His128 on the distal side is essential and it works in conjunction with Asn201, which is important but not absolutely essential. In BLC, the active site His is located above ring III of the heme group (Murshudov *et al*, 1996), whereas in HPII and CatF the heme is flipped by 180°, placing the active site His above ring IV (Bravo *et al*, 1999 and Carpena *et al*, 2003). The orientation of the heme in both orientations is stabilized by van der Waals interactions with protein residues surrounding the heme, in particular with the methyl and vinyl groups on rings I and II (Chelikani *et al*, 2004). HPII catalyzes the oxidation of heme b to heme d by a mechanism requiring the formation of CpdI as the first step. Formation of the spirolactone is linked to reduction of CpdI and formation of the unique His392-Tyr415 bond (Bravo *et al*, 1997). Interestingly, the His392Gln variant of HPII retained

almost wild type activity in spite of having heme b in the active site (Bravo *et al*, 1997) showing that heme conversion was not required for catalysis.



Heme b

Heme d

Heme b type catalases
(BLC, CATA, PMC, MLC, CatF)

Heme d type catalases
(HPII, PVC)

Fig 1.2. The structure of heme b and heme d (Bravo *et al*, 1997).

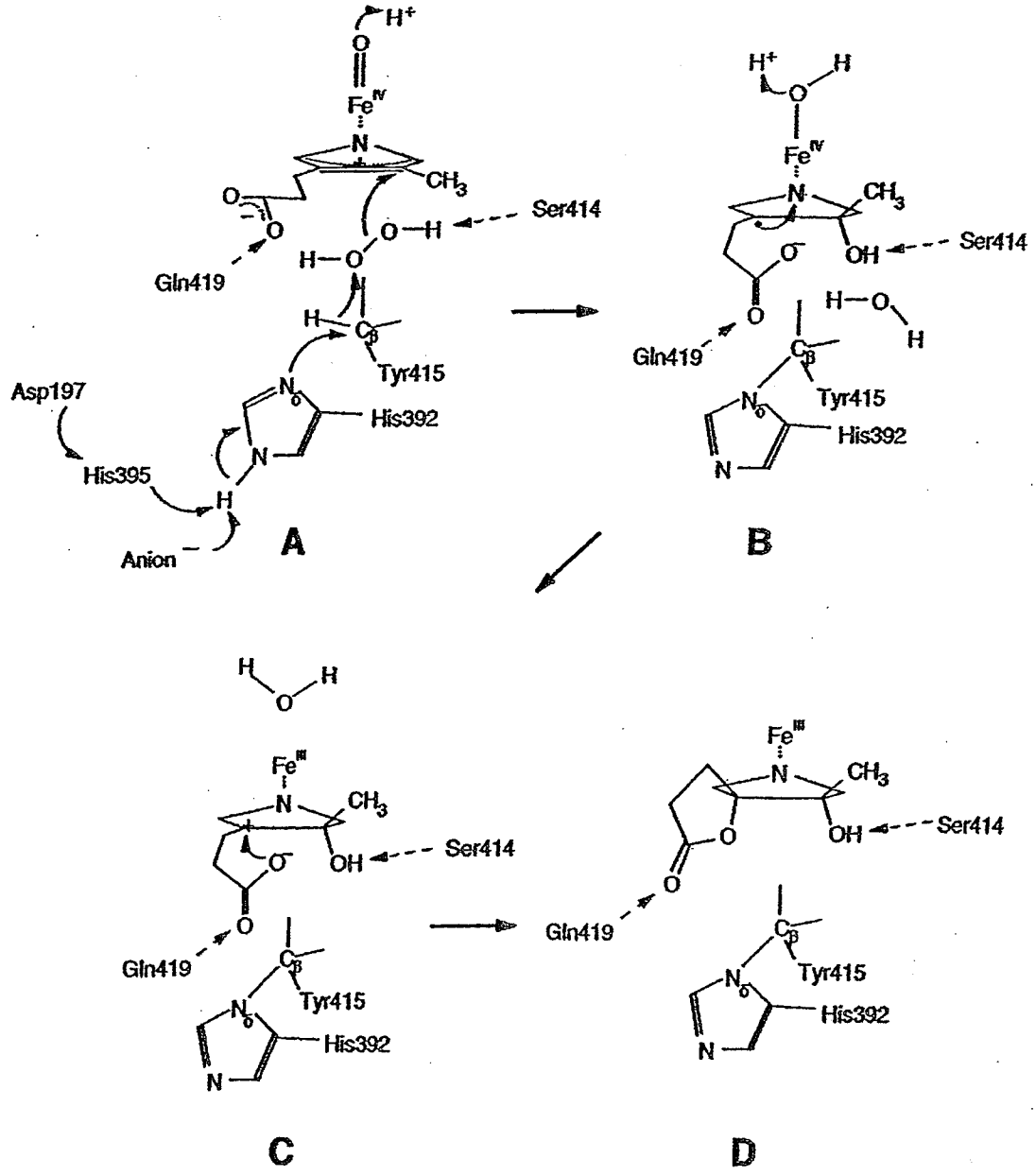


Fig 1.3. The hypothesized steps for the conversion of the heme b to heme d, involving the formation of His393-Tyr312 covalent bond (Bravo *et al*, 1997).

Structure of HPII subunit

The secondary structure of HPII subunit includes 18 α helices and 16 β-strands (Bravo *et al*, 1995). The subunit can be subdivided into five domains: the amino terminal domain, the antiparallel β barrel domain, which is the core of the enzyme including the active site, the wrapping domain, the helical domain and the carboxyl terminal domain (Bravo *et al*, 1997). The N-terminal domain includes 127 residues up to the active site histidine, although the terminal 27 residues are not visible in the crystal structure. The only hypothesized function for this domain is to maintain the stability of the tertiary structure of the protein. The β barrel core domain consists of 250 residues forming two groups of four β strands. The wrapping domain includes 110 residues connecting the β barrel and the helical structure. A large portion of this domain is involved in subunit-subunit interactions and helix α 9 forms part of the proximal side heme pocket. The helical domain consists of about 60 residues in four α helices (α10-13). The four helices are closely associated with helices α 3-5 of the core domain and presumably maintain stability of the structure. The C-terminal domain shows extensive secondary structure including 4 α helices and 8 β strands. No significant function has been identified for this domain, despite its flavodoxin like structure (Bravo *et al*, 1999).

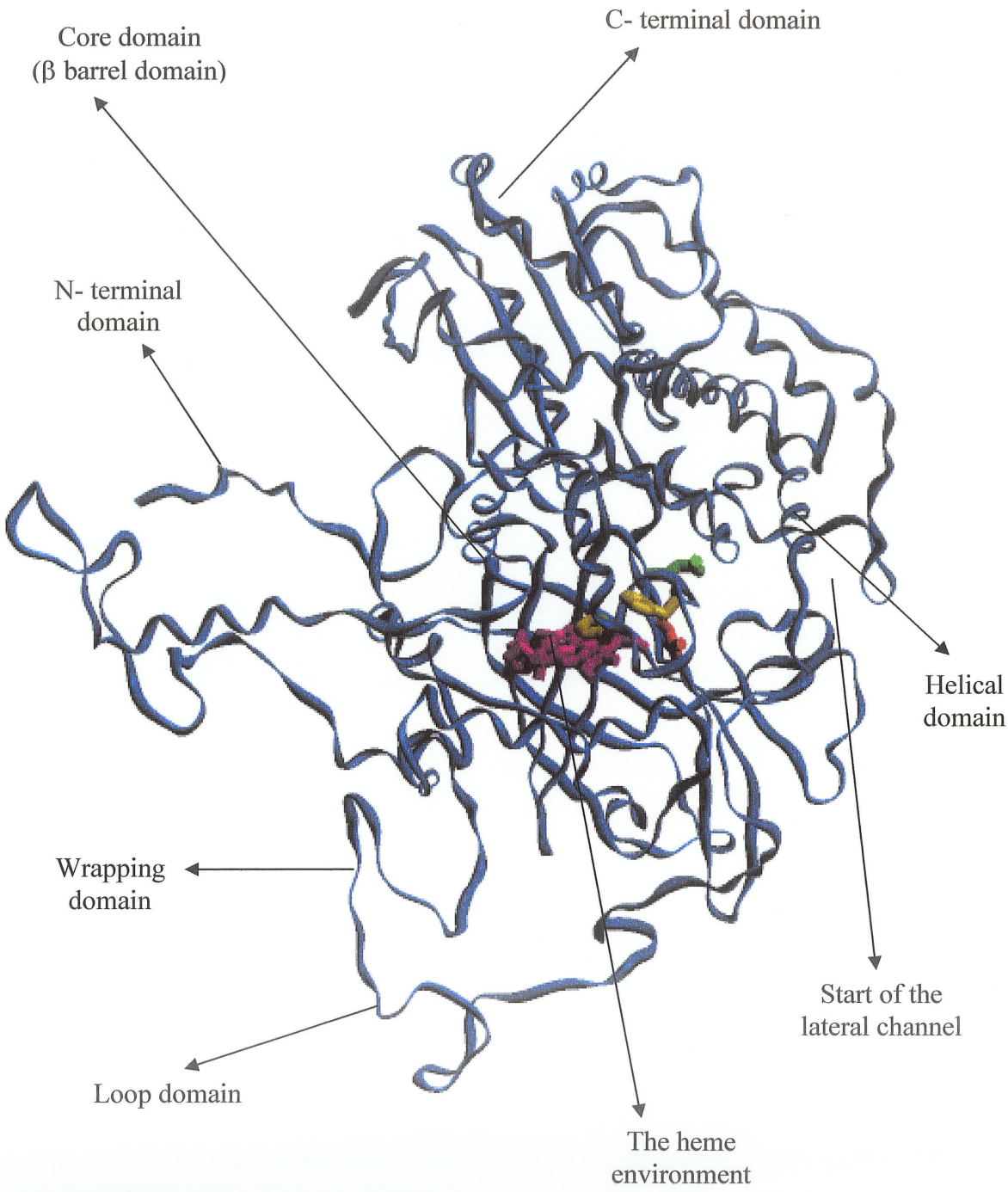


Fig 1.4. A cartoon for the structure of HPII subunit showing the different domains, active site environment and the start of the lateral channel

HPII folding

The functional form of HPII is as a tetramer with a total molecular weight of ~336 kDa. The dimensions of the molecule are 90 Å, 70 Å and 140 Å along the P, Q and R axes respectively, larger than small subunit enzymes only along the R axis. Folding of the tetramer requires the N- terminus to protrude through a loop in the wrapping domain of the corresponding Q-related subunit providing interactions between the two domains along a stretch of 80 residues. This "inter-woven" structure of subunits, A and C, and B and D, was initially thought to be responsible for the enhanced stability of the protein (Bravo, 1999), but this has recently been disproved (Chelikani *et al*, 2004).

From work with yeast catalase, the heme group appears to bind early in the folding process of individual subunits. Subsequently, subunits may associate to form dimers and dimers associate to form tetramers (De Duve, 1973).

Channels and Substrate flow in HPII

Because the active site of HPII, and catalases in general, is deeply buried in the core of the protein, substrate molecules must travel a minimum of 30 Å – 50 Å from the molecular surface of the protein to reach the active site. In addition, products H_2O and O_2, must be exhausted from the site. Despite these distances, the k_{cat} value or turnover rate can be as high as ~10^6/sec (Obinger *et al*, 1997b and Switala and Loewen, 2002). To provide access for substrate and products a network of channels within the protein exists including the main and the lateral channels and a third channel leading to the central cavity.

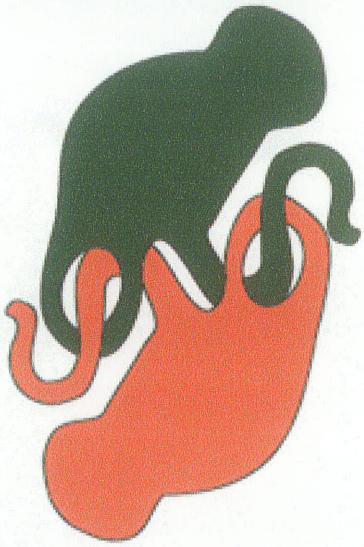


Fig 1.5. A diagram showing the interwoven folding pattern involving two adjacent subunits of HPII, in which the N- terminal of each subunit is inserted in the wrapping domain of the adjacent subunit.

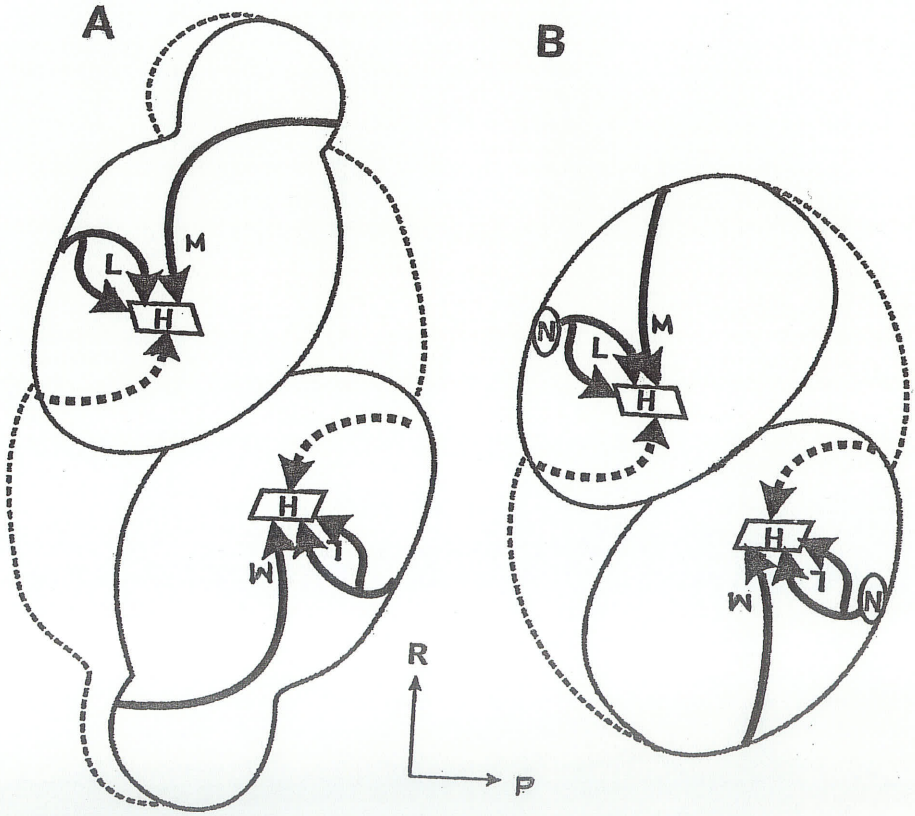


Fig 1.6. A diagram showing the main channel (M) and lateral channel (L) in catalases with either large subunit (HPII) or small subunit (BLC) showing NADPH location. The third proposed channel is also shown (Nicholls, *et al* 2001).

The Main Channel

The main channel of a subunit starts with the carboxyl-terminal domain of the R related subunit and extends ~50 Å before reaching the active site heme. Substrate molecules initially pass through the interface between the core domain and the C-terminal domain of the R subunit to reach a bend where the channel extends directly to the heme (Bravo *et al*, 1999). Molecular dynamics modeling suggests the major channel as the principal pathway for substrate molecules (Kalko *et al*, 2001 and Bravo *et al*, 1995). Crystallographic and biochemical studies of wild type HPII and its variants have confirmed the roles of a number of essential residues in the catalytic process as well as the solvent occupancy within the channel (Melik-Adamian *et al*, 2001).

Substrate flow through the main Channel

Electron density maps of HPII have revealed the presence of solvent molecules in the main channel, interacting with the heme group and with catalytic residues. Two water molecules that are hydrogen bonded to each other also link His128 and Asn201. W1, closest to the heme group is of lower occupancy and acts as hydrogen donor to His128 while W2 is of higher occupancy and interacts with the NH_2 of Asn201. Changing either of these residues substantially changes the distribution of solvent molecules in the channel. Moving "up" the channel from the heme, another three water molecules are found, but they are not protein bound because they lie in the hydrophobic portion of the channel. They are dependent on interactions with other waters and form a continuous water matrix extending up the channel. The next protein bound water molecule is interacting with Asp181, which is 12 Å away from the active site (Melik-Adamian *et al*,

2001). Recent mutagenesis studies targeting the negatively charged Asp181 suggest that an electrical potential field exists between it and the positively charged iron in the heme group which is required for the positioning of solvent and peroxide molecules in an optimum orientation for the interaction in the heme active site (Chelikani *et al*, 2003).

The lateral Channel

The lateral channel is approximately 30 Å in length extending from a funnel shape opening on the molecular surface near residues 590-595 to the heme edge near residue I274 (Melik-Adamian *et al*, 2001). In catalases that bind NADPH, this channel is blocked by the cofactor, while in HPII the cofactor is absent, but is replaced by a portion of the protein chain. Arg260 is located at the upper branch of the lateral channel and is ionically bound to Glu270 (Sevinc *et al*, 1999); a bridge that may limit accessibility to the heme active site through the channel. Structural and biochemical studies targeting Arg260 suggested that the lateral channel was used by inhibitors and by either products or substrate to access or leave the active site (Sevinc *et al*, 1998).

Isoleucine 274

Ile274 in HPII is located at the start of the lateral channel only 3.5 Å below the methyl group on ring I of the heme group. Ile274 is conserved in *Penicillium vitale* and corresponds to Ser169 in BLC and *P. mirabilis* catalase (PMC) (Murshudov *et al*, 1996). Ile274, together with Pro356 and Lys407 (in HPII), are in Van derWaals contact with the heme group and may control or maintain its orientation. The strategic location of Ile274 at the start of the channel raises the possibility that it may have a role in controlling the

movement of substrate and products in the lateral channel. Molecular dynamics studies in PMC suggested that Ser169 has a role in coordinating substrate/product accessibility to the heme active site (Mate *et al*, 1999 and Amara *et al*, 2001).

Phenylalanines 206&214

Phenylalanine is a hydrophobic amino acid that is a common component of the surface of channels in catalases creating an environment which will not interfere with solvent flow. In HPII the active site is surrounded with four Phe residues at positions 206, 207, 214 and 217, of which Phe206 and 214 are adjacent to the heme (von Ossowski *et al*, 1991). Phe 214 is located at 3.4 Å from the heme group and Phe206 is 5.6 Å from methyl group on ring I of the heme.

Glutamate 270

HPII does not bind NADPH, a cofactor often found in catalases likely because part of the linker to the C- terminal domain wraps into the putative binding site (von Ossowski *et al*, 1991). In catalases such as BLC, NADPH interacts with four key residues, of which two are not present in HPII, Glu270 and Glu362 replace Asp212 and His304 in BLC (Sevinc *et al*, 1998). Glu270 is located ~9 Å from the heme active site, on the top of the lateral channel and is ionically bound to Arg260. Previous studies disrupting the ionic bond between R260 and E270 increased the specific activity, while introducing BLC counterparts into HPII didn't influence binding of the cofactor to the variant protein (Sevinc *et al*, 1998).

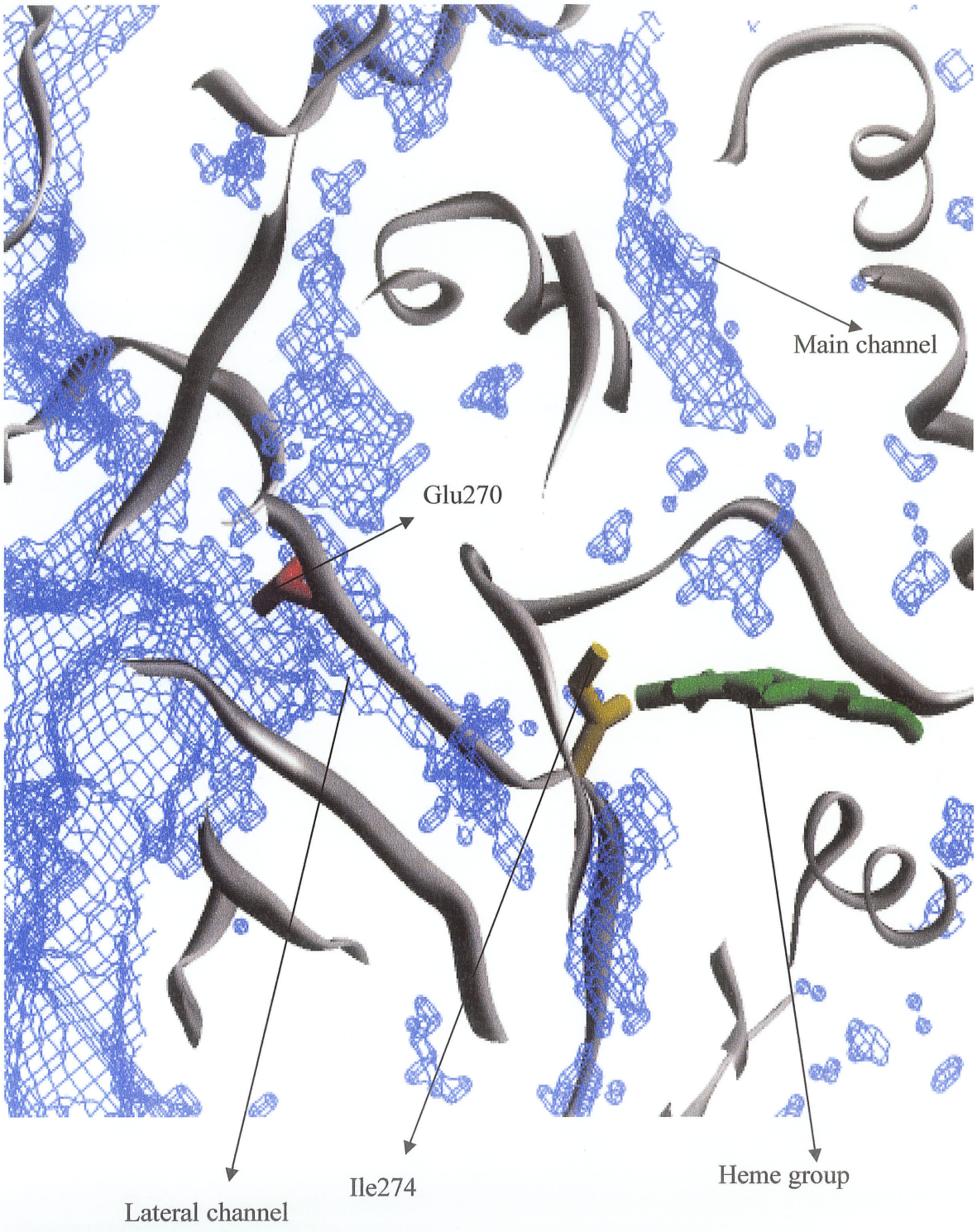


Fig 1.7. A surface diagram showing the channels leading to the heme active site. The blue wire mesh indicates water accessible surface and the portions extending into the subunit are channels.

Objectives

The object of the work described in this thesis is to study the structure function relationship of residues in the lateral channel of the *E. coli* catalase HPII. The residues targeted for study included Ile274, Phe214 and Phe206, close to the heme, and Glu270, in the middle of the lateral channel. A second object was to determine if the orientation of the heme could be changed by modifying residues making contact with it.

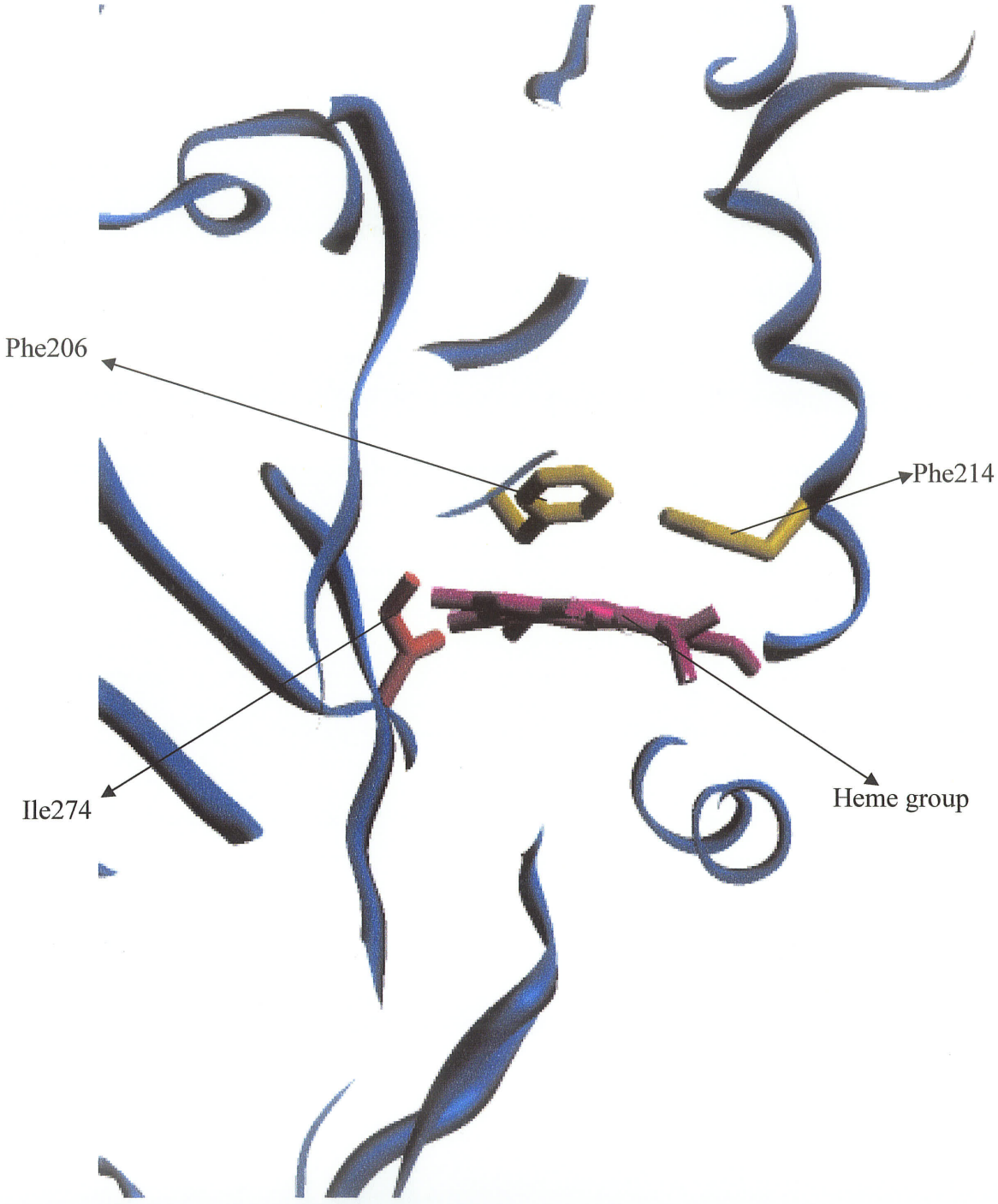


Fig 1.8. A diagram showing relationship of the heme to residues isoleucine 274, phenylalanine 206 and phenylalanine 214

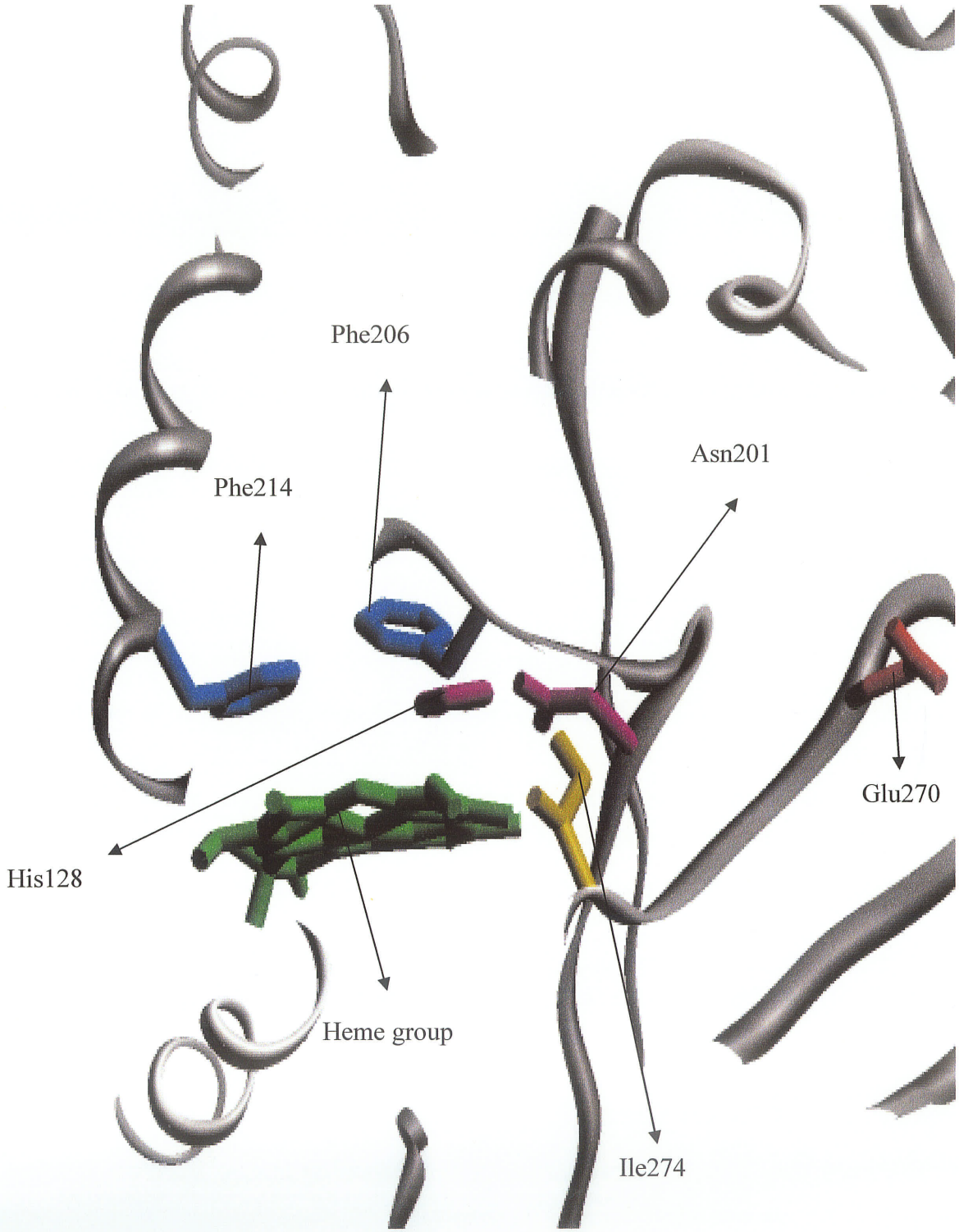


Fig 1.9. A diagram showing the relationship of I274, E270, F206, F214, H128 and N201 to the heme.

2. MATERIALS AND METHODS

2.1. *Escherichia coli* strains, plasmids and bacteriophage

The lists 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 harbored the subclone of pAMkatE72, pKS^{-}H-E and pKS^{+}E-C (Fig 2.1), to be 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 to generate single stranded DNA. Strain UM255 was used for expression of catalase from various plasmids harboring wild type and variant proteins. Strain JM109 was used for production of plasmid DNA for sequencing.

2.2. Media, growth conditions and storage of cultures

E. coli cultures were grown in standard LB (Luria-Bertani) medium that contained 10 g/L tryptone, 5 g/L yeast extract, 5 g/L sodium chloride. Solid media (hard top agar) contained 14 g/L agar. Ampicillin was added to 100 μg/ml for the growth of the plasmid-hosting cells. 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 sulfoxide. Bacteriophage R408 was stored at +4°C in culture supernatant.

Table 2.1. List of *Escherichia coli* strains, plasmids and bacteriophage

	Genotype/Phenotype	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 *lacy 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
Bacteriophage		
R408 (helper phage)		Stratagene Cloning Systems

2.3. Site directed mutagenesis

2.3. a. Plasmid DNA isolation and purification

Plasmid DNA was isolated according to Sambrook *et al.* (1989), modified as described below. Plasmid containing cells from a 3-5 ml culture were pelleted by centrifugation and resuspended in a buffer containing 50mM Tris, pH 7.5, 10mM Na-EDTA and 100μg/ml RNAse. The cells were 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 minutes incubation at room temperature, the mixture was centrifuged twice to eliminate cell debris. 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.

2.3. b. Uracil-containing single stranded DNA isolation

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 were 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 minutes and centrifuged to pellet the phage particles. The pellet was resuspended in TE buffer (10mM 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. An equal volume of 7.5 M ammonium acetate, pH 7.5 and 4 volumes of ice-cold absolute ethanol were added and the mixture was kept at -20°C until further use.

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 30 minutes. DNA was added followed by incubation for 30-45 minutes and heat-shock at 42°C for 90 seconds. The cells were then added to 1.0 ml LB medium and incubated at 37°C for 1 hour without aeration. The mixture was plated on ampicillin-containing LB plates.

2.3. d. Preparation of synthetic oligonucleotides

Oligonucleotides used for site-directed mutagenesis were purchased (GIBCO-BRL) and 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 units of T4 kinase were incubated in the manufacturer's buffer at 37°C for 30 minutes. T4 kinase was inactivated by incubation at 65°C for 5 minutes.

2.3. e. 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 simplified restriction map of *katE* that also indicates the locations of subclones is shown in Fig 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 Fig 2.2. The sequences of oligonucleotides used in mutant construction are listed in Table 2.2. Mutagenesis reactions were performed by annealing the phosphorylated mutagenic- oligonucleotides to the uracil-containing single-stranded DNA, obtained from the appropriate Bluescript phagemid subclone. Complementary strands were synthesized using unmodified T7 DNA polymerase (New England Biolabs) and ligated using 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 sequencing analysis with the Sanger method. Once the mutation was detected, the complete sequence of the subclone was confirmed to ensure no other base changes had occurred during DNA manipulation.

2.3. f. 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 μl volume with 0.5-2.0 μg DNA incubated for 2-3 hours at 37°C.

2.3. g. 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 40-60 mA constant current until the blue dye in the stop buffer migrated ~4 cm from the loading well. Samples of 10 μl were mixed with 2 μl stop buffer (40% (v/v) glycerol, 10 mM EDTA pH 8.0, 0.25% (w/v) bromphenol blue). The 1 Kilo base DNA ladder (GIBCO-BRL) was used as molecular weight size markers. DNA bands were visualized with ultraviolet light and photographed using the Bio-Rad Gel Doc 1000.

2.3. h. 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. i. Reconstruction of *katE* variant gene

Mutant subclones were subsequently used to reconstruct the complete *katE* gene. Appropriate DNA bands were excised from agarose gels and purified using the GENECLEAN DNA extraction kit (Bio/Can Scientific Inc.). Purified DNA strands were ligated and transformed into UM255 for determination of enzyme activity and visualization of protein in crude extracts by SDS-PAGE. Clones that expressed detectible levels of HPII-like protein were then grown in large scale (4-8 liters) for purification and characterization of the enzyme.

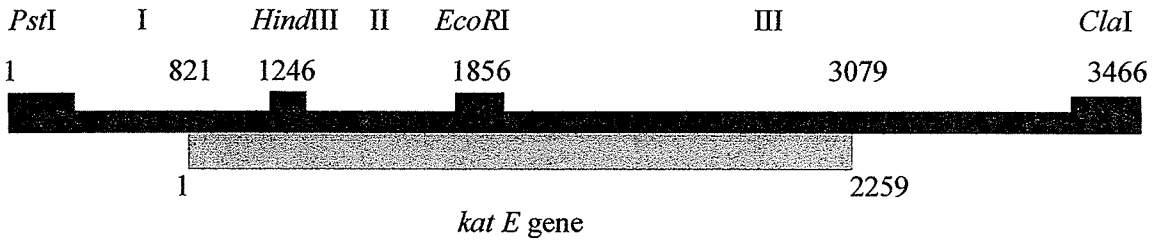


Fig 2.1. Simplified restriction map of the cloned 3466 bp chromosomal fragment in pAMKatE72. The three subclones are shown (I, II, III) and the 2259 nucleotides *katE* open reading frame is shown in grey.

Fig 2.2. The DNA sequence of *katE* gene within the chromosomal insert in pAMkatE72. The sequence of the mutagenic primers are shown in red, the major restriction sites are in blue, Shine Dalgarno (S.D) sequence and putative promoter sequence (-35, -10) are highlighted in red. The residues studied were highlighted in green.

PstI
CTGCAGCCTT TCTTTAAAAG AGTCGAAAGC CAGGCTTTTA ATATTTAAAT CACCATAATT 60
ACTCTGTATT AAGTTTGTAG AAAACATCTC CCGCCTCATA TTGTTAACAA AATTATTATC 120
TCATTTAAAT CTAAGTCATT TACAATATAA GTTTAAGAGC GACGCCACAG GATGAACTAT 180
CAAAAATAGC TCATCATGAT TAGCAAAACT TAACCATTTT AAAATAAATA AACAATTAAA 240
AAAAAAGATC ACTTATTTAT AGCAATAGAT CGTCAAAGGC AGCTTTTTGT TACAGGTGGT 300
TTGAATGAAT GTAGCAACGA AATACAGAAT TTCAGGTCAT GTAACTCCCG GCAAACCGGG 360
AGGTATGTAA TCCTTACTCA GTCACTTCCC CTTCCTGGCG GATCTGATTT GCCCAACGTT 420
GGGCAGATTC AGGCACAGTA AACGCCGGTG AGCGCAGAAA TGACTCTCCC ATCAGTACAA 480
CGCAACATAT TTGCCACGCA GCATCCAGAC ATCACGAAAC GAATCCATCT TTATCGCATG 540
TTCTGGCGGC GCGGGTTCCG TGCGTGGGAC ATAGCTAATA ATCTGGCGGT TTTGCTGGCG 600
GAGCGGTTTC TTCATTACTG GCTTCACTAA ACGCATATTA AAAATCAGAA AAACTGTAGT 660
TTAGCCGATT TAGCCCCTGT ACGTCCCGCT TTGCGTGTAT TTCATAACAC CGTTTCCGAA 720
-35 -10

TAGTCTCCGA AGCGGGACTG GCTGGTGGTC TATAGTTAGA GAGTTTTTTG ACCAAACAGC 780

M S Q H N (5)
ACAGCGGCCC TTTCAGTAAT AAATTAAGGA GACGAGTTCA ATG TCG CAA CAT AAC 840
SD

E K N P H Q H Q S P L H D S S (20)
GAA AAG AAC CCA CAT CAG CAC CAG TCA CCA CTA CAC GAT TCC AGC 880

E A K P G M D S L A P E D G S (35)
GAA GCG AAA CCG GGG ATG GAC TCA CTG GCA CCT GAG GAC GGC TCT 925

H R P A A E P T P P G A Q P T (50)
CAT CGT CCA GCG GCT GAA CCA ACA CCG CCA GGT GCA CAA CCT ACC 970

A P G S L K A P D T R N E K L (65)
GCC CCA GGG AGC CTG AAA GCC CCT GAT ACG CGT AAC GAA AAA CTT 1015

N S L E D V R K G S E N Y A L (80)
AAT TCT CTG GAA GAC GTA CGC AAA GGC AGT GAA AAT TAT GCG CTG 1060

T T N Q G V R I A D D Q N S L (95)
ACC ACT AAT CAG GGC GTG CGC ATC GCC GAC GAT CAA AAC TCA CTG 1105

R A G S R G P T L L E D F I L (110)
CGT GCC GGT AGC CGT GGT CCA ACG CTG CTG GAA GAT TTT ATT CTG 1150

R E K I T H F D H E R I P E R (125)
CGC GAG AAA ATC ACC CAC TTT GAC CAT GAG CGC ATT CCG GAA CGT 1195

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 I   V   H   A   R   G   S   A   A   H   G   Y   F   Q   P  (140)
ATT GTT CAT GCA CGC GGA TCA GCC GCT CAC GGT TAT TTC CAG CCA  1240

 Y   K   S   L   S   D   I   T   K   A   D   F   L   S   D  (155)
TAT AAA AGC TTA AGC GAT ATT ACC AAA GCG GAT TTC CTC TCA GAT  1285
      HindIII

 P   N   K   I   T   P   V   F   V   R   F   S   T   V   Q  (170)
CCG AAC AAA ATC ACC CCA GTA TTT GTA CGT TTC TCT ACC GTT CAG  1330

 G   G   A   G   S   A   D   T   V   R   D   I   R   G   F  (185)
GGT GGT GCT GGC TCT GCT GAT ACC GTG CGT GAT ATC CGT GGC TTT  1375

 A   T   K   F   Y   T   E   E   G   I   F   D   L   V   G  (200)
GCC ACC AAG TTC TAT ACC GAA GAG GGT ATT TTT GAC CTC GTT GGC  1420

 N   N   T   P   I   F   F   I   Q   D   A   H   K   F   P  (215)
AAT AAC ACG CCA ATC TTC TTT ATC CAG GAT GCG CAT AAA TTC CCC  1465
             -------AAC--F206N
                                             -------CAC--F214H

 D   F   V   H   A   V   K   P   E   P   H   W   A   I   P  (230)
GAT TTT GTT CAT GCG GTA AAA CCA GAA CCG CAC TGG GCA ATT CCA  1510

 Q   G   Q   S   A   H   D   T   F   W   D   Y   V   S      (245)
CAA GGG CAA AGT GCC CAC GAT ACT TTC TGG GAT TAT GTT TCT CTG  1555

 Q   P   E   T   L   H   N   V   M   W   A   M   S   D   R  (260)
CAA CCT GAA ACT CTG CAC AAC GTG ATG TGG GCG ATG TCG GAT CGC  1600

 G   I   P   R   S   Y   R   T   M   E   G   F   G   I   H  (275)
GGC ATC CCC CGC AGT TAC CGC ACC ATG GAA GGC TTC GGT ATT CAC  1645
                         -------GCA--E27A
                         -------TCA--E270S
                         -------ATT--E270I

                                         -------GCT--I274A
                                         -------GGT--I274G
                                         -------GTT--I274V
                                         -------TTT--I274F

 T   F   R   L   I   N   A   E   G   K   A   T   F   V   R  (290)
ACC TTC CGC CTG ATT AAT GCC GAA GGG AAG GCA ACG TTT GTA CGT  1690

 F   H   W   K   P   L   A   G   K   A   S   L   V   W   D  (305)
TTC CAC TGG AAA CCA CTG GCA GGT AAA GCC TCA CTC GTT TGG GAT  1735

 E   A   Q   K   L   T   G   R   D   P   D   F   H   R   R  (320)
GAA GCA CAA AAA CTC ACC GGA CGT GAC CCG GAC TTC CAC CGC CGC  1780

 E   L   W   E   A   I   E   A   G   D   F   P   E   Y   E  (335)
GAG TTG TGG GAA GCC ATT GAA GCA GGC GAT TTT CCG GAA TAC GAA  1825

 L   G   F   Q   L   I   P   E   E   D   E   F   K   F   D  (350)
CTG GGC TTC CAG TTG ATT CCT GAA GAA GAT GAA TTC AAG TTC GAC  1870
                                         EcoRI
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 F   D   L   L   D   P   T   K   L   I   P   E   E   L   V  (365)
TTC GAT CTT CTC GAT CCA ACC AAA CTT ATC CCG GAA GAA CTG GTG  1915

 P   V   Q   R   V   G   K   M   V   L   N   R   N   P   D  (380)
CCC GTT CAG CGT GTC GGC AAA ATG GTG CTC AAT CGC AAC CCG GAT  1960

 N   F   F   A   E   N   E   Q   A   A   F   H   P   G   H  (395)
AAC TTC TTT GCT GAA AAC GAA CAG GCG GCT TTC CAT CCT GGG CAT  2005

 I   V   P   G   L   D   F   T   N   D   P   L   L   Q   G  (410)
ATC GTG CCG GGA CTG GAC TTC ACC AAC GAT CCG CTG TTG CAG GGA  2050

 R   L   F   S   Y   T   D   T   Q   I   S   R   L   G   G  (425)
CGT TTG TTC TCC TAT ACC GAT ACA CAA ATC AGT CGT CTT GGT GGG  2095

 P   N   F   H   E   I   P   I   N   R   P   T   C   P   Y  (440)
CCG AAT TTC CAT GAG ATT CCG ATT AAC CGT CCG ACC TGC CCT TAC  2140

 H   N   F   Q   R   D   G   M   H   R   M   G   I   D   T  (455)
CAT AAT TTC CAG CGT GAC GGC ATG CAT CGC ATG GGG ATC GAC ACT  2185
                        SphI
 N   P   A   N   Y   E   P   N   S   I   N   D   N   W   P  (470)
AAC CCG GCG AAT TAC GAA CCG AAC TCG ATT AAC GAT AAC TGG CCG  2230

 R   E   T   P   P   G   P   K   R   G   G   F   E   S   Y  (485)
CGC GAA ACA CCG CCG GGG CCG AAA CGC GGC GGT TTT GAA TCA TAC  2275

 Q   E   R   V   E   G   N   K   V   R   E   R   S   P   S  (500)
CAG GAG CGC GTG GAA GGC AAT AAA GTT CGC GAG CGC AGC CCA TCG  2320

 F   G   E   Y   Y   S   H   P   R   L   F   W   L   S   Q  (515)
TTT GGC GAA TAT TAT TCC CAT CCG CGT CTG TTC TGG CTA AGT CAG  2365

 T   P   F   E   Q   R   H   I   V   D   G   F   S   F   E  (530)
ACG CCA TTT GAG CAG CGC CAT ATT GTC GAT GGT TTC AGT TTT GAG 2410

 L   S   K   V   V   R   P   Y   I   R   E   R   V   V   D  (545)
TTA AGC AAA GTC GTT CGT CCG TAT ATT CGT GAG CGC GTT GTT GAC  2455

 Q   L   A   H   I   D   L   T   L   A   Q   A   V   A   K  (560)
CAG CTG GCG CAT ATT GAT CTC ACT CTG GCC CAG GCG GTG GCG AAA  2500

 N   L   G   I   E   L   T   D   D   Q   L   N   I   T   P  (575)
AAT CTC GGT ATC GAA CTG ACT GAC GAC CAG CTG AAT ATC ACC CCA  2545

 P   P   D   V   N   G   L   K   K   D   P   S   L   S   L  (590)
CCT CCG GAC GTC AAC GGT CTG AAA AAG GAT CCA TCC TTA AGT TTG  2590

 Y   A   I   P   D   G   D   V   K   G   R   V   V   A   I  (605)
TAC GCC ATT CCT GAC GGT GAT GTG AAA GGT CGC GTG GTA GCG ATT  2635

 L   L   N   D   E   V   R   S   A   D   L   L   A   I   L  (620)
TTA CTT AAT GAT GAA GTG AGA TCG GCA GAC CTT CTG GCC ATT CTC  2680

 K   A   L   K   A   K   G   V   H   A   K   L   L   Y   S  (635)
AAG GCG CTG AAG GCC AAA GGC GTT CAT GCC AAA CTG CTC TAC TCC  2725
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 R   M   G   E   V   T   A   D   D   G   T   V   L   P   I (650)
CGA ATG GGT GAA GTG ACT GCG GAT GAC GGA ACG GTG TTG CCT ATA  2770

 A   A   T   F   A   G   A   P   S   L   T   V   D   A   V (665)
GCC GCT ACC TTT GCC GGT GCA CCT TCG CTG ACG GTC GAT GCG GTC  2815

 I   V   P   C   G   N   I   A   D   I   A   D   N   G   D (680)
ATT GTC CCT TGC GGC AAT ATC GCG GAT ATC GCT GAC AAC GGC GAT  2860

 A   N   Y   Y   L   M   E   A   Y   K   H   L   K   P   I (695)
GCC AAC TAC TAC CTG ATG GAA GCC TAC AAA CAC CTT AAA CCG ATT  2905

 A   L   A   G   D   A   R   K   F   K   A   T   I   K   I (710)
GCG CTG GCG GGT GAC GCG CGC AAG TTT AAA GCA ACA ATC AAG ATC  2950

 A   D   Q   G   E   E   G   I   V   E   A   D   S   A   D (725)
GCT GAC CAG GGT GAA GAA GGG ATT GTG GAA GCT GAC AGC GCT GAC  2995

 G   S   F   M   D   E   L   L   T   L   M   A   A   H   R (740)
GGT AGT TTT ATG GAT GAA CTG CTA ACG CTG ATG GCA GCA CAC CGC  3040

 V   W   S   R   I   P   K   I   D   K   I   P   A   *     (753)
GTG TGG TCA CGC ATT CCT AAG ATT GAC AAA ATT CCT GCC TGA      3082
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TGGGAGCGCG CAATTGCGCC GCCTCAATGA TTTACATAGT GCGCTTTGTT TATGCCGGAT 3142
GCGCGTGAAC GCCTTATCCG GCCTACAAAA CTGTGCAAAT TCAATATATT GCAGGAAACA 3202
CGTAGGCCTG ATAAGCGAAG CCATCAGGCA GTTTTGCGTT TGTCAGCAGT CTCAAGCGGC 3262
GGCAGTTACG CCGCCTTTGT AGGAATTAAT CGCCGGATGC AAGGTTCACG CCGATCTGGC 3322
AAACATCCTC ACTTACACAT CCCGATAACT CCCCAACCGA TAACCACGCT GAGCGATAGC 3382
ACCTTTCAAC GACGCTGATG TCAACACATC CAGCTCCGTT AAGCGTGGGA AACAGTAAGC 3442
ACTCTGACGG ATAGTATTAT CGAT                                       3466
                    ClaI
```

Table 2.2. Sequences of the oligonucleotides used for site-directed mutagenesis.

Primer Name	Position w/in *katE*	Primer Sequence	Sub-clone
I274A (ATT-GCT)	1631-1651	GGCTTCGGT**GCT**CACACCTTC	II
I274G (ATT-GGT)	1631-1651	GGCTTCGGT**GGT**CACACCTTC	II
I274V (ATT-GTT)	1631-1651	GGCTTCGGT**GTT**CACACCTTC	II
I274F (ATT-TTT)	1631-1651	GGCTTCGGT**TTT**CACACCTTC	II
E270A (GAA-GCA)	1610-1630	CGCACCATG**GCA**GGCTTCGGT	II
E270S (GAA-TCA)	1610-1630	CGCACCATG**TCA**GGCTTCGGT	II
E270I (GAA-ATT)	1610-1630	CGCACCATG**ATT**GGCTTCGGT	II
F214H (TTC-CAC)	1453-1473	GCGCATAAC**CAC**CCCGATTTG	II
F214L (TTC-TTA)	1453-1473	GCGCATAAC**TTA**CCCGATTTG	II
F206N (TTC-AAC)	1427-1447	ACGCCAATC**AAC**TTTATCCAG	II

Table 2.3. Sequences of the oligonucleotides used for sequences confirmation.

Primer Name	Position w/in *katE*	Oligonucleotide Sequence
C	1107-1123	5'-GTGCCGGTAGCCGTGGT
D	1356-1372	5'-TGCGTGATATCCGTGGC
D2	1430-1447	5'-CCAATCTCTTTATCCAG
E	1606-1620	5'-CCCCCGCAGTTACCG

2.3. j. DNA Sequencing

Sequencing of DNA was carried out manually according to Sanger *et al.* (1977), using the primers shown in Table 2.3. Reactions were prepared using Pharmacia T7 Sequencing kit and 5-15 μCi [α-^{35}S] 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 a film (Kodak X-OMAT AR) to visualize the DNA bands.

2.4. Purification of HPII Catalase

For small scale whole cell assays of HPII and variant activities, cells containing desired plasmids were grown in 30 ml LB medium in 125 ml flasks at 37°C and 28°C. Cells were harvested in stationary phase (A_{600} >4.0) and used for enzyme assays and visualization of proteins by electrophoresis on sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE).

For large-scale preparations, UM255 cells harboring plasmids containing wild type or mutated *katE* were grown in LB medium supplemented with 100 μg/ml ampicillin for 18-20 hours at 37°C or 22-24 hours at 28°C with shaking in 8 to15 2 L flasks (500 ml per flask). Cells were then harvested by pelleting and stored at -60°C. Isolation of HPII was done according to Loewen & Switala (1986) with some modifications as follows. Pelleted cells were thawed in 37°C water bath, resuspended in 20-300 ml of 50 mM potassium phosphate buffer, pH 7.0 containing 5 mM EDTA, and 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 resuspended in 15-20ml of 50 mM potassium phosphate buffer, pH 7.0 (buffer A) and were tested separately for catalase activity. Two fractions were combined only if specific activities of both were within 80% of each other. Otherwise only the fraction with the highest activity was used for further steps. A small volume of the selected fraction(s) was then tested for heat resistance at 53°C for 20 minutes. The activity was compared to that of the unheated fraction and if less than 20% of the activity was lost, the whole fraction was heat-treated and centrifuged, and the supernatant was dialyzed against 2 L of buffer A overnight.

2.4. a. Ion-Exchange column chromatography

The dialyzed sample was loaded onto a 2.5 x 23 cm column packed with DEAE-cellulose A-500 (Amicon Matrix Cellophane, CHISSO Corp. Japan) equilibrated in buffer A. The column was washed with buffer A or 50 mM NaCl in buffer A until the absorbance A_{280} of column fractions was below 0.050. HPII and its variants were then eluted by a 50 to 500 mM NaCl linear gradient in buffer A. Fractions of 5ml were collected and assayed for catalase activity. Those with highest activity were pooled, concentrated using a protein concentrator (Amicon), and dialyzed against 1 liter of buffer A overnight. The purity of catalase was estimated spectrophotometrically from A_{407}/A_{280} ratio (heme/protein) and by SDS-PAGE. If the ratio was below 0.7, the protein was loaded on a second smaller column packed with DEAE-cellulose A-500 or

hydroxyapatite HP (BIO RAD). Whenever hydroxyapatite was used, the proteins were eluted by a gradient of 50 to 400 mM of phosphate buffer pH 7.0. Aliquots of the enzyme were stored at -70°C.

2.4. b. Flow pressure liquid chromatography (FPLC)

For a final purification step, HPII and its variants were dialyzed overnight in 50 mM Tris buffer pH 7.0, concentrated to ~20mg/ml, and loaded on a 24 ml (1x30cm) Superose 12 column attached to FPLC Biologic workstation. Desired fractions were collected and stored at -70°C.

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 mg/ml SDS, 1.28 M 2-mercaptoethanol, 0.36 g/ml urea and 0.15% bromophenol blue) and boiled for 3 minutes prior to loading. Samples were run at 150 V in a vertical BIO-RAD Protean II electrophoresis system. Samples were stained in a solution containing 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 RØrth & 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 minute in a 60 mM H_2O_2 at 37°C, pH 7.0. Appropriately diluted samples were incubated for 0.5-1.0 minutes at 37°C in 1.8 ml buffer A 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 buffer A 50 mM potassium phosphate, pH 7.0. Specific activity was expressed as units per mg purified protein or units per mg dry cell weight. Protein concentration (mg/ml) was estimated spectrophotometrically (Layne *et al*, 1957). Specific activity in whole cells (unit/mg dry cell weight) was determined by converting the A_{600} values to dry cell weight. Specific activity was always determined as the average of 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 analyzed using SigmaPlot software (v. 5).

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 SigmaPlot software (v. 5) for preparation of elution profiles.

2.9. Specific Activity and Kinetic Properties of Catalase HPII and Variants.

The dependence of the enzyme reaction rates on substrate concentration was examined, with saturation curves generated by fitting the data with the hyperbola, two parameter equation found in the SigmaPlot software (v. 5) (Jandel, San Rafael, CA.). The apparent V_{max} (the maximal initial reaction velocity) and apparent K_m (the substrate concentration at which the initial reaction velocity is equivalent to half the maximum initial reaction velocity) values were obtained from these curves. The turnover rate, k_{cat}, was also calculated.

2.10. Inhibition Studies

The effect of various inhibitors on HPII or variants was studied by following enzyme activity and spectral changes. Various concentrations of sodium cyanide, sodium azide, hydroxylamine and O-methyl hydroxylamine were incubated with HPII at 37°C for 1 minute in buffer A prior to the addition of H_2O_2 to the reaction cell. To study the effect of cyanide and azide on the visible spectra of HPII or variants, appropriate concentrations of each individual protein were incubated with 5mM of cyanide or azide at 37°C for 15 minutes. Visible spectra were obtained as described above.

3. RESULTS

3.1. Construction of Catalase HPII Variants

HPII variants were constructed by site directed mutagenesis using mutagenic primers to introduce point mutations into *katE* subclone II. All mutant genes were sequenced to confirm the presence of the desired mutation and to confirm the absence of any other mutations. Ten variants were constructed including Ile274 conversion to Ala, Gly, Val and Phe, Glu270 conversion to Ala, Ile and Ser, Phe214 conversion to His, and Phe206 conversion to Asn. During the course of preparation of Ile274Ala, an accidental mutation Phe214 to Leu was detected. The two mutations were separated and recloned independently allowing the variant protein Phe214Leu to be included in the study. Approximately 50% of the products from a mutagenesis contained the desired mutation.

3.2. Effect of Temperature on the Expression Catalase HPII and its Variants

To determine the optimum temperature for expression of the constructed variants, HPII and its variants were grown at three temperatures 37°C, 28°C and room temperature (RT). Two measures were considered: the catalase activity of the crude extracts (Table 3.1) and the accumulation of HPII like protein on SDS-PAGE. All variants showed decreased catalase activities compared to the WT protein when grown at the three temperatures. Six variants presented less than 20% of WT activity when grown at 37°C, and four variants presented 30% to 70% of WT activity. At 28°C, two of the variants presented activity similar to WT. At RT, no significant increase in catalase activity was detected in WT HPII or any of its variants. Ratios between activities at 28°C/37°C were calculated for each variant and wherever the ratio exceeded 1.0, the variant protein was

prepared at 28°C (Table 3.1) including, six out of eight variants: I274A, I274G, E270A, E270S, F214H and F206N. Two variants were prepared at 37°C: I274V and F214L. The two remaining variants I274F and E270I didn't present any activity or detectable HPII-like bands on SDS-PAGE at either temperatures and are not considered in any further experiments.

3.3. Purification and Characterization of HPII and its Variants

The purification protocol for HPII and its variants is described in the Methods section and, in brief, involves cell lysis, cell debris removal, streptomycin sulfate precipitation and ammonium sulfate precipitation and column chromatography. The specific activities of the purified variants were determined (Table 3.2) and the proteins were tested for heat resistance at 52°C for 18 minutes; and for heme content, heme b or heme d. Figure 3.1 shows the SDS-PAGE for the purified WT HPII and its variants.

3.3. a. Isoleucine 274 Variants

Isoleucine 274 was converted to Ala, Gly, Val and Phe. The four constructed variants I274A, I274G, I274V, and I274F all showed decreases in the catalytic activity and a decrease in expression by SDS-PAGE. The I274V, in spite of the decrease in the catalytic activity of cell extracts, the variant showed similar properties to WT protein concerning expression, specific activity and sensitivity towards different inhibitors. However, heme characterization by HPLC as well as spectral data revealed a combination of heme b and heme d (Fig 3.2, Fig 3.3). The electrophoretically pure Gly variant showed ~20% of the WT activity and A_{407}/A_{280} ratio of 0.6 and the yield of heme

extraction from this variant was always less than wild type using equal amounts of protein from both. The pure HPII and its variants usually show a ratio close to 1.0. Heme characterization by HPLC as well as spectral analysis showed a combination of heme b and heme d (Fig 3.3). The I274G variant was heat sensitive but generally more resistant to different inhibitors especially to hydroxylamine (Fig 3.8), for which the concentration needed to achieve 50% inhibition, was 10 fold more than that needed for 50% inhibition of the WT protein (Table 3.4).

The pure I274A variant exhibited 40% of W.T specific activity and A_{407}/A_{280} ratio of 0.74. Spectral and HPLC analyses revealed a protein very similar to wild type HPII although the heme spectrum did not resemble that of either heme b or d (Fig 3.2, Fig 3.3).

3.3. b. Glutamate 270 Variants

Three mutations were introduced at position E270, Ala, Ser, and Ile. For E270A and E270S the maximum yield and activity were achieved by growing cells at 28°C for 24 hours. Purified E270A had a specific activity slightly higher than WT, while E270S had a specific activity similar to WT. The heme profiles and spectral analysis of both variants were similar to WT HPII with typical peaks for heme d (Fig 3.2, Fig 3.3). E270I did not accumulate HPII-like protein suggesting a folding problem.

3.3. c. Phenylalanine 206 and Phenylalanine 214 Variants

Phe206 was converted to aspargine while Phe214 was converted to leucine and histidine. All three variants showed reduced activities in crude extracts compared to WT

regardless of growth temperature. A comparison of activities at 28°C and 37°C (Table 1) resulted in F206N and F214L being grown at 28°C and F214L being grown at 37°C.

The three electrophoretically pure variants presented activities that were 10 to 15% of WT activity, and A_{407}/A_{280} ratios below 1.0. The absorbance spectra of the three proteins alone suggested a mixture of heme b and heme d (Fig 3.2, Fig 3.7) but heme characterization by HPLC revealed the presence of unknown heme species in each variant (Fig 3.3). Very little heme was extractable from F214H, suggestive of a covalent attachment (Fig 3.3). HPLC analysis of the heme from F214L revealed a predominant peak of heme d but with nearly equal amounts of the *cis* and *trans* isomers and a small heme b peak.

3.4. Properties of HPII and its Variants

The kinetic properties of HPII and catalases in general do not follow the conventional Michaelis-Menten pattern. The enzyme/substrate saturation curve needed to calculate the kinetics constants of Michaelis-Menten's equation cannot be obtained for catalases because the active site can never be saturated and activities may continue to increase up to 5M H_2O_2. Such high concentrations cause inactivation. The apparent V_{max} and K_m values were calculated by fitting the data obtained at low [H_2O_2] and are included in Table 3.3.

Table 3.1. Effects of growth temperature on the production of WT HPII and its variants

Variant	Act*. at 28°C	Act*. at 37°C	Act*. at R.T	Ratio 28°/37°
W.T HPII	410 ± 40	550 ± 60	390 ± 30	0.75
I274A	230±25	100±6	160±5	2.3
I274G	180±3	60±4	125±7	3.0
I274V	240±90	450±40	320±30	0.53
I274F	< 0.01	< 0.01	< 0.01	n/a
E270A	380 ± 30	190± 6	270 ± 15	2.0
E270I	< 0.01	< 0.01	< 0.01	n/a
E270S	390 ± 25	170 ± 15	320 ± 30	2.3
F214L	140±22	130±10	90±15	1.1
F214H	210±16	80±7	n/d	2.6
F206N	60±8	30±3	n/d	2.0
UM255	< 0.01	< 0.01	< 0.01	n/a

* catalase activity in units/mg dry cell wt.
n/d: not determined
n/a: not applicable

Table 3.2. Comparison of the physical properties of purified WT HPII and its variants

Variant	Specific activity units/mg	Ratio Abs A_{407}/A_{280}	Heme group	Heat resistance
W.T HPII	19000±900	0.98	d	R
I274A	7700±490	0.74	d+b	R
I274G	4300±710	0.52	d+b	S
I274V	18000 ±1400	0.97	d+b	R
E270A	24000±1200	0.99	d	R
E270S	20500±400	0.96	d	R
F214H	3000±150	0.71	b	R
F214L	3600±220	0.6	b+d	R
F206N	2700±400	0.51	b	R

R: Heat resistant
S: Heat sensitive
d: Variant contains heme d
d+b: Variant contains heme d and heme b
b+d: Variant contains heme d and heme b.
b: Variant contains heme b.

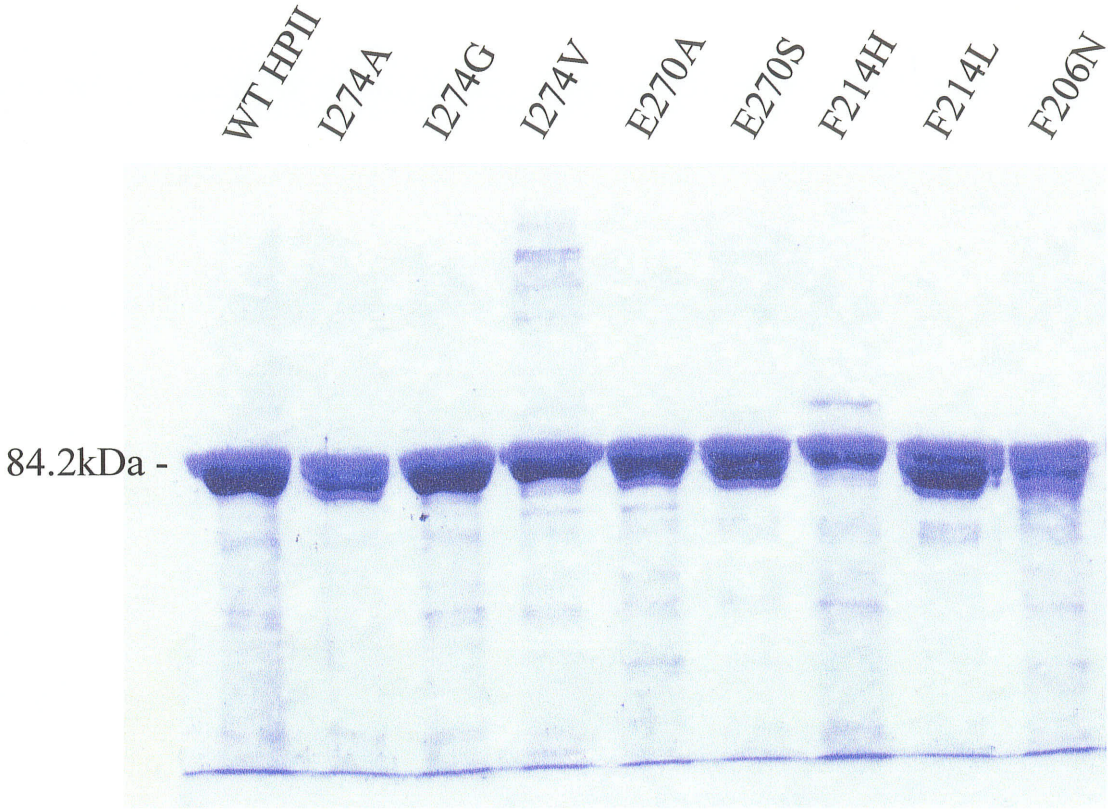
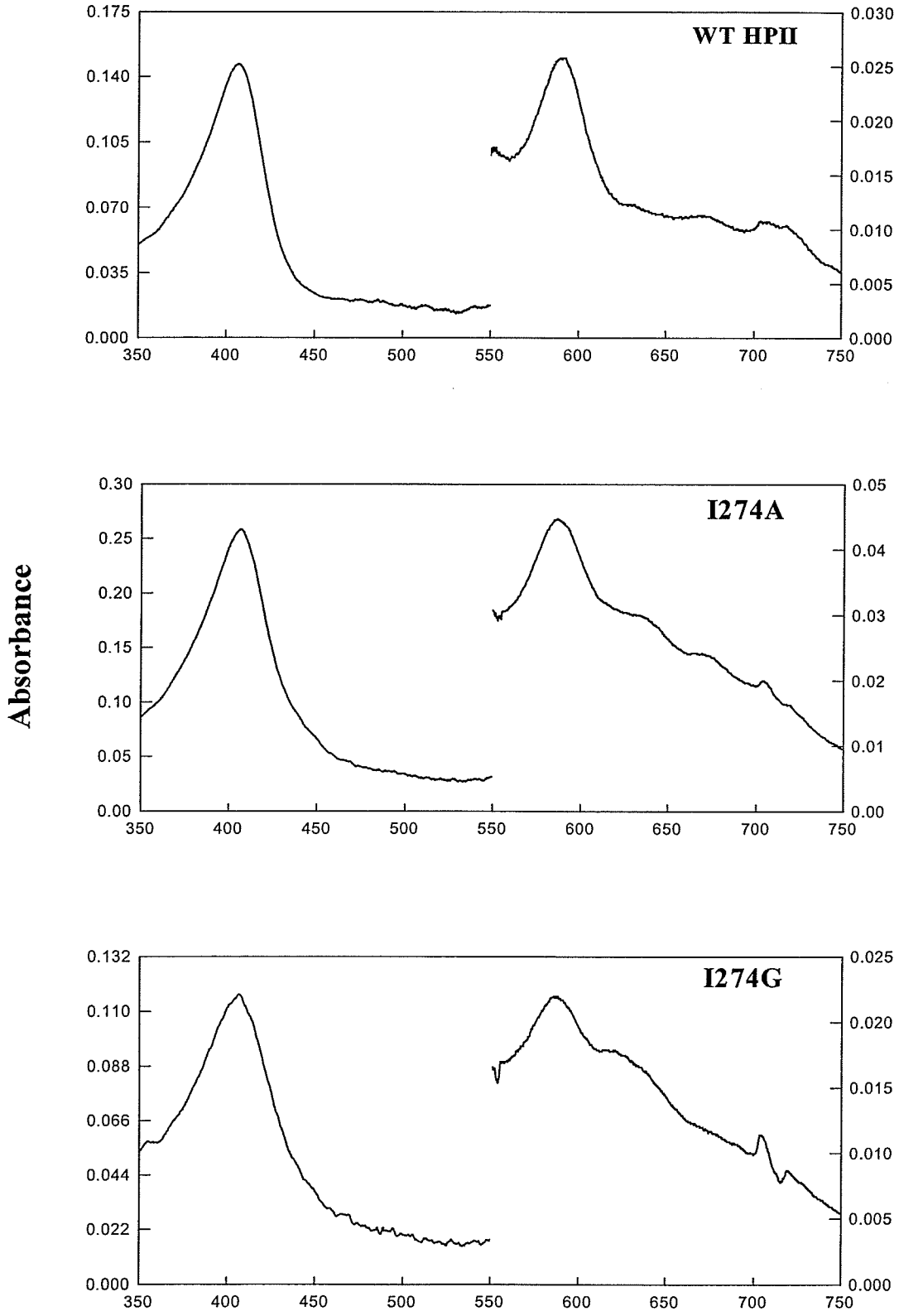
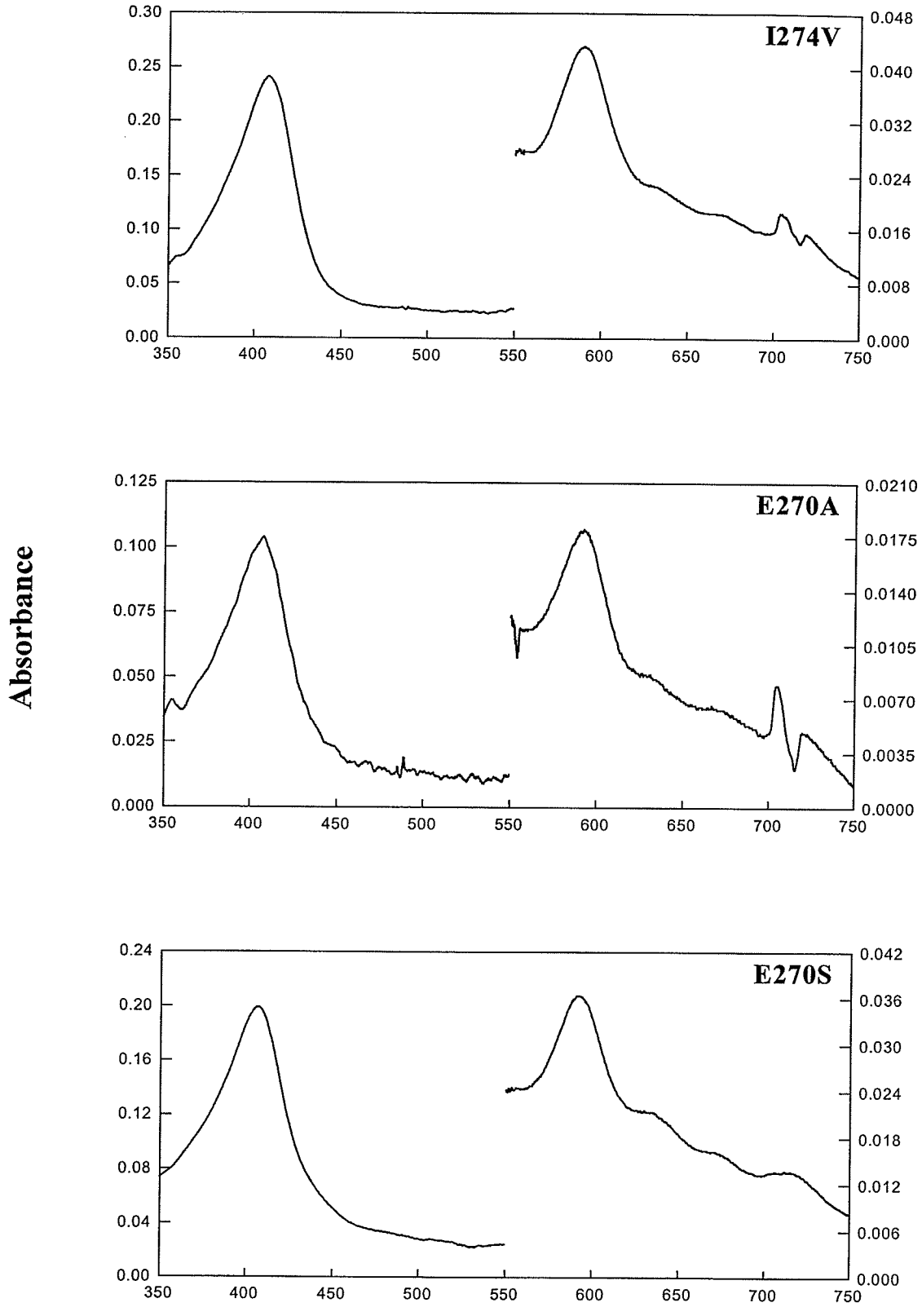


Fig 3.1. SDS-PAGE of purified WT HPII and its variants. Samples of approximately 5µg were loaded on 8% gel and stained with Coomassie Brilliant Blue

Fig 3.2. The visible spectra of purified WT HPII and its variants. Appropriate dilutions of each purified protein in 50 mM potassium phosphate buffer, pH 7.0 were scanned using a Milton Roy MR3000 spectrophotometer with 1 cm pathlength quartz cell at room temperature. 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


WT HPII
I274A
I274G
Absorbance
Wavelength
0.175
0.140
0.105
0.070
0.035
0.000
0.030
0.025
0.020
0.015
0.010
0.005
0.000
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0.05
0.04
0.03
0.02
0.01
0.00
0.132
0.110
0.088
0.066
0.044
0.022
0.000
0.025
0.020
0.015
0.010
0.005
0.000
350
400
450
500
550
600
650
700
750


I274V
E270A
E270S
Absorbance
Wavelength

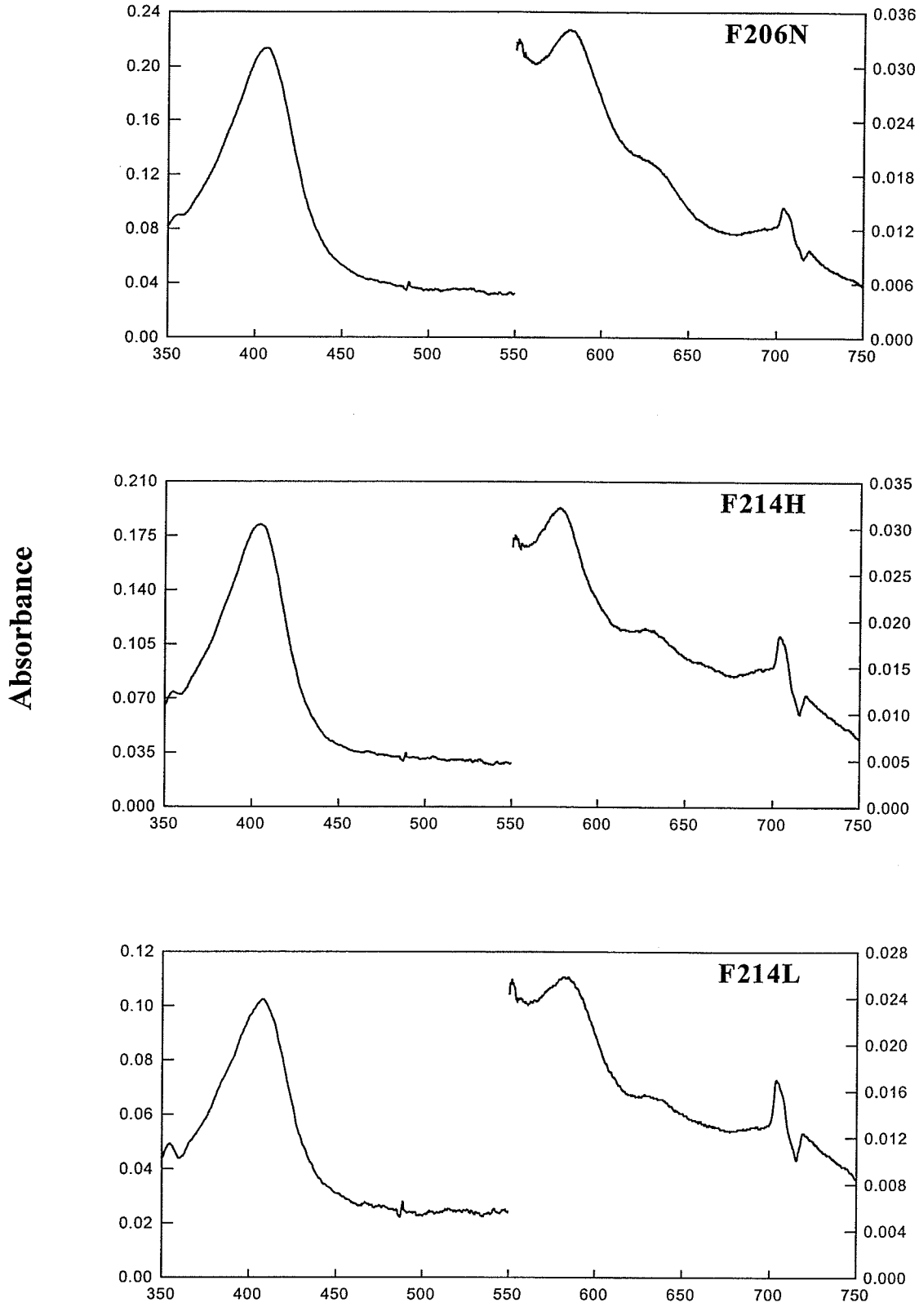
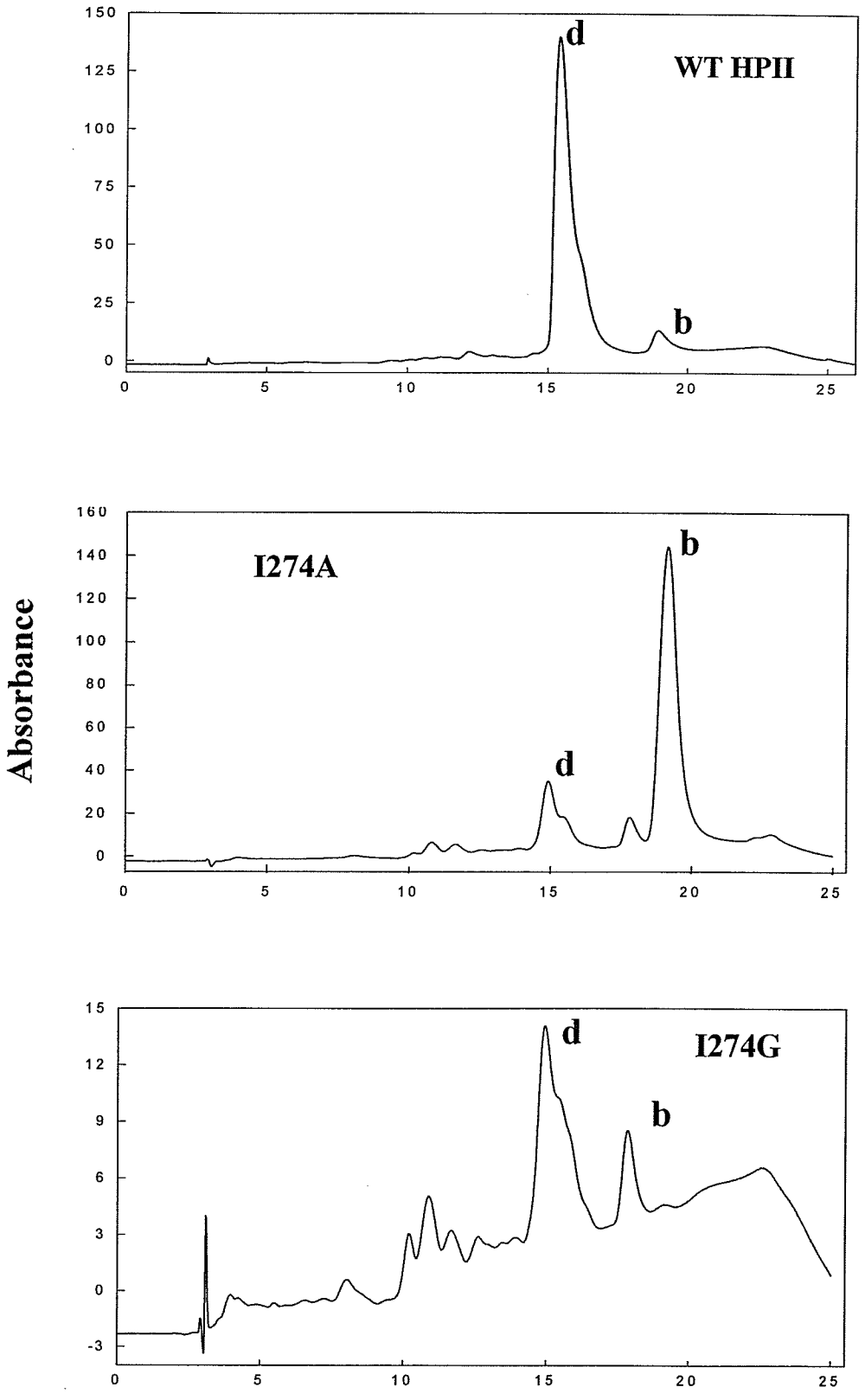
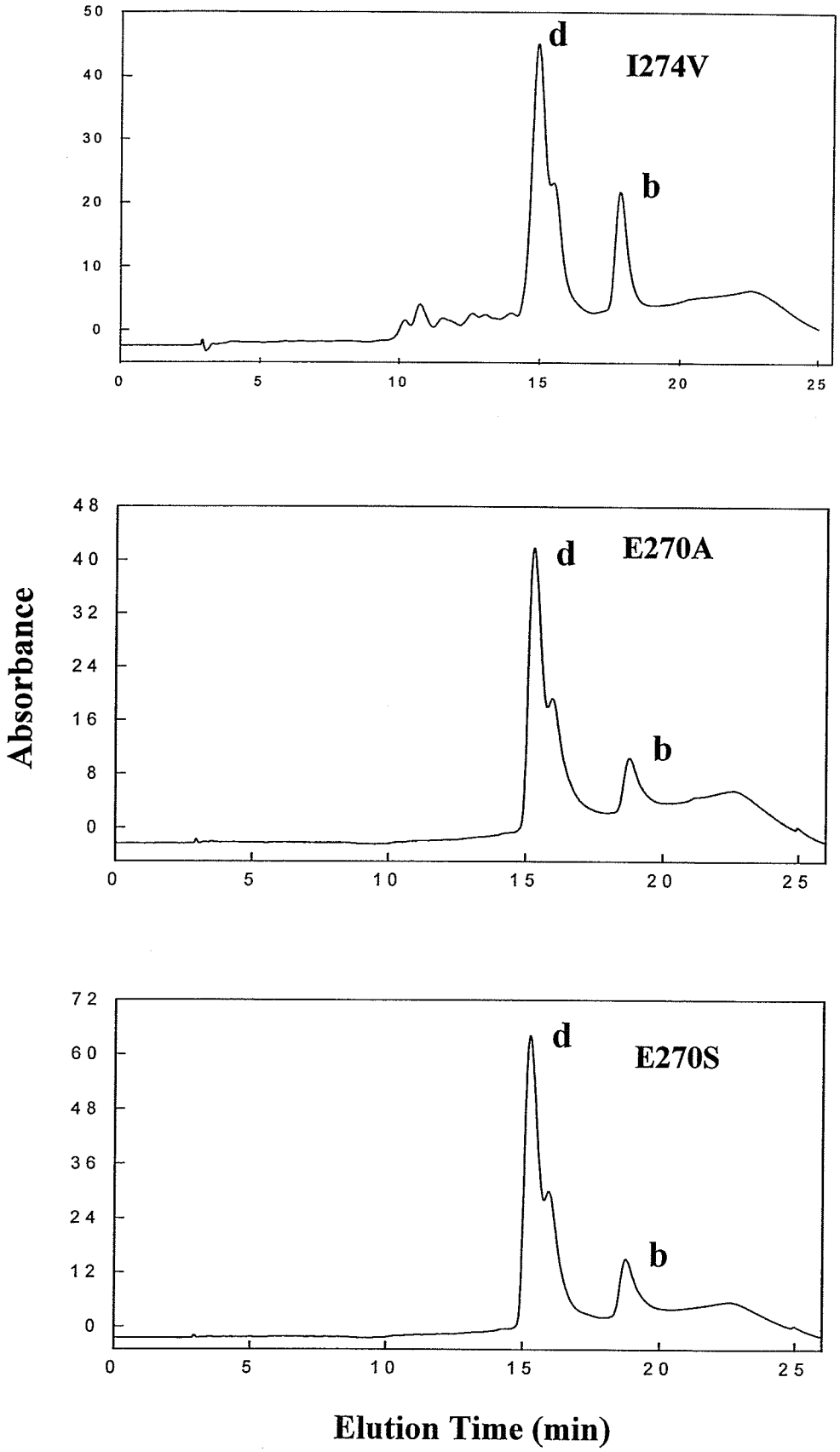

F206N
F214H
F214L
Absorbance
Wavelength

Fig 3.3. Elution profiles from C18 reverse phase HPLC of heme extracted from 1 mg protein of purified WT HPII and its variants. The heme was extracted according to Loewen *et al,* 1993. Samples were 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.


WT HPII
d
b
I274A
d
b
I274G
d
b
Absorbance
Elution Time (min)


I274V
d
b
E270A
d
b
E270S
d
b
Absorbance
Elution Time (min)

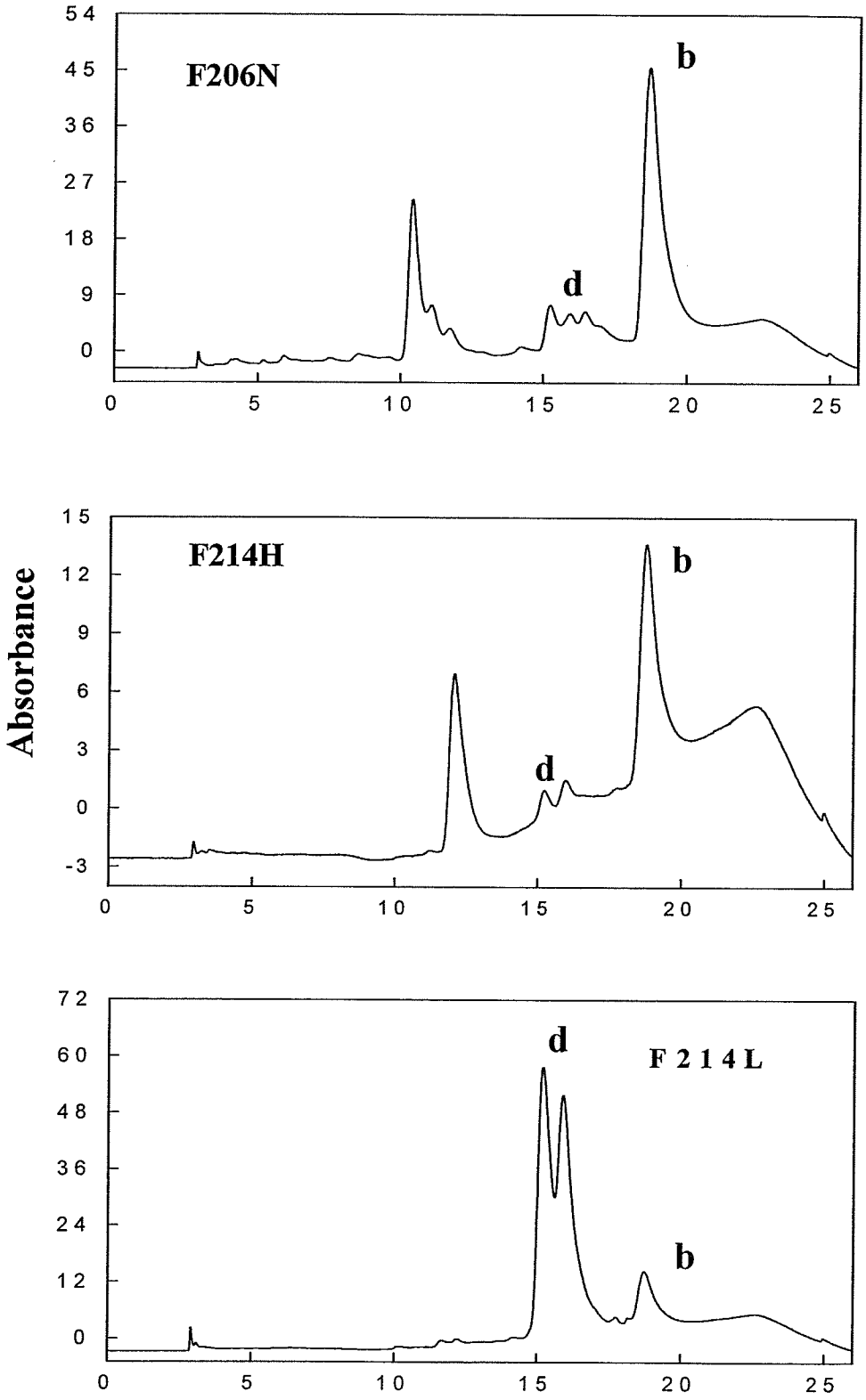
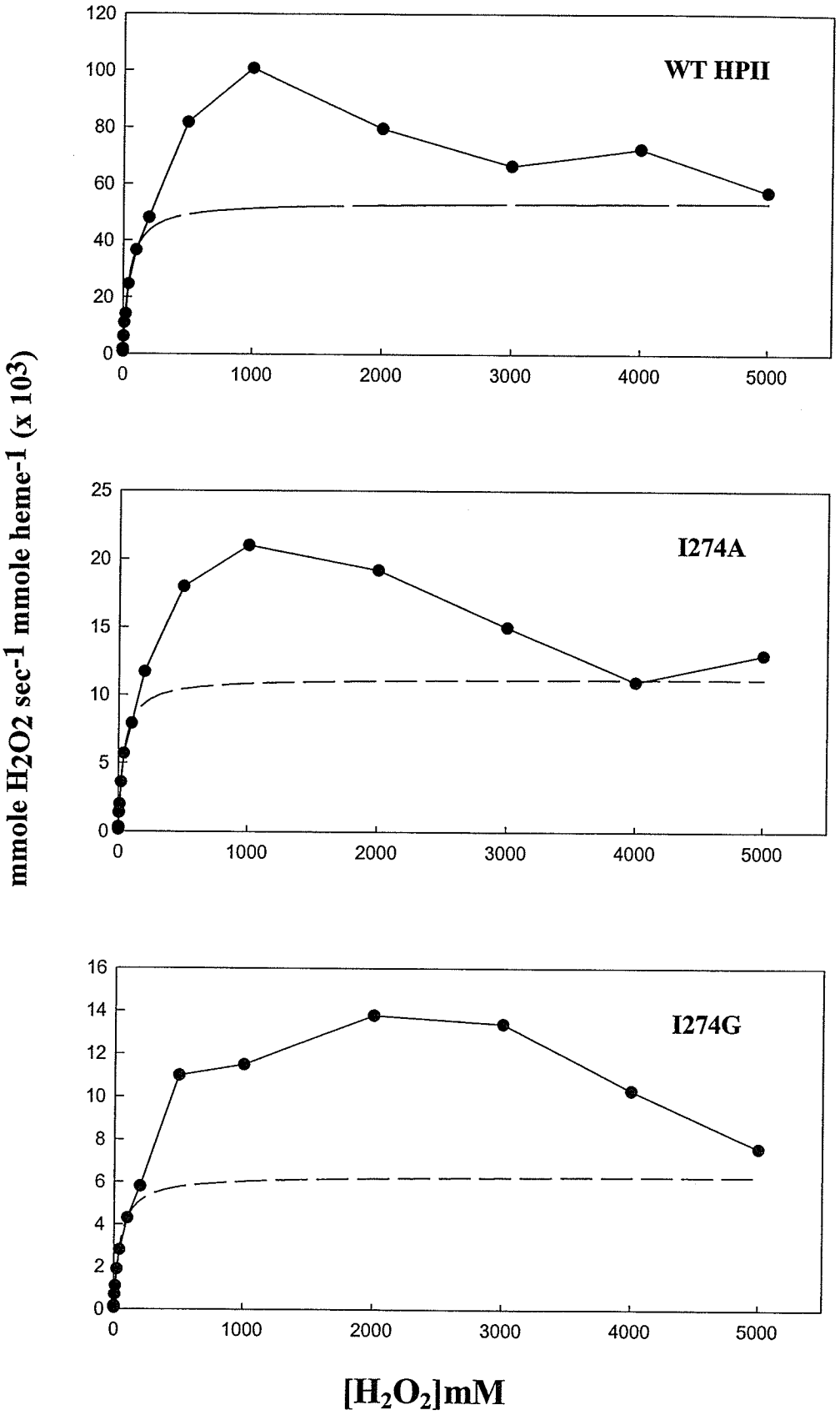

F206N
b
d
F214H
b
d
F214L
d
b
Absorbance
Elution Time (min)

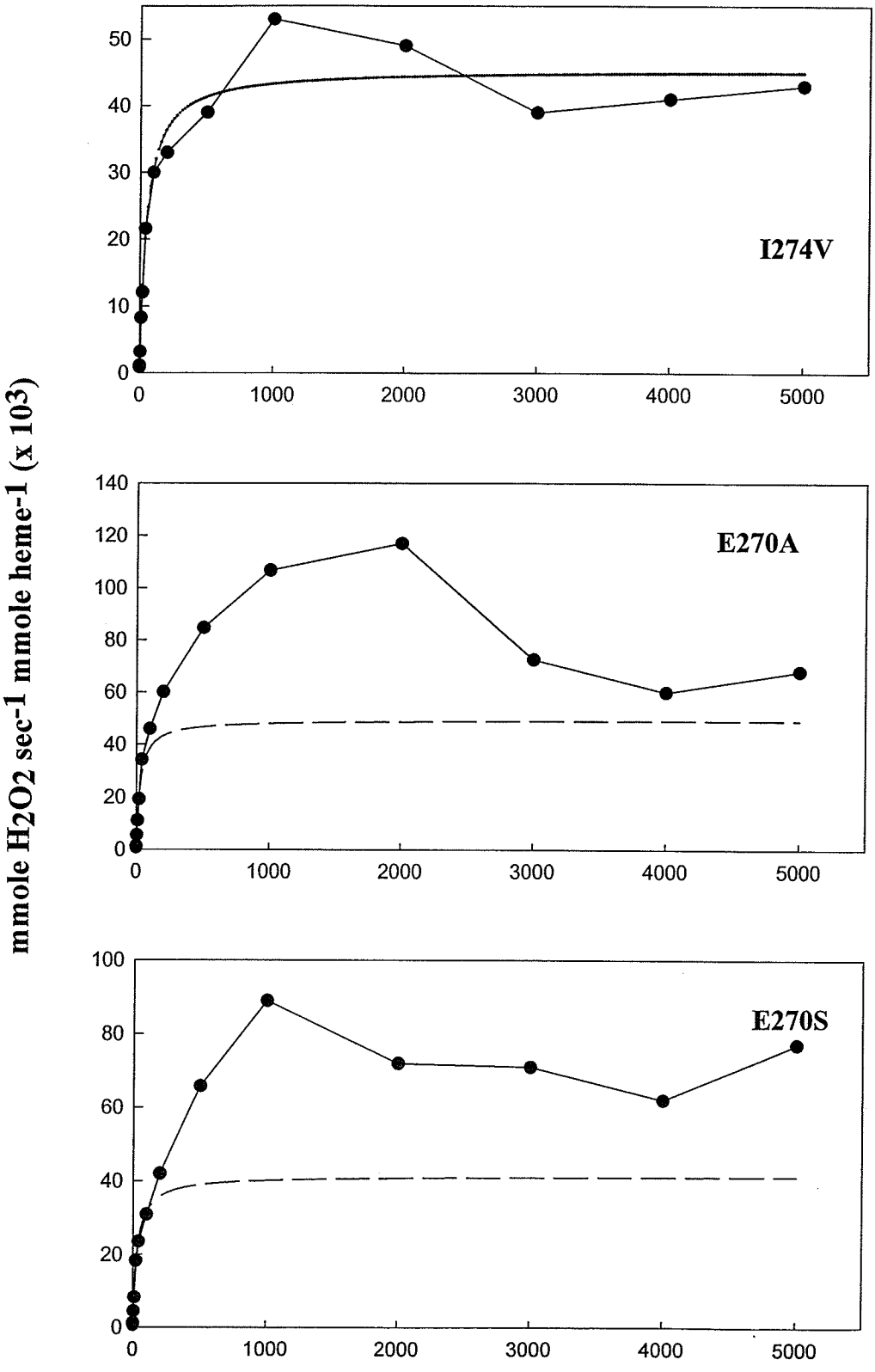
Table 3.3. Specific activities and kinetic parameters of purified WT HPII and its variants showing calculated and observed values

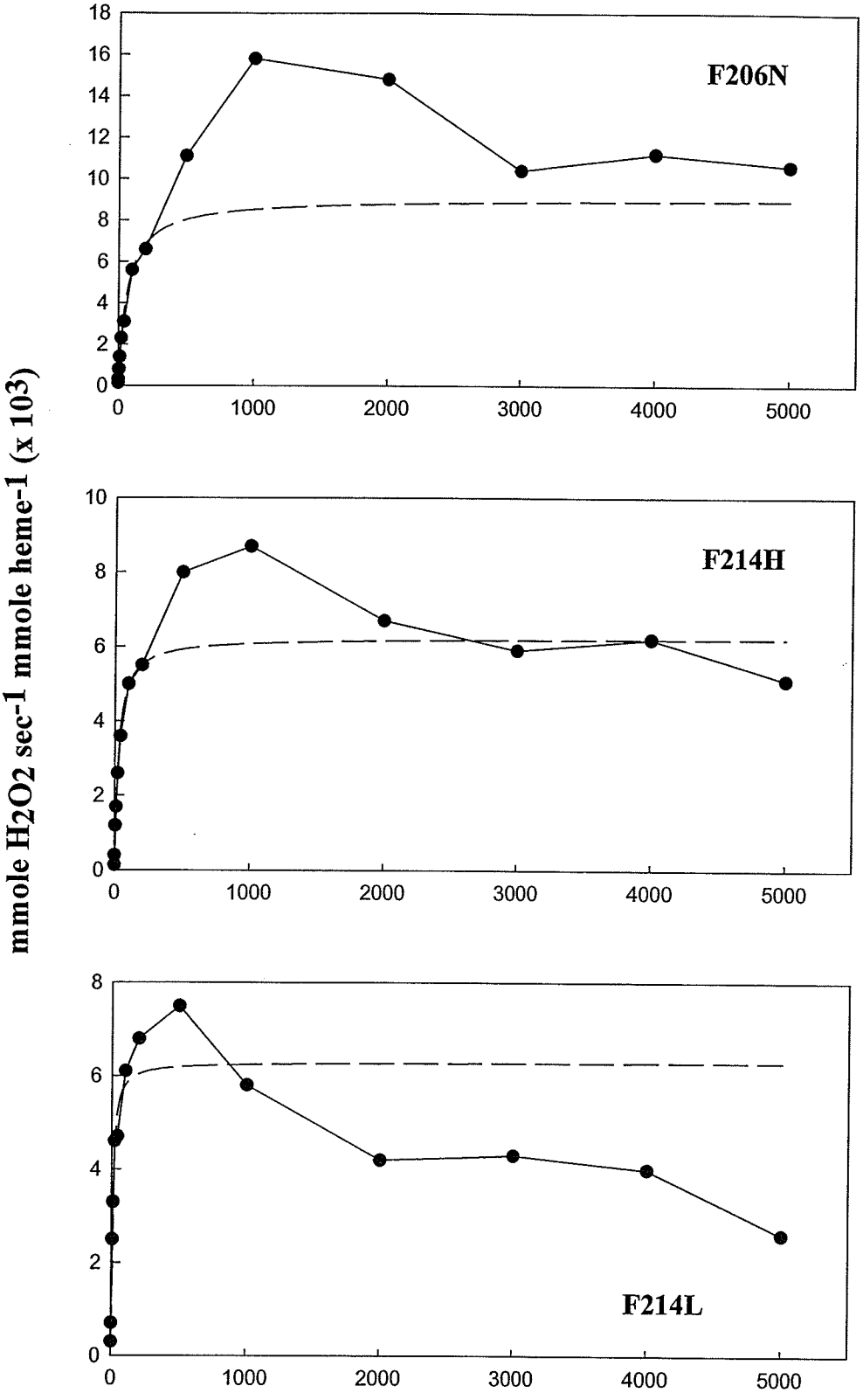
Variant	Calculated[a]			Observed	
	V_{max}[b]	K_m (mM)	k_{cat} (sec^{-1})	V_{max}[b]	$[H_2O_2]$ at $V_{max}/2$ mM
Wild Type	54,000	47	1.21×10^6	100,800	221
I274A	11,200	41	1.56×10^5	20,900	500
I274G	6,300	47	4.5×10^4	13,700	1000
I274V	45,000	48	8.7×10^5	53,000	500
E270A	49,200	28	1.4×10^6	116,900	250
E270S	41,100	31	8.7×10^5	89,100	1000
F214L	6,300	8.5	1.1×10^5	7,532	250
F214H	6,200	26.5	1.7×10^5	8,700	500
F206N	9000	63.2	3.2×10^4	15,800	250

a: Calculated for $H_2O_2 < 100$mM
b: V_{max} in μmol H_2O_2 μmol $heme^{-1}s^{-1}$

Fig 3.4. Comparison of the effect of different hydrogen peroxide concentrations on the initial velocities (V_i) of WT HPII and its variants, showing the observed (———) and calculated (-----)V_{max} for each purified protein.


WT HPII
I274A
I274G
mmole H2O2 sec-1 mmole heme-1 (x 103)
[H2O2]mM


mmole H2O2 sec-1 mmole heme-1 (x 103)
I274V
E270A
E270S
[H2O2]mM


F206N
F214H
F214L
mmole H2O2 sec-1 mmole heme-1 (x 103)
[H2O2]mM

3.5. Effect of Inhibitors on HPII and its Variants

3.5. a. Effect of Cyanide and Azide on variants of HPII.

Azide and cyanide have been reported to reversibly inhibit catalase by binding to the heme iron (Beyer and Fridovich, 1988). Both inhibitors alter HPII's visible absorbance spectrum, including a red shift in both the Soret band and A_{590} peak and an increase in the A_{630} peak. In contrast, addition of cyanide to variants of HPII with a heme b prosthetic group results in a red shift of the Soret band, as well as the appearance of an absorbance peak at 545 nm with a shoulder at 590 nm, and a significant increase in the 630 nm peak. Azide compounds do not induce changes in the spectra of variants containing heme b. A comparison of the inhibitory effects of azide and cyanide on HPII and its variants is presented in Table 3.4. The effect of both inhibitors on the visible spectra of HPII and its variants is shown in Fig 3.5.

3.5. b. Effect of NH_2OH and CH_3ONH_2 on variants of HPII.

Hydroxylamine has been reported to react with compound I of catalase to form nitric oxide (Marcocci, 1994), which reversibly reacts with heme to cause inhibition of the enzyme (Doyle *et al,* 1981 and Brown, 1995) by binding in the heme distal side pocket (Zhao, 1998). The inhibitory effects of hydroxylamine (NH_2OH) and O-methyl hydroxylamine were studied, the concentrations needed for each inhibitor to achieve 50% inhibition for HPII and variants were calculated and are shown in Table 3.4.

Table 3.4. Determination of the 50% inhibitory concentrations of different catalase inhibitors for WT HPII and its variants

Variant	Concentration needed for 50% inhibition			
	NaN_3 (μM)	NaCN (μM)	NH_2OH (nM)	NH_2OCH_3 (mM)
I274A	175	16	175	6
I274G	210	18	1000	5
I274V	150	10	116	6
E270A	75	9	123	4
E270S	90	8	108	4
F214H	100	13	154	6
F214L	80	16	120	1.5
F206N	70	17	102	6
WT HPII	130	10	120	5.5

Fig 3.5. Comparison of the effects of sodium azide on WT HPII and its variants. Appropriate dilutions of each purified protein were incubated for 1 min in the reaction chamber with different concentrations of NaN_3 before adding H_2O_2.

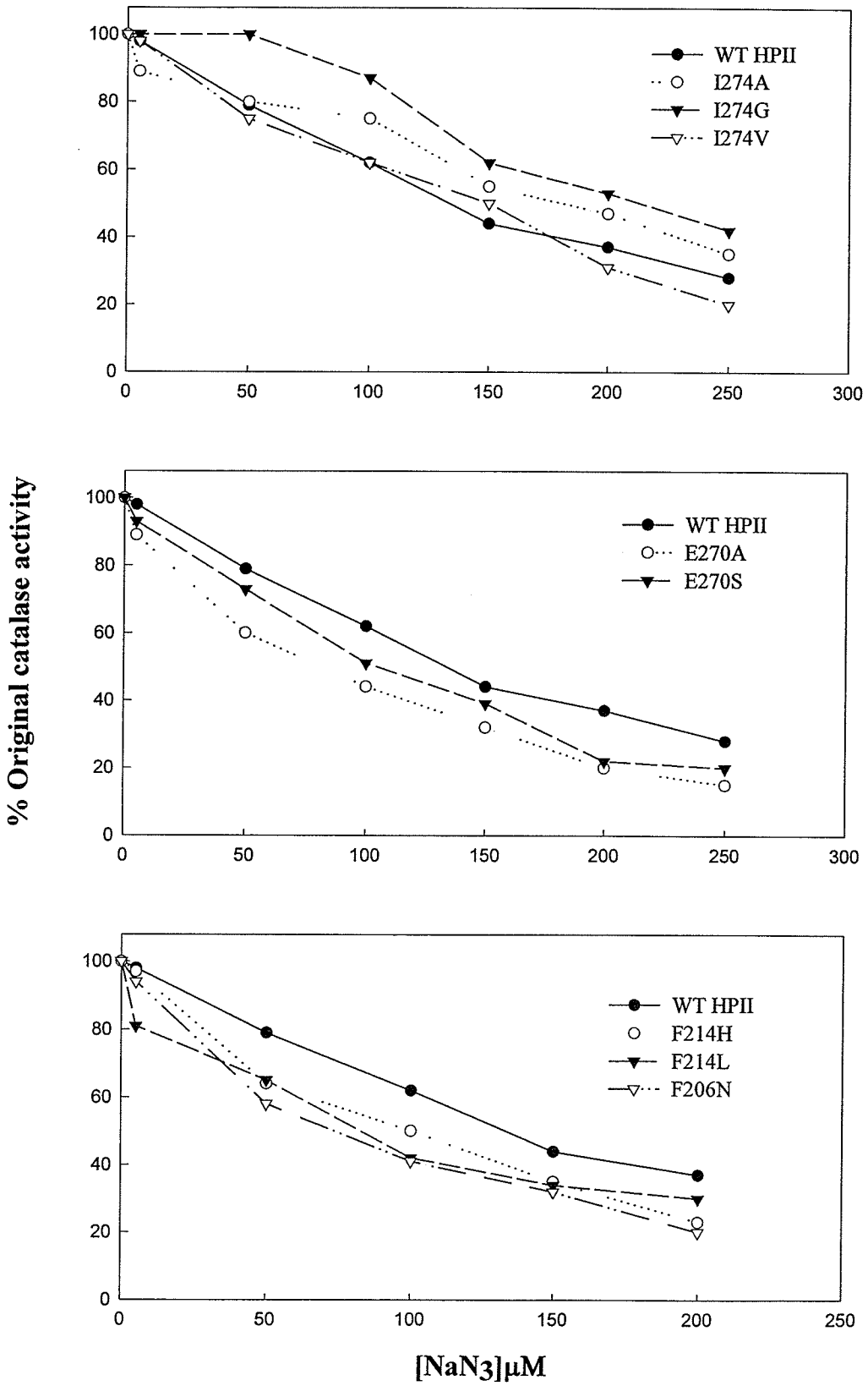

% Original catalase activity
WT HPII
I274A
I274G
I274V
WT HPII
E270A
E270S
WT HPII
F214H
F214L
F206N
[NaN3]μM

Fig 3.6. Comparison of the effects of sodium cyanide on WT HPII and its variants. Appropriate dilutions of each purified protein were incubated for 1 min in the reaction chamber with different concentrations of NaCN before adding H_2O_2.

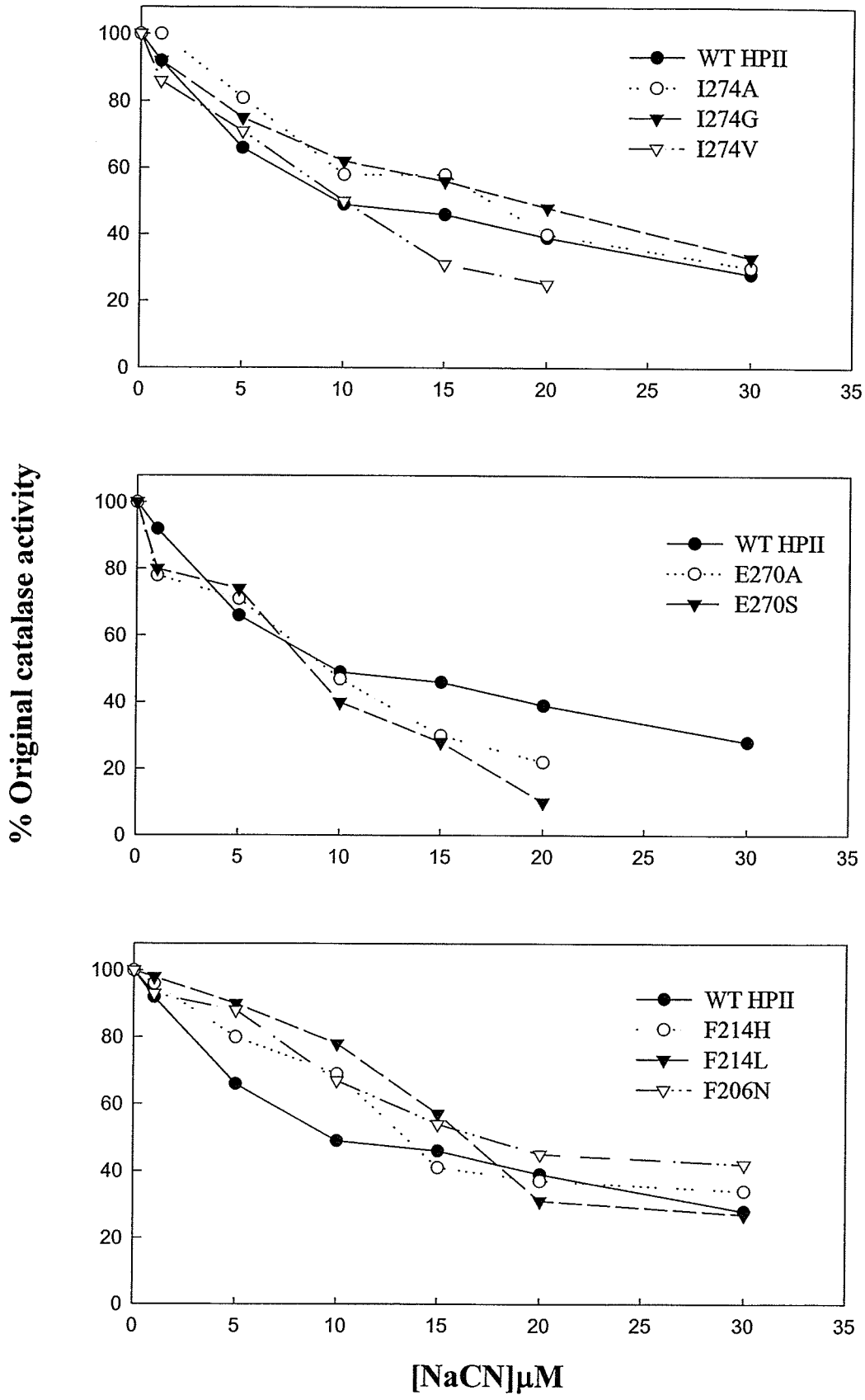
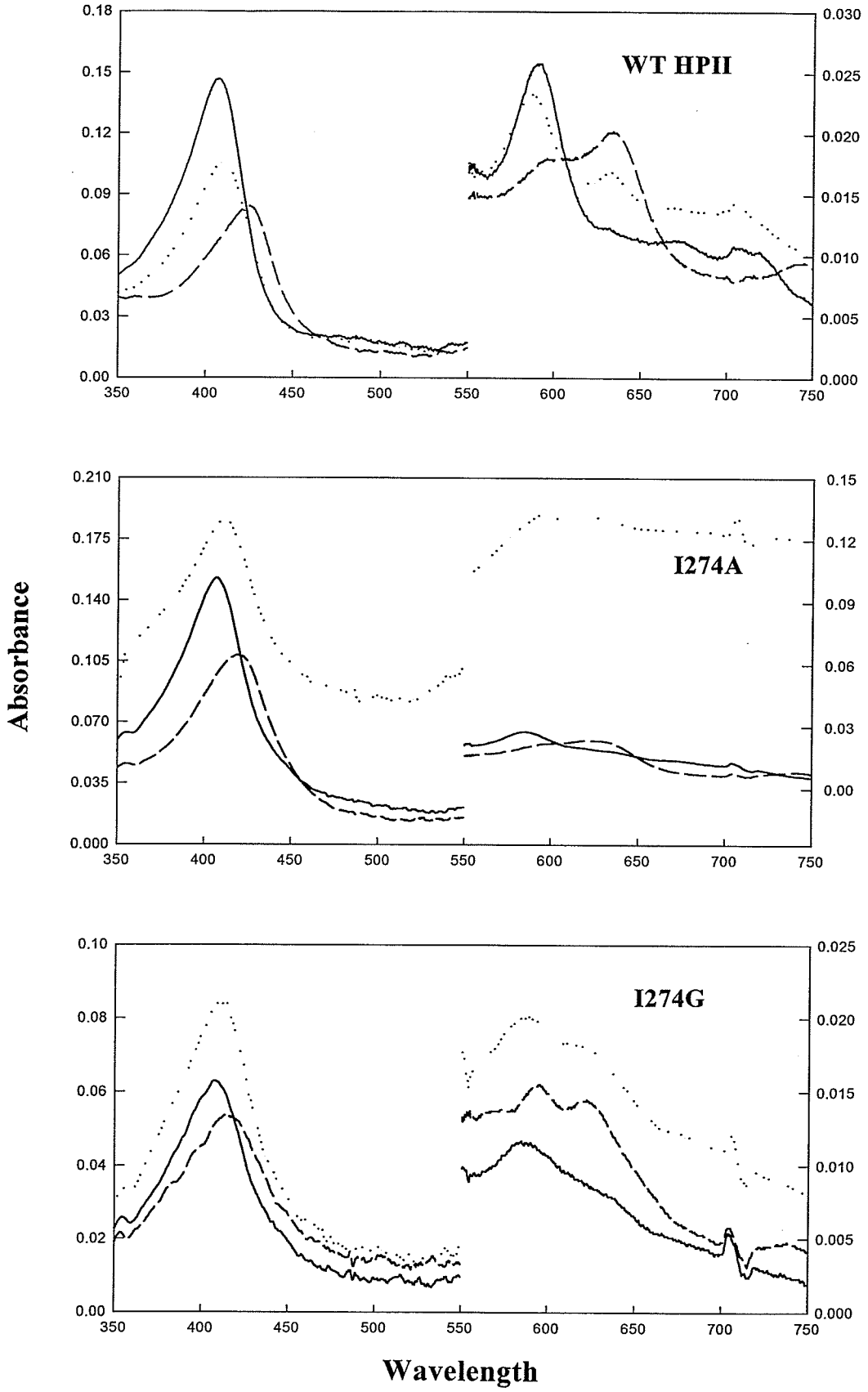
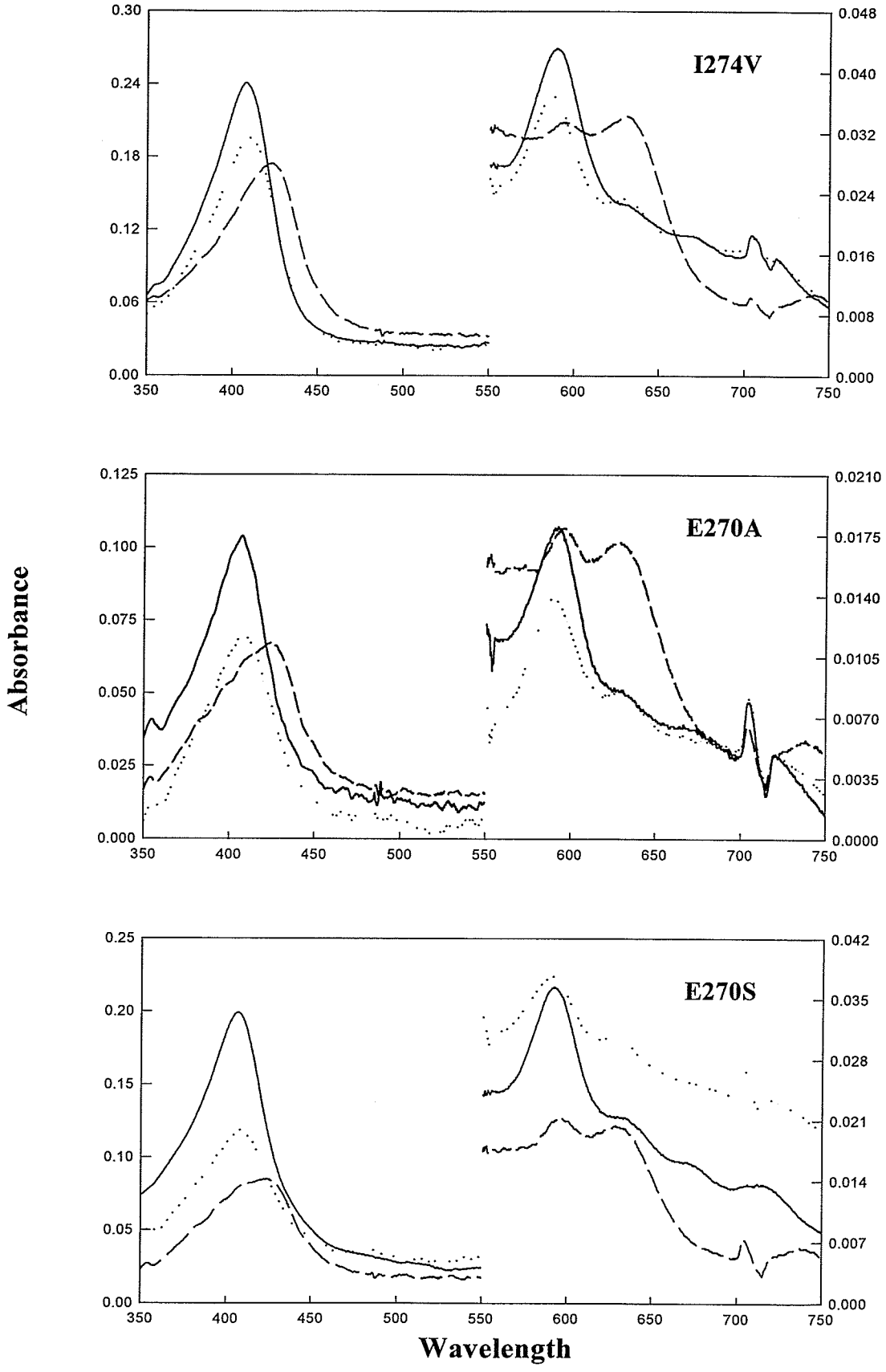

% Original catalase activity
WT HPII
I274A
I274G
I274V
WT HPII
E270A
E270S
WT HPII
F214H
F214L
F206N
[NaCN]μM

Fig 3.7. The visible spectra of WT HPII and its variants in presence or absence (——) of NaN_3 and NaCN. Each protein was incubated with 1mM NaN_3 (……) or 0.5 mM NaCN (------) at room temperature for 15 minutes prior to spectral analysis. The left axis is for the range from 350 to 550 nm and the right axis is for the range from 550 to 750 nm


WT HPII
I274A
I274G
Absorbance
Wavelength


I274V
E270A
E270S
Absorbance
Wavelength

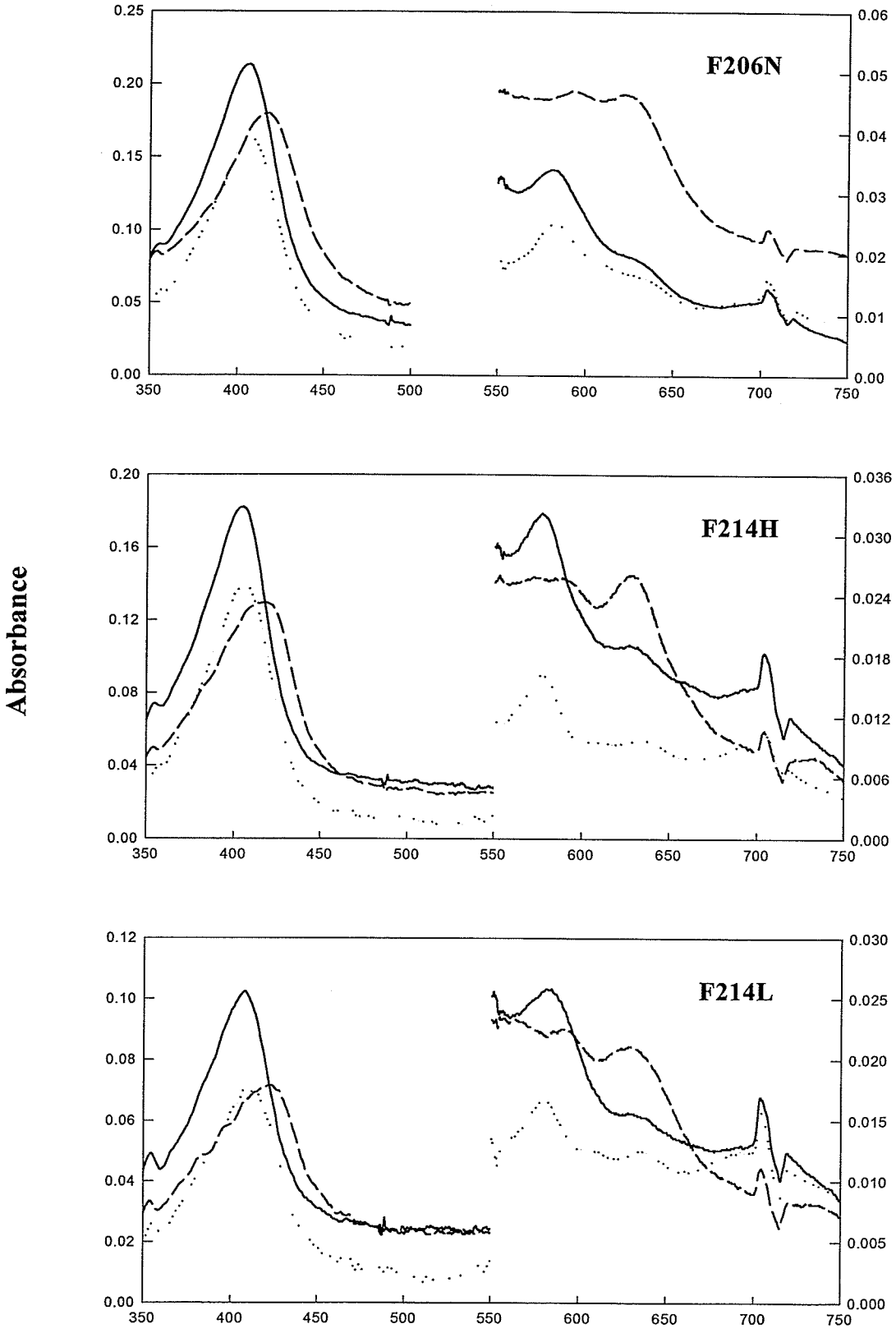

F206N
F214H
F214L
Absorbance
Wavelength

Fig 3.8. Comparison of the effects of hydroxylamine on WT HPII and its variants. Appropriate dilutions of each purified protein were incubated for 1 min in the reaction chamber with different concentrations of NH_2OH before adding H_2O_2.

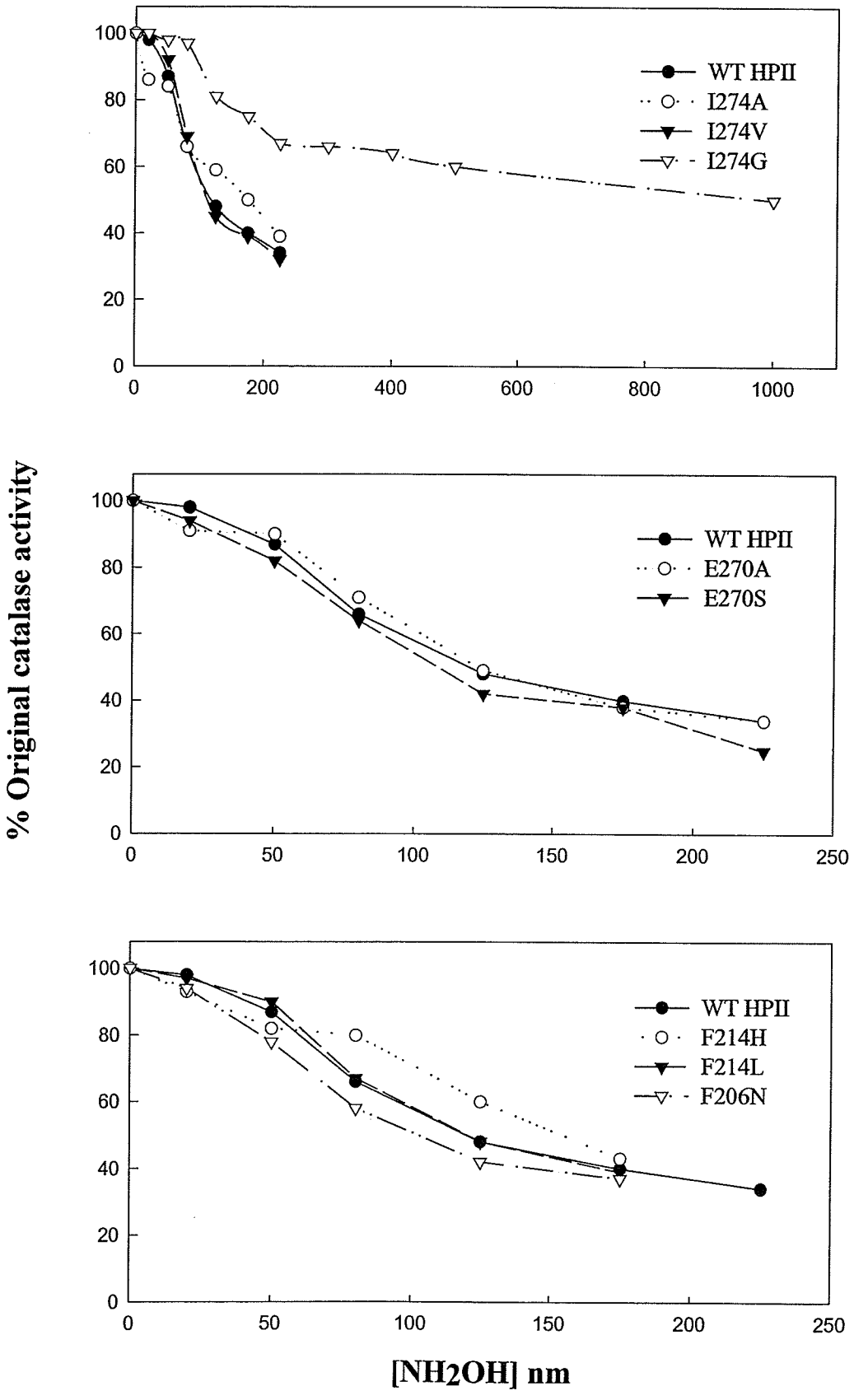
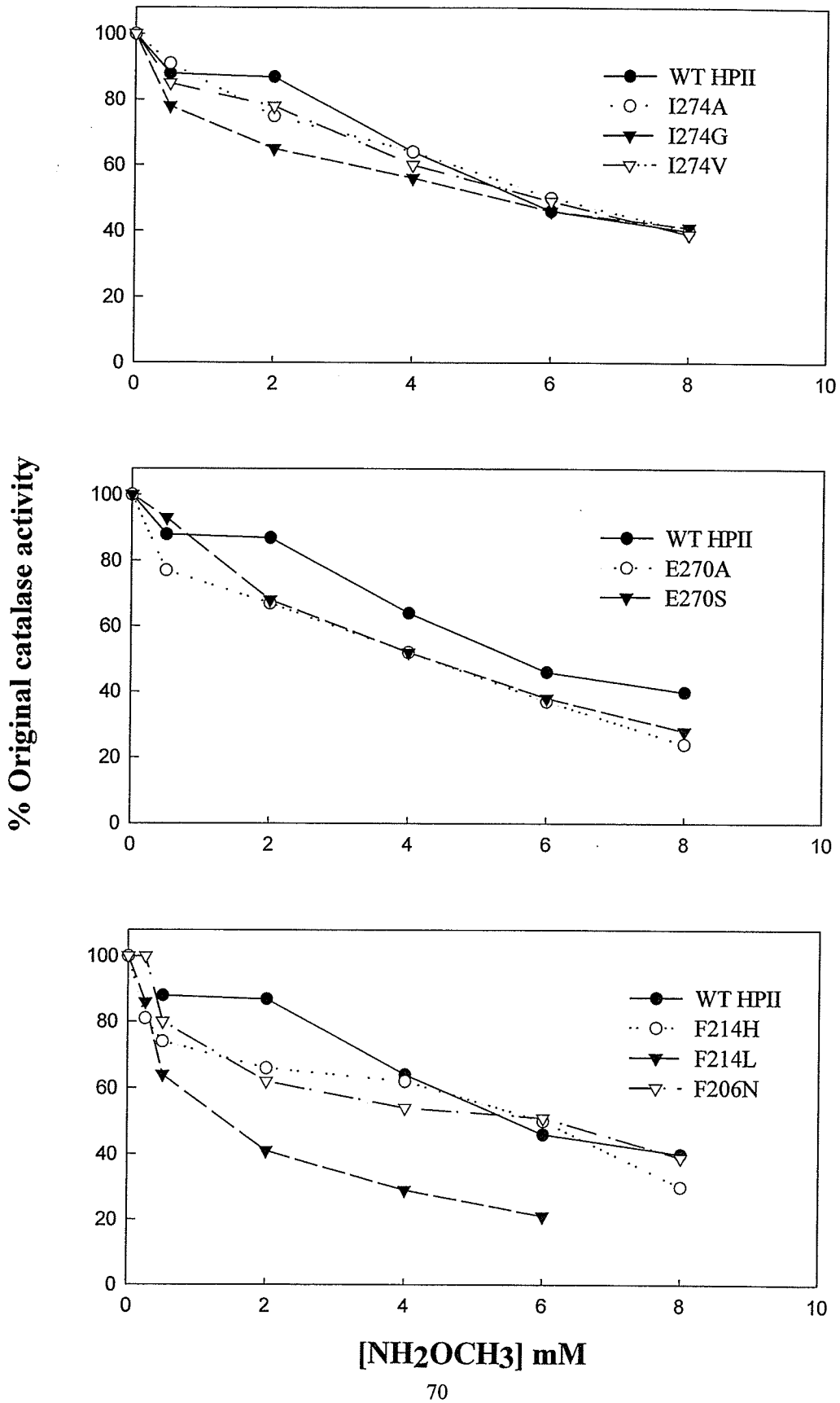

% Original catalase activity
WT HPII
I274A
I274V
I274G
WT HPII
E270A
E270S
WT HPII
F214H
F214L
F206N
[NH2OH] nm

Fig 3.9. Comparison of the effects of O-methyl hydroxylamine on WT HPII and its variants. Appropriate dilutions of each purified protein were incubated for 1 min in the reaction chamber with different concentrations of NH_2OCH_3 before adding H_2O_2.


% Original catalase activity
100
80
60
40
20
0
0
2
4
6
8
10
WT HPII
I274A
I274G
I274V
WT HPII
E270A
E270S
WT HPII
F214H
F214L
F206N
[NH2OCH3] mM

4. CONCLUSIONS AND DISCUSSION

4.1. Isoleucine 274 Variants

Because of the close proximity of Ile274 to the heme (3.8 Å) and its location at the start of the lateral channel on the side of the heme that is opposite to the heme b to d oxidation, two possible roles have been suggested for the residue including the maintenance of the heme orientation and control of the passage of solutes, substrate, products and solvent through the lateral channel. Ile274 was converted to four hydrophobic amino acids (Gly, Ala, Val and Phe) to study the effect of changing the size of the hydrophobic group at this location. I274F variant protein did not accumulate any protein suggesting that the large side chain of the Phe interfered with the folding of the protein. The three variants (Ala, Gly and Val) with smaller R groups were isolated in sufficient amounts for study, indicating that smaller side chains could be accommodated without a significant disruption in structure. In spite of the standard culture conditions, the I274V variant contained a mixture of heme d and heme b, which was difficult to rationalize, because the other properties of the variant were similar to the WT protein. The I274A variant exhibited lower activity consistent with the presence of a mixture of heme b, heme d and a predominant peak for an unidentified heme species with greater apparent hydrophobicity than heme b. I274A also exhibited a reduced A_{407}/A_{280} ratio which could be explained as due to a different heme environment (lower extraction coefficient) or a different heme species, but no firm conclusions can be drawn until the unidentified heme species is fully characterized. The I274G variant was produced in low yield and was heat sensitive most likely due to the structural instability introduced by the isoleucine to glycine change. The low yield of the extracted heme together with the

reduced A_{407}/A_{280} ratio of 0.6 suggest a lower than normal heme/ protein content or a change in the electronic environment of the heme. Further characterization of this variant is needed. Both the Ala and Gly variants were more resistant than WT towards cyanide, azide and NH_2OH, suggesting structural changes in the heme environment, consistent with the spectral and HPLC data.

Introduction of smaller R groups in the entrance to the lateral channel did not result in an increase of the protein's activity suggesting that the diameter of the lateral channel is not a determinant of activity. The possibility of reduced heme composition is consistent with the decrease in the activity and may be interfering with the effect of a larger channel entrance. If the I274G variant is found to contain less heme it will be the first example of a tetrameric catalase with less than a full complement of four heme groups.

4.2. Glutamate 270 Variants

Of the three variants of E270, E270A, E270S and E270I only the Ile variant did not accumulate any protein, most likely because of steric interactions caused by the bulkier side chain that interfered with the protein folding, while E270A and E270S were as active as or slightly more active than WT. E270 interacts ionically with R260 in the upper chamber of the lateral channel narrowing the diameter of the channel. The ionic bond will be disrupted in E270A, creating a larger diameter for the channel and this is consistent with the kinetic constants and the increased sensitivity towards catalase inhibitors. Curiously the activity of E270A, while higher than WT, still it is not as high as that of R260A in which the E-R ionic association is also disrupted (Sevinc *et al*, 1999).

This suggests that the side chain of R260 plays a bigger role in blocking the channel than does the side chain of E270. The properties of E270S are consistent with this disposition, presenting a turnover rate equivalent to WT, confirming that changes in residue 270 have little effect on the dimensions of the channel.

4.3. Phenylalanine 206 and Phenylalanine 214 Variants

The phenyl ring of Phe214 is in the distal pocket just 3.4 Å from the heme group and 6.3Å from the essential His128. Phe206 is also located in the distal pocket about 5.6 Å from the heme. The phenyl ring is hydrophobic presenting only weak opportunities for interaction with water molecules. Having the channel and active site surrounded by such residues may effectively direct the substrate to the polar active site residues. There were two objects to study those residues. The first was to determine how important these residues are in catalysis and structure integrity. The second was to determine if there was any change in heme orientation. Changes in the heme species and possibly in the amount of heme together with the difficulties in extracting the heme suggested the possibility of covalent attachment of the heme to the protein in F214H. The three variants F214H, F214L and F206N all exhibited decreased activity. At the moment, the only explanation is that a change in the electronic integrity of the heme has affected the interaction with the substrate H_2O_2. It is unclear if the mutations have caused a change in heme orientation but at least one new heme species is formed, but not identified. The F206N and F214H variants could be used to construct the double mutants F206N/ N201F and F214H/ H128F with the object of putting more pressure to flip the heme by 180°. The appearance of unidentified heme species also suggests that F206 and F214 are important in maintaining

the integrity of the heme active site. Unfortunately it is hard to draw more firm conclusions in the absence of a crystal structure determination.

5. SUMMARY

The structure/function relationship of residues in the lateral channel of the *E. coli* catalase HPII have been studied. The targeted residues were Ile274, close to the heme group at the start of the lateral channel, Phe206 and Phe214, on the distal side of the heme and Glu270 in the middle of the lateral channel. Ile274 was converted to Ala, Gly, Val and Phe, Glu270 was converted to Ala, Ser and Ile, Phe206 was converted to Asn and Phe214 was converted to His and Leu. Out of the 10 variants, two, I274F and E270I, did not accumulate any protein and were not included in purification and characterization experiments. For Ile 274 variants, I274V exhibited activity and kinetic profiles similar to wild type and was grown at 37°C. I274A and I274G both exhibited decreased activities, were grown at 28°C to improve the yield of expressed protein and exhibited higher resistance towards catalase inhibitors. These properties are consistent with reduced heme content, something that was not observed in any other tetrameric catalase indicating the importance of the isoleucine side chain in stabilizing the heme association with the protein. This is surprising given that the equivalent residue is glycine in CAT A and serine in BLC.

Of the Glu270 variants, E270A and E270S had properties similar to WT and E270I did not accumulate protein. The slightly higher activity of E270A is probably due to an increase in the diameter of the channel resulting from the disruption of the ionic bond between E270 and R260, but the increase in activity is small compared to that of R260A (Sevinc, 1999). The three fold increase in activity of R260 indicates that removing the arginine residue must have a much large effect on channel architecture than the removal of E270.

F206N, F214H and F214L all exhibited lower activity compared to WT and contained unusual mixtures of heme species with possible cross linking of the heme to the protein in F214H. The changes in apparent heme content and sensitivity to inhibitors suggest an important role for these residues in maintaining the integrity of the heme pocket.

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