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Characterization of NfsA, the Major Nitroreductase from *Escherichia coli*

by

Peter Koziarz, B.Sc.

A thesis submitted to

the Faculty of Graduate Studies and Research

in partial fulfilment of the requirements

for the degree of Master of Science

Ottawa-Carleton Institute of Biology

Faculty of Science

Department of Biology

Carleton University

Ottawa, Ontario

May 2\textsuperscript{nd}, 2000
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Submitted by
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Chair, Department of Biology

Thesis Supervisor

Carleton University
Abstract

NfsA, the major nitroreductase in *Escherichia coli*, has been characterized with respect to its genetic and biochemical characteristics, and its role in the development of bacterial resistance to nitrofuran derivatives. Examination of the *nfsA* gene in a collection of first- (n=24) and second-step (n=57) furazolidone-resistant mutants revealed mutations within the *nfsA* gene in all mutants, with a large contribution to the mutational spectrum provided by integration of bacterial IS elements (71 %). This high rate of transposition was observed in two different strains of *E. coli*, and was reduced to 5 % when an extrachromosomal source of the *nfsA* gene was used, indicating the importance of local chromosomal factors in promoting transposition. Plasmid pQEnfsA, an expression construct for NfsA bearing a N-terminal His tag, was constructed and used for rapid purification of NfsA. Characterization of the enzyme showed that His-tag NfsA catalyzed reduction of nitrofurazone through a ping-pong Bi-Bi mechanism with $V_{\text{max}}$ of 43 mol·min$^{-1}$·mg enzyme$^{-1}$ and $K_M$ (NADPH) and $K_M$ (nitrofurazone) of 22 μM and 72 μM, respectively. To examine the structural features of NfsA, a model based on a crystal structure of its sequence homolog, the major NADPH-flavin oxidoreductase from *Vibrio harveyi* (Frp), was constructed. Plasmid pQEnfsA was mutagenized with NH$_2$OH and the mutants were characterized with respect to sequence of the *nfsA* gene, nitroreductase activity, and spectral characteristics of the FMN cofactor. Examination of several mutations produced in NfsA, in the structural context provided by the model, was successful in rationalizing their impact on protein functionality and supports the accuracy of the model.
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I would like to express my gratitude to Dr. Iain Lambert for his patience and support throughout the course of this project. I would also like to thank all my colleagues for their advice and assistance during my stay in our laboratory, in particular, Reza Nokhbeh, Jacqui Whiteway, Dr. Suzanne Paterson and Craig Carroll.
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<td>FAD</td>
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<td>FMN</td>
<td>flavin mononucleotide</td>
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Chapter 1

General Introduction
Nitroaromatic and nitroheterocyclic compounds have found widespread use in pharmaceutical industry as well as in manufacturing of agrochemicals, dyes, plastics, explosives and pesticides (Hallas and Alexander, 1983; Kedderis et al., 1988; Bryant and DeLuca, 1991; Nishino and Spain, 1993; Somerville et al., 1995). In addition, nitrated polycyclic aromatic hydrocarbons have been found in photocopy toners, coal fly ash and are formed during a variety of combustion processes involving fossil fuels, food and tobacco products (Rosenkranz et al., 1980; Rafii et al., 1991). The increasing prevalence of nitro compounds within the human environment and the resulting concern has stimulated a large number of studies focusing on the adverse health effects associated with exposure to these chemicals. A particular class of nitroheterocyclics, 5-nitrofurans and their derivatives, has received a great deal of attention, due to their mutagenic, clastogenic and cytotoxic effects in bacteria and cultured mammalian cells (Bryant and McCalla, 1980) as well as oncogenic activity in various organs in experimental animals such as rats, mice, hamsters and dogs (Erturk et al., 1971, Cohen, 1978). Several derivatives of 5-nitrofurans, including nitrofurazone, furazolidone and nitrofurantoin have been in use for many years as therapeutic agents for topical infections, bacterial enteritis and urinary and gastrointestinal tract infections (Breeze and Obaseiki-Ebor, 1983), and some are still employed as general antimicrobial agents.

The nitro substituent at the 5-position of the furan ring is generally believed to be required for oncogenic and mutagenic activities of nitrofurans, since analogs lacking this function do not exhibit biological activity (Hatcher et al., 1995). In addition, it has been demonstrated in many studies that nitrofurans per se are not particularly toxic and metabolic
activation involving one or more enzymatic transformations is required for these compounds to exert their adverse effects (McCalla et al., 1970; Bryant and DeLuca, 1991). A key step in this activation is the reduction of the nitro moiety by enzymatic nitroreductase functions involving incremental transfer of one or two electrons to produce biologically active metabolites. Metabolic activation proceeding through successive transfer of single electrons results in the formation of a nitroaromatic anion free radical (Figure 1.1) which has been found to be rapidly reoxidized to the parent compound in the presence of molecular oxygen (Peterson et al., 1979). This short-lived free radical species does not appear to react directly with protein or DNA, but may yield reactive oxygen species that have the capacity to cause biological damage. However, since two electron transfer is known to produce intermediates that account for the biological activity of nitrofurans (see below), the contribution of radical species to the toxic action of nitrofurans is believed to be minor.

Reduction of the nitro group under aerobic conditions involves a transfer of two electrons and initially yields a transient nitroso derivative which is subsequently converted to a 4-electron reduction product, N-hydroxylamine (Bryant and DeLuca, 1991). Formation of the hydroxylamine derivative of the parent nitro compound is believed to represent a key transformation in the biological activation pathway, since this derivative can undergo protonation and elimination of water to form a nitrenium ion which can react with DNA (Debnath et al., 1993), or rearrange to form an open chain acrylonitrile derivative (Vroomen et al., 1988; Lambert et al., 1991) which has been shown to form conjugates with glutathione, mercaptoethanol and the thiol residues of proteins (reviewed in Bertenyi and Lambert, 1996) as well as to have mutational capacity (Vroomen et al., 1988). The end-
FIGURE 1.1. Reductive metabolism of nitroheterocyclic and nitroaromatic compounds (adapted from Bertenyi and Lambert, 1996).
products of the two-electron reductive pathway include aminofurans and open chained nitriles, both of which are biologically inactive.

In *Escherichia coli*, several enzymes are responsible for metabolic activation of nitrofurans to their toxic forms. These have been classified as type I and type II reductases based on their sensitivity to oxygen as dictated by the mode of electron transfer during the reduction process (McCalla *et al.*, 1975). Type II enzymes catalyze one-electron transfer reduction which, as described above, is followed by rapid reoxidization of the nitro anion radical to the original nitro compound in the presence of oxygen; therefore type II enzymes are termed “oxygen-sensitive” nitroreductases. In the absence of oxygen, type II enzymes will form reduced nitroso- hydroxylamine- and amino- derivatives through successive single electron transfers. Type I enzymes, on the other hand, are “oxygen-insensitive” nitroreductases and are able to reduce nitrofurans through a two-electron transfer route and yield products which are not readily reoxidizable.

In order to identify the genes and enzymes responsible for these nitroreductase activities, nitrofurazone-resistant mutants of *E. coli* were isolated by growing the bacteria in the presence of nitrofurazone or other nitrofuran derivatives. While the mutants deficient in type I reductase activity were easily obtainable under treatment with moderate levels of nitrofurazone, no mutants involving the loss of type II nitroreductase activity have been isolated even at treatments with high concentration of nitrofurazone under anaerobic conditions (McCalla *et al.*, 1978). Acquisition of type I resistance to nitrofurazone was demonstrated to be a two-step process, with the first step yielding mutants exhibiting roughly threefold resistance to nitrofurazone, relative to the wild-type, and the second step producing
mutants that were six- to sevenfold more resistant than the wild-type (McCalla et al., 1970). Genetic analysis of both the first- and second-step mutants suggested the presence of two resistance genes, named \textit{nfsA} and \textit{nfsB}, responsible for oxygen-insensitive nitroreductase activity (McCalla et al., 1978). This finding was corroborated by biochemical analysis of both mutant and wild-type strains of \textit{E. coli} in which a total of three distinct type I reductase activities were identified: a major activity (IA), and two minor activities (IB\textsubscript{1} and IB\textsubscript{2}), (Bryant et al., 1981). The activities varied in their cofactor requirements; the major activity was dependent solely on NADPH as the reductive source, while both minor activities utilized either NADH or NADPH. The stepwise acquisition of resistance to nitrofurazone under aerobic conditions was postulated to first involve the loss of the functional product of the \textit{nfsA} gene, which would remove the IA activity and produce first-step mutants; subsequent inactivation of the product of the \textit{nfsB} gene (IB\textsubscript{1} activity) would yield second-step mutants. Interestingly, both first- and second-step mutants still retained the IB\textsubscript{2} activity, ascribed to a putative \textit{nfsC} gene, which remains unidentified. Although no mutants involving the loss of oxygen-sensitive nitroreductase activity have ever been isolated, as mentioned above, at least four reductase II components are believed to exist in \textit{E. coli}, based on fractionated cell-extract nitrofurazone reduction studies (McCalla et al., 1978). To this day no genetic evidence has been produced to support this notion.

Both \textit{nfsA} and \textit{nfsB} genes have been cloned, purified and partially characterized in \textit{E. coli} (Zenno et al., 1996a, Zenno et al., 1996b). The \textit{nfsA} gene was found to be located at 19 min. on the \textit{E. coli} map (Zenno et al. (1996b). The product of the \textit{nfsA} gene is an enzyme with a calculated molecular weight of 26,799 which is tightly associated with FMN.
as a prosthetic group and displays a broad electron acceptor spectrum. NfsA reduces nitrofurazone according to the ping-pong biproduct-bisubstrate mechanism (Segal, 1975) and is highly selective for NADPH as a source of reducing power (Zenno et al., 1996b). Therefore, NfsA represents the major oxygen-insensitive nitroreductase activity in *E. coli*. Examination of the amino acid sequence of NfsA reveals a high degree of identity (51%) to that of Frp, a NADPH-flavin oxidoreductase from *Vibrio harveyi* (Lei et al., 1994), and several other reductases identified among bacteria (Figure 1.2). Frp is a well-described enzyme whose crystal structure has been solved (Tanner et al., 1996) and which shares some of the characteristics of NfsA as it uses a tightly bound FMN cofactor and is also selective for NADPH in reductive reactions. In addition, it is able to accept a wide variety of electron acceptors that are reducible by NfsA, including nitrofurazone (Zenno et al., 1998). A notable difference between kinetic profiles of NfsA and Frp is their flavin reductase activity, which is approximately 10 times higher in the latter. This activity, however, can be augmented in NfsA to a level above that of Frp by a single amino acid substitution in the putative active site of NfsA which emphasizes close evolutionary relationships between the two enzymes (Zenno et al., 1998). Another structural feature that might be extended to NfsA is the homodimeric state of native Frp (Liu et al., 1997). Although initial DMP-cross-linking experiments failed to produce any dimeric structures of NfsA, gel exclusion chromatography of purified NfsA indicated the presence of a potential 47-kDa dimer (Zenno et al., 1996b). It is therefore conceivable, given the functional and structural similarities that exist between Frp and NfsA, that NfsA may also exist as a homodimer in its native state.
| NfsA | 1 | -- | TPTI LCC-HE TDE | EAPREA | KS R STS | SC CS | SR R | K
| SnrA | 1 | -- | TPTI LCC-HE TDE | EAPREA | NCR RS STS | SC CS | SR R | R
| Frp | 1 | -- | SNTI TLA-KE TA | DQ EORQT | QA LAAS | SM WV | PV R | S
| Bac | 1 | -- | SNTI TLA-KE TA | DQ EORQT | KS QAAS | SY L A Y | G | P
| Cr | 1 | -- | MK LRY | DGN KEI ELE IAT QMAA | HAY A Y | W | E
| Nox | 1 | MEATL I DAK AALR | RER R | KPY EGGLE | RES A | LRAF | W | P

| NfsA | 55 | ALB ED VTL TGC K HH AQA | EFW | A | FN | HLO | CPEAQ LG | -- | AQ LG | GV V
| SnrA | 55 | ALB EDVSSTLTGK HH AQA | EFW | A | FN | HLO | CPEAQ LG | -- | AQ LG | GV V
| Frp | 55 | EKR ENTA AQ F | NAY | ESA | EFL | I YQ | HAT | NPDV QA D | -- | TL T GAV
| Bac | 55 | EKR EN AT VSV LA | NPY | TKE | NHH | FTA | AL Y | HQQ | ZAEK G | ESEL | NENG | S L
| Cr | 53 | EKR EK GML S | NNP QYTE | GA F | V | FK | HLS QAK L | GD | V | V A | V D | A | EN | GA
| Nox | 61 | ATKR AEA AAFG | AH | EAPVVL | LYA | LED A | L | DEV | HPG | CQER | REA | KQ A | QRA F

| NfsA | 108 | TQ | -- | M A T | A | -- | L | G V | N | NIA | TK | K | QHVLP |
| SnrA | 108 | TQ | -- | M A T | A | -- | L | G V | N | NIA | TK | K | QHVLP |
| Frp | 108 | S | -- | M A T | A | -- | L | G V | N | NIA | TK | K | QHVLP |
| Bac | 114 | A | -- | M A T | A | -- | L | G V | N | NIA | TK | K | QHVLP |
| Cr | 110 | V | -- | F | E | -- | P | I | C | K | F | E | SE | FN | Y | F | E
| Nox | 121 | A | P | Q | SEQ | ARKAWAS | S | P | TLY | T | L | LE | V | LGS | P | MG | D | P | R | R | R | A | S | SRAI PAL

| NfsA | 155 | DC | W ADNPFL | L A | ASI | W | E | -- | PLDKG A | AC | DEQ | A | E | LT | GSNR | RDT
| SnrA | 155 | DC | W ADNPFL | L A | ASI | W | E | -- | PLDKG A | AC | DEQ | A | E | LT | GSNR | RDT
| Frp | 155 | DC | W ADNPFL | L A | ASI | W | E | -- | PLDKG A | AC | DEQ | A | E | LT | GSNR | RDT
| Bac | 161 | DAV | H | LNLGK | K | H | KQA | Y | E | E | HNVNT DD FR | T | N | DT | SR | EKT | NPGK | EET
| Cr | 157 | ETI | V | ARNEV | H | V | W | E | -- | TE | KEY | ELP | P | A | NTD | EAA | N | EN | SS | NR | IDN |
| Nox | 181 | D | A | Y | E | E | GY | PS | L | LE | V | LWR |

| NfsA | 212 | T | D | HRT | IKE | P | PE | LDK | HK | W | TR
| SnrA | 212 | T | D | HRT | IKE | P | PE | LDK | HK | W | TR
| Frp | 212 | T | D | HRT | IKE | P | PE | LDK | HK | W | TR
| Bac | 221 | D | DOL | L | K | Q | K | Q | P | T | Y | K | E | FN | K
| Cr | 215 | T | K | Q | A | D | IE | Q | P | H | K | D | AK | FN | W
| Nox | | | | | | | | | | | | |

**Figure 1.2.** Multiple amino acid sequence alignment of the major nitroreductase from *Escherichia coli*, NfsA, and several identified sequence homologs: SnrA - nitroreductase A homolog from *Salmonella typhimurium* (Lambert et al., GenBank entry AF117952), Frp - NAD(P)H-oxidoreductase from *V. harveyi* (Lei et al., 1994), Bac - nitro/flavin reductase nfrA1 from *B. subtilis* (Glaser et al., 1993), Cr - chromium (VI) reductase from *Pseudomonas sp.* (Suzuki, GenBank entry D83142) and Nox - NADH dehydrogenase from *Thermus aquaticus* (Park et al., 1993). The sequences were aligned using MultAlin software package and shaded using Boxshade server. Black fields indicate the fractions of sequences where identity of residues relative to the column consensus exceeds 0.8; grey fields represent regions where similarity (as defined in the internal parameter file of the server) exceeds 0.8.
The locus corresponding to the *nfsA* gene had been previously identified and described as *mdaA* gene in a study aimed at identifying bacterial gene products that modulate the sensitivity of bacteria to tumoricidal agent DMP 840 and related compounds (Chatterjee and Sternberg, 1995). Overexpression of *mdaA* in a bacterial host was observed to impart resistance upon the host to a mammalian topoisomerase inhibitor, adriamycin, presumably through a drug-detoxification mechanism. A second gene identified in the study, *mdaB*, conferred resistance to both adriamycin and etoposide possibly by modulation of topoisomerase IV activity, as suggested by the authors, given its chromosomal locus between the loci of two topoisomerase IV subunits. While the mode of action of *mdaB* remains speculative, the gene does demonstrate some weak similarity to bacterial and mammalian NAD(P)H-oxidoreductases/dehydrogenases. For instance, MdaB protein exhibits 18 % identity and 29 % similarity to a putative NAD(P)H oxidoreductase from *Bacillus subtilis* (Kasahara *et al.*, SwissProt entry P96674).

In support of the proposed role of *nfsA/mdaA* in cellular detoxification mechanisms, the *nfsA* gene has been recently identified as a member of the *soxRS* regulon involved in cellular defence against oxidative stress (Liochev *et al.*, 1999a). The response mediated by *soxRS* is inducible upon exposure to redox cycling agents and includes several enzymes acting in concert which relieve oxidative stress by neutralizing harmful O$_2^\cdot$ free radicals, participating in DNA repair, and through other mechanisms. A possible role for NfsA in this response may be a divalent reduction of offending chemical agents to prevent their participation in redox cycling and O$_2^\cdot$ generation.
The nitroreductase encoded by the \textit{nfsB} gene represents the minor (IB) nitroreductase activity in \textit{E. coli}. The \textit{nfsB} gene is situated at 13.0 min on the \textit{E. coli} map and gives rise to a polypeptide, having a calculated molecular weight of 23,904, that is capable of forming a homodimer and is tightly associated with FMN as a prosthetic group (Zenno et al., 1996a). Similarly to NfsA, NfsB nitroreductase displays a broad electron acceptor specificity and catalyzes reduction of nitrofurazone through a ping-pong Bi-Bi mechanism. NfsB can utilize either NADH and NADPH as a source of reducing equivalents and accepts FAD as an effective substitute for FMN (Zenno et al., 1996a). As demonstrated in Figure 1.3, several sequence homologues of NfsB exist among bacteria, most notable of which are classical nitroreductases from 	extit{Salmonella typhimurium} (Watanabe et al., 1990) and \textit{Enterobacter cloacae} (Bryant et al., 1991) with identity exceeding 80% between the three sequences. The major NAD(P)H-flavin oxidoreductase from \textit{Vibrio fischeri}, FRase I (Zenno et al., 1994) is also an identified sequence homologue of NfsB (32% identity) whose crystal structure has been solved (Koike et al., 1998). Interestingly, there is no appreciable sequence similarity between the \textit{nfsA} and \textit{nfsB} genes.

NfsA and NfsB, along with their sequence homologs, belong to a class of proteins known as NAD(P)H-flavin oxidoreductases, and form two separate families based on the sequence data. This class of enzymes is ubiquitous among microorganisms and higher organisms and in many cases their enzymatic function is redundant in nature as it is performed by multiple enzymes within a single cell (Fieschi et al., 1995). The luminous bacterium, \textit{V. harveyi}, contains at least three types of FMN reductases (Lei et al., 1994), whereas in \textit{E. coli} at least two other types of flavin reductases other than NfsA and NfsB are
FIGURE 1.3. Multiple amino acid sequence alignment of the minor nitroreductase from E. coli, NfsB, and several identified sequence homologs: Sal - NAD(P)H-nitroreductase from S. typhimurium (Watanabe et al., 1990), Ent - NAD(P)H-nitroreductase from Enterobacter cloacae (Bryant et al., 1991), Fre - FRase I, minor NAD(P)H-oxidoreductase from V. fischeri (Zenno et al., 1974), Bsu - putative NAD(P)H-oxidoreductase from B. subtilis (Kunst et al., GenBank entry Z99115) and Hel - NADPH oxidoreductase from Helicobacter pylori (Kwon et al., GenBank entry AF183174). The sequences were aligned using MultAlin software package and shaded using Boxshade server. Black fields indicate the fractions of sequences where identity of residues relative to the column consensus exceeds 0.8; grey fields represent regions where similarity (as defined in the internal parameter file of the server) exceeds 0.8.
known to exist. The most abundant flavin reductase in *E. coli*, Fre, does not bear any significant sequence similarity to either NfsA or NfsB and given its specificity towards a flavin isoalloxazine ring, it appears to be primarily concerned with reduction of free flavins rather than nitrosubstituted compounds (Fieschi *et al.*, 1995). As in the case of NfsA and NfsB, Fre has several sequence homologues in other bacteria including *V. harveyi* and *V. fischeri* (Zenno and Saigo, 1994). This functional redundancy and ubiquity demonstrated by NAD(P)H-flavin oxidoreductases underscores their importance in cellular metabolism.

Bacterial nitroreductases have several interesting applications in biotechnology including development of new antimicrobial agents and mutagen detection strains, and as enzyme component of antibody-directed enzyme prodrug therapy (ADEPT) and gene-directed enzyme prodrug therapy (GDEPT). Nitroreductase-overproducing *S. typhimurium* strains are extremely sensitive to the mutagenic effects of nitroarenes (Watanabe *et al.*, 1998), thus increasing the detection level for toxicity testing of nitrosubstituted compounds. A unique and novel approach utilizing nitroreductive metabolism in cancer treatment is employed in ADEPT and GDEPT in which a non-toxic prodrug is administered globally and is metabolized to a cytotoxic derivative by an enzyme targeted to the site of tumour either through an antitumour antibody-enzyme conjugate (ADEPT) or through specific expression within tumour cells (GDEPT). A prodrug CB 1954 and its derivatives coupled with a bacterial nitroreductase have demonstrated considerable efficacy in several studies concerned with this therapeutic approach (Knox *et al.*, 1993, Bridgewater *et al.*, 1995, Bailey *et al.*, 1996, McNeish *et al.*, 1998). CB 1954, an otherwise benign agent in humans, can be metabolized to a highly cytotoxic derivative through bacterial nitroreduction and subsequent
coupling to a thioester, such as AcetylCoA, to yield a DNA crosslinking species which results in a 100,000-fold increase in toxicity relative to the parent compound (Knox et al., 1993). The rather benign character of CB 1954 towards human tissues is a result of a low efficiency of bioactivation by the human enzyme NAD(P)H-quinone oxidoreductase, i.e. DT diaphorase, which is about 100 times slower in this regard than its bacterial counterpart (Knox et al., 1992). This difference in bioaffinity towards CB 1954 translates into a more precise compartmentalization of cytotoxicity, allowing higher dosing of the agent with lower side effects. Several other prodrug-enzyme combinations have been proposed for ADEPT and GDEPT including thymidine kinase/gancyclovir, carboxypeptidase/mustard compounds and cytosine deaminase/5-fluorocytosine to name a few with each demonstrating certain advantages and limitations (Nishihara et al., 1998). A potential benefit offered by the nitroreductase/CB 1954 combination is a relatively high degree of “bystander killing” i.e. killing of tumour cells neighbouring those expressing the bioactivating gene (GDEPT), or those where antibody-enzyme complex has localized (ADEPT). Given the rather low yields of target cells expressing the bacterial enzyme that can be attained with the current targeting/transfection methods, this effect is considered highly desirable, making the nitroreductase/CB 1954 combination an attractive candidate for this approach. Current efforts aimed at improving the therapeutic efficiency of this combination concentrate mainly on the design of more potent prodrugs (Friedlos et al., 1997). However, future solutions may include direct modification of activity and substrate specificity of the nitroreductase through genetic alteration. Since modification studies are dependent on thorough understanding of the catalytic mechanism as well as structural and functional features of the enzyme,
characterization of bacterial nitroreductases constitutes an important area of research.

The primary objective of this thesis was to characterize, at both the genetic and biochemical level, the \textit{nfsA} gene and its nitroreductase product. In the first part of the study, the role of the \textit{nfsA} gene in development of bacterial resistance to nitrofuran derivatives was studied in a collection of first- and second-step nitrofurazone-resistant mutants in order to provide genetic evidence for its involvement in nitrofuran resistance as inferred from biochemical data produced by McCalla \textit{et al.} (1978). The second part of the study was concerned with molecular aspects of function of NfsA enzyme. An expression construct of NfsA bearing a N-terminal His tag was constructed to allow for rapid purification of the enzyme, and the properties of NfsA were investigated. These included the $V_{\text{max}}$ and $K_M$ parameters of the reaction as well as the characteristics of the bound flavin cofactor. Random mutagenesis of the expression construct was performed using hydroxylamine to generate amino acid substitutions in the enzyme in an attempt to identify critical residues in NfsA. The mutants were described with respect to nitroreductase activity, characteristics of the FMN cofactor and the level of resistance to nitrofurazone. The results were analyzed in a structural context of the enzyme provided by a computer-generated model of the tertiary and quaternary structure of NfsA.
Chapter 2
2.1 Introduction

Wild-type *Escherichia coli* cells acquire resistance to nitrofurans in a stepwise manner upon exposure to these agents. In the case of nitrofurazone, resistance has been shown to be a two-step phenomenon with the first-step mutants exhibiting roughly threefold resistance and the second-step mutants having six- to sevenfold levels of resistance relative to wild type (McCalla *et al.*, 1970). Acquisition of resistance to nitrofurans and derivatives has been postulated to involve the loss of functional products of the *nfsA* and *nfsB* oxygen-insensitive nitroreductase genes with mutation in *nfsA* being an obligatory first step and removing the major fraction of type I reductase activity thus producing first-step mutants; a subsequent mutation in *nfsB* yields second-step mutants in which type I reductase activity is further decreased. A third nitroreductase component, ascribed to a putative gene, *nfsC*, has been identified in cell extracts of wild-type *E. coli* but no genetic evidence for its existence has been produced and it has not yet been implicated in the development of resistance to nitrofurans and their derivatives.

The involvement of *nfsA* and *nfsB* genes in modulating resistance to nitrofurans has been documented primarily on the basis of biochemical analysis of fractionated cellular extracts from nitrofurazone-resistant mutants (McCalla *et al.*, 1978). Prior to this thesis, no corroborating sequence-based genetic data in support of these findings had been produced. The aim of this study was to provide molecular evidence for the role of the *nfsA* gene in development of resistance to nitrofurans. To achieve this, collections of both first- and second-step nitrofuran-resistant mutants were generated and *nfsA* gene was amplified from the mutants and analyzed by DNA sequencing. Since *nfsB* has been a subject of a similar
analysis being conducted by other workers in the laboratory, it will be possible to test the proposed mechanism for development of resistance to nitrofurans against genetic evidence.

2.2 Methods and Materials

2.2.1 Bacterial strains, media and growth conditions

*E. coli* K-12 strains AB1157, NFR402, NFR502, NFR5021 and SILA1 were provided by D. Bryant, McMaster University, Hamilton, Canada. AB1157 (F\(^{-}\) thr-1 leu-6 thi-1 supE44 lacY1 galK2 ara-14 xyl-4 mtl-1 proA2 his-4 argE3 str-31 tsx-33 λ\(^{+}\) sup37) is the parental strain of nitrofurazone-resistant mutants NFR402, NFR502 and NFR5021. DH5α [supE44 lacU169 (φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1] is a standard laboratory strain used for most plasmid preparations and propagation. INVαF\(^{+}\) [F\(^{-}\) endA1 recA1 hsdR17 (rK\(^{-}\) mK\(^{-}\)) supE44 thi-1 gyrA96 relA1 φ80lacZΔM15 Δ(lacZYA-argF)U169 λ\(^{-}\)] was a part of The Original TA Cloning Kit obtained from Invitrogen. The strain NR3835 [F\(^{-}\) prolacP lacZL8; Δ(prolac) ara thi trpE9777] has previously been used in 5-nitrofuran mutagenesis studies in our laboratory. The plasmid used in this study was pCR®2.1 and was included in The Original TA Cloning Kit (Invitrogen). Bacterial strains were routinely grown at 37 °C with shaking at 225 rpm; growth medium was Luria-Bertani (LB) broth (1 % tryptone, 0.5 % yeast extract, 10 g/L NaCl, pH 7.0) or LB agar (LB medium containing 1.5 % agar) obtained as premixed powder from Canadian Life Technologies (Burlington, Ontario, Canada). The media was supplemented with the following where indicated: kanamycin (50 μg/mL) to maintain the construct pTAufsA, X-Gal (40 μg/mL) to allow for blue/white screening, and furazolidone (1.5 μg/mL) and P-Gal (75 mg/mL) to select for *nfsA*
and lacI mutants, respectively. Furazolidone was a kind gift from D. Bryant, McMaster University. These and all other reagents used in this study were of highest purity commercially available.

2.2.2 Isolation of furazolidone-resistant strains and lacI mutants

Furazolidone-resistant strains (JVxx) were selected previously by other workers in our laboratory (Whiteway et al., 1998). Other furazolidone-resistant mutants as well as lacI mutants were selected in this study from strains AB1157 and NR3835 for the purpose of calculating the mutation frequency and size analysis of the nfsA and lacI genes. Briefly, the selection was done as follows. A fresh colony of AB1157 or NR3835 was inoculated into 6 mL of LB broth and grown for 10-12 hrs. Serial dilutions (10^0-10^-5) were made from the culture and 100 μL aliquots were spread in triplicate on both standard (LB agar) and selective media (LB agar supplemented with 1.5 μg/mL of furazolidone and 75 mg/mL of P-Gal for nfsA and lacI mutants, respectively). The plates were incubated at 37 °C for 16-20 hrs (48 hrs if required for P-Gal plates) and scored for colony counts. The counts from triplicate plates of each dilution were averaged and used to obtain the mutation frequency (average count from selective media/average count from standard media). Mutant colonies were picked from selective media plates and used directly to amplify the gene of interest as described in section 2.2.4.3.
2.2.3 Isolation of bacterial DNA

Bacterial cultures were prepared by inoculating 6 mL of LB from frozen stocks and growing overnight (10-12 hrs). The next day, the culture was divided into 2 mL Eppendorf tubes and the cells were harvested by spinning at 8000 X g for 5 min at 4 °C (Sorvall Refrigerated Centrifuge Model RC-5B, DuPont Instruments). After completely removing the supernatant the residue was resuspended in an equivalent volume of SET buffer (20 % sucrose, 50 mM Tris-HCl, 50 mM EDTA, pH 7.6) to wash the cells, and spun again as above. The supernatant was removed again and the contents were resuspended in 1 mL of SET buffer and placed in the -20 °C freezer for 2-3 min to weaken the cell wall. Once the suspension was warmed up in a 37 °C water bath, 50 μL of freshly prepared lysozyme solution (5 μg/μL) in TE buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA)) and 25 μL of RNAs A in water (10 μg/μL) were added and the mixture was kept on ice for 15 min. Subsequently, 37.5 μL of 10 % SDS (w/v) were added; this was followed by incubation at 37 °C for 4-6 hrs and at the end of this incubation period 75 μL of proteinase K (2 μg/μL) were added along with enough CI (24:1 chloroform : isoamyl alcohol) to bring the volume in the 2 mL Eppendorf tube to about 1.8 mL. The contents were shaken gently and incubated at 37 °C overnight. The next morning, the volume in the tubes was adjusted to about 1.8 mL with CI, the tubes were shaken gently for 5 min and centrifuged in a standard benchtop centrifuge (10,000g; Eppendorf Centrifuge 5412) for 6 min to separate the fractions. Using a wide-mouthed pipette tip, the dense aqueous fraction was transferred into a clean 2 mL Eppendorf tube, and after returning the volume to 1.8 mL with PCI (25 : 24 : 1 phenol : chloroform : isoamyl alcohol), the contents were extracted twice with PCI. The final
aqueous fraction was combined with 2.5-3 X volumes of ice-cold 95 % ethanol and the tubes were incubated at -20 °C for at least 20 min. Next, the contents were gently inverted several times, spun in a benchtop centrifuge for 6 min and the ethanol was gently decanted off. The pellet was rinsed twice with 1 mL of ice-cold 95 % ethanol (v/v) and the tubes were placed in a 50 °C vacuum oven with the caps open for 30 min or until the alcohol was evaporated off completely. Each bacterial DNA pellet was then resuspended in 100 μL of TE buffer and stored at -20 °C.

2.2.4.1 Amplification of the nfsA gene

Amplification of the nfsA gene from genomic DNA of both first- and second-step nitrofurazone-resistant mutants was accomplished by PCR using the Expand High Fidelity PCR System (Boehringer Mannheim Canada). A 100 X dilution of bacterial genomic DNA in TE buffer was prepared and used as the DNA source. Two mixtures were prepared in separate 1.5 mL Eppendorf tubes containing the following amount per single 100 μL PCR reaction: Mix 1 - 8 μL of 2.5 mM dinucleotide phosphate mix (dATP, dGTP, dCTP, dTTP) and 3 μL of each 10 mM primer; Mix 2 - 10 μL of 10 X buffer with MgCl₂, 63.5 μL of sterile ddH₂O and 0.5 μL (1.7 U) of Expand enzyme (added last). A 24 μL aliquot of Mix 1 and 74 μL of Mix 2 were then combined with 2 μL of source DNA in a small PCR tube and placed in the PCR machine (Thermolyne Model Amplitron II) dwelling at 94 °C with hot-top turned on. Subsequently, the amplification cycle was initiated and consisted of a single 3 min pre-dwell at 94 °C, 30 cycles of denaturation (94 °C for 15 sec), annealing (55 °C for 30 sec), elongation (72 °C for 1 min) and, following completion of the
amplification cycles, a single post-dwell at 72 °C for 7 min. Completed PCR reactions were either allowed to remain in the machine at 4 °C overnight or were purified immediately.

2.2.4.2 Purification of PCR reactions

Prior to DNA sequencing analysis, amplified nfsA reactions were subjected to purification using a High Pure PCR Product Purification Kit (Boehringer Mannheim Canada) as follows. A 500 μL aliquot of binding buffer (3 M guanidine-thiocyanate, 10 mM Tris-HCl, 5 % ethanol (v/v), pH 6.6) was mixed with 100 μL of PCR reaction and the mixture was transferred to a High Pure filter column with a wash collector and centrifuged in a benchtop centrifuge for 30 sec. The flow-through was discarded and the column was washed once with 500 μL and then once with 200 μL of wash buffer (20 mM NaCl, 80 % ethanol (v/v), 2 mM Tris-HCl, pH 7.5). DNA was eluted from the column with 50 μL of elution buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.5) and stored at -20 °C. DNA concentration was assessed visually through agarose-gel electrophoresis (0.7-0.8 %) in the presence of ethidium bromide against a standard of known concentration.

2.2.4.3 Amplification of nfsA and lacI genes from bacterial colonies

Colonies from furazolidone or P-Gal selection plates (section 2.2.2) were picked from the plates with sterile toothpicks and each colony was suspended by vortexing in 12 μL of colony suspension solution (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 50 μg/mL of proteinase K) in a 1.5 mL Eppendorf tube. The contents were incubated for 15 min at 55 °C and then 15 min at 80 °C to lyse the cells, and subsequently chilled on ice for 1 min. The tubes were
then vigorously vortexed for 3 min; 1-2 μL of produced lysate were used for DNA amplification.

Amplification of the *nfsA* and *lacI* genes was carried out using the *Taq* DNA Polymerase Kit (Canadian Life Technologies). A master mix was prepared in a single tube for the desired number of reactions and placed on ice. The master mix contained the following per single 100 μL reaction: 10 μL of 10 X PCR buffer minus Mg (200 mM Tris-HCl, pH 8.4, 500 mM KCl), 2 μL of 10 mM dNTP mixture, 3 μL of 50 mM MgCl₂, 3 μL of each 10 μM primer, 73.5 μL of sterile ddH₂O and 0.5 μL (2.5 u) of *Taq* DNA polymerase (added last). A 99 μL aliquot of the mix was then added to each tube containing an appropriate amount of the colony lysate and the amplification was initiated according to the parameters described in section 2.2.3.1.

2.2.5.1 Preparation of electrocompetent cells

One liter of LB broth was inoculated with 6 mL of overnight culture of the appropriate bacterial strain and grown with shaking at 225 rpm at 37°C until the exponential phase of growth was reached (O.D.₆₀₀ = 0.5-0.7). The cells were harvested by spinning at 8000 X g for 15 min at 4°C, the supernatant was decanted off and after an additional spin the supernatant was removed completely by suction. To progressively concentrate the cells, the cells were washed with 1 X, 0.5 X and 0.25 X volumes of 10 % ice-cold glycerol (v/v) and were kept on ice at all times. Each wash cycle consisted of a complete resuspension of the cell pellets in a small volume of 10 % glycerol to facilitate dissolution of the pellets, addition of 10 % glycerol to the desired volume, and centrifugation as above. After the final
wash, the cells were resuspended in 10 % ice-cold glycerol to a total volume of 4 mL and 40 μL aliquots were dispensed into 100 ice-cold 1.5 mL Eppendorf tubes. The cells were then transferred immediately to a -80 °C freezer and stored until used. This protocol was scaled down appropriately as needed by adjusting all the relevant volumes, except the final aliquot.

2.2.5.2 Electroporation

Competent cells (40 μL) were thawed on ice and 0.5-3 μL of DNA (~0.1 μg) were added. After stirring gently with the pipette tip, the contents were transferred to the bottom of an ice-cold electroporation cuvette (0.2 cm) and allowed to incubate on ice for 1 min. The contents were subsequently pulsed at 2.5 KV field strength (E. coli Pulser, Bio-Rad); 960 μL of ice-cold SOC medium (2 % tryptone (w/v), 0.5 % yeast extract (w/v), 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, 20mM glucose) were added immediately and the suspension was shaken at 37 °C for 15-30 min at 150 rpm. Dilutions (10⁶ - 10⁻³) of the culture were made and 100 μL of each dilution were spread on selective media plates and allowed to incubate overnight at 37 °C.

2.2.6 AT cloning

Cloning of the nfsA gene was accomplished with the Original TA Cloning Kit (Invitrogen) as follows. The nfsA gene along with its putative regulatory regions was amplified from the strain AB1157 with the primers Nfs0 and Nfs3 using The Expand High Fidelity PCR System, as described above. The 3' A overhangs necessary for cloning into pCR® 2.1 vector supplied with the cloning kit were added through a 10 min incubation of the
completed PCR product at 72 °C with 1 U of Taq DNA Polymerase (Canadian Life Technologies). The ligation mix was set up in a 1:3 ratio for vector:insert and consisted of 1 µL (25 ng) of the pCR®2.1 vector, 2 µL (20 ng) of the PCR product, 0.5 µL of 10 X ligation buffer for DNA ligase, water up to 4.75 µL, and 0.25 µL (100 U) of T4 DNA ligase. The ligation mixture was incubated at 16 °C overnight, quick-spun in a standard benchtop centrifuge and transformed into INVαF’ cells as follows. A 2 µL aliquot of 0.5 M β-mercaptoethanol was added to the ligation mix and, after gently stirring with the pipette tip, 2 µL of the mix were combined with 50 µL of INVαF’ competent cells completely thawed on ice. The contents were gently stirred with the pipette tip and allowed to incubate on ice for 30 min. Cells were then transferred into a small ice-cold glass test tube and heat-shocked by placing in a 42 °C water bath for 2 min; subsequently 250 µL of SOC medium were added and the contents were shaken at 37 °C for 1 hr at 225 rpm. Transformants were then applied onto selective media plates (LB containing 50 µg/mL of kanamycin and 40 µg/mL of X-Gal) by spreading 200 µL and 50 µL aliquots of the transformation mixture. The plates were incubated at 37 °C for 15-20 hrs and white colonies containing putative pTAnfsA construct were selected for screening. The sequence of the cloned nfsA gene was verified in the final pTAnfsA construct by DNA sequencing.

2.2.7 Preparation of plasmid DNA for analysis

Plasmid DNA was routinely harvested for size screening and sequence analysis using the Wizard™ Plus Miniprep DNA Purification Systems (Promega). An overnight (10-12 hrs) bacterial culture of the strain harboring the plasmid of interest was grown at 37 °C in
60 mL of LB medium supplemented with appropriate antibiotics, divided into two SS-34 centrifugation tubes and harvested by spinning at 8000 X g for 15 min at 4 °C. The supernatant was then decanted off and completely removed by suction, and each pellet was resuspended in 1.2 mL of Cell Resuspension Solution (50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 100 µg/mL RNase A) and divided into 400 µL aliquots in three 2 mL Eppendorf tubes. Into each tube, 400 µL of Cell Lysis Solution (0.2 M NaOH, 1 % SDS) were added and after gently inverting several times, the tubes were allowed to stand at room temperature for 5 min. At the end of this period, 400 µL of Neutralization Solution (1.32 M potassium acetate, pH 4.8) were added to each tube and, after the contents were shaken vigorously several times, the tubes were centrifuged in a benchtop centrifuge for 5 min. During the centrifugation period an apparatus, consisting of a custom suction unit, Wizard™ minicolumns and 3 mL syringe barrels (1 per Eppendorf tube), was assembled and the supernatant from the centrifuged tubes was pipetted into the barrels. Into each barrel, 0.9 mL of the Wizard™ Purification Resin were then ejected vigorously from the pipette to allow mixing, and suction was turned on to effect the passage of the contents through the minicolumn. Once all the resin particulate collected in the minicolumn and liquid passage was complete, the suction was turned off and 2 mL of Column Wash Solution (80 mM potassium acetate, 8.3 mM Tris-HCl, pH 7.5, 40 µM EDTA, 55 % ethanol (v/v)) were added into each syringe barrel and the suction was resumed. The suction was maintained for 30 sec more after the passage of fluid through the column was complete; the columns were detached from the apparatus and centrifuged in a benchtop centrifuge for 30 sec to remove any remaining liquid from the column. Elution of DNA from the column was accomplished
with 50 μL of TE (pH 7.4) and quick-spinning in a benchtop centrifuge. The amount of plasmid DNA (3-4 kb in size) harvested in this manner was usually in the range of 30-50 μg which allowed for complete sequencing of the nfsA gene. In cases where only amounts of plasmid sufficient for size screening were necessary, this protocol was scaled down with the following volume changes: 3 mL of starting bacterial culture, 200 μL of each of Cell Resuspension, Cell Lysis and Neutralization solutions and 20 μL elution volume.

2.2.8 Preparation of single-stranded plasmid templates

Plasmid DNA minipreps were routinely converted to single-stranded form to facilitate DNA sequencing as follows. A desired amount of purified plasmid DNA was brought up to 90 μL with ddH₂O in a 1.5 mL Eppendorf tube and 10 μL of 2 M NaOH were added (final NaOH concentration of 0.2 M). The contents were then incubated for 30 min in a 37 °C water bath. At the end of this incubation period, 11 μL of 3 M sodium acetate (pH 5.0) were added and the contents were vortexed vigorously for 30 sec. DNA precipitation was effected by addition of 350 μL of ice-cold 0.16 M sodium acetate in ethanol and 10 μL of glycogen (2 μg/μL), incubation at -20 °C overnight, and centrifugation at 15,000 X g for 15 min at 4 °C (Eppendorf Centrifuge 5403). Subsequently, the pellet was washed once with 200 μL of ice-cold 70 % ethanol, dried in a centrovap for 5 min and brought up to a desired volume with TE.
2.2.9 DNA sequencing

DNA Sequencing was carried out using the T7 Sequenase DNA Sequencing Kit v.2.0 (Amersham Life Science). The amount of 2-4 µg of DNA prepared as described in sections 2.2.6 and 2.2.7 was used in each single reaction. When sequencing PCR products, each annealing reaction was set up by mixing 7 µL of double-stranded template DNA (2-4 µg), 1 µL of primer (15 pmol) and 2 µL of ddH₂O in separate 1.5 mL Eppendorf tubes. The tubes were then boiled for 5 min, quickly cooled on ice and quick-spun at 10,000 X g at 4 °C to collect the liquid at the bottom of the tubes. The tubes were kept on ice until they were used in the sequencing reaction. In the case of single-stranded templates, the annealing reaction was set up in the same way as above and the contents were incubated at 80 °C for 5 min and slowly cooled down to 30 °C. The sequencing reactions were carried out in microtiter plates containing 2.5 µL of ddATP, ddTTP, ddGTP, ddCTP sequence terminators in separate slots for each reaction, and were pre-warmed to 37 °C. Two master mixes were prepared on ice containing the following per single sequencing reaction - Mix 1: 2 µL of Sequenase Reaction Buffer (5 X: 200 mM Tris-HCl, pH 7.5, 5 mM DTT), 1 µL of 0.1 M DTT, 0.4 µL of 5 X labeling mix (prepared from 40 X stock; at 5 X strength contains 37.5 µM dGTP, dCTP, dTTP), 0.5 µL of [α-³⁵S] dATP (Amersham Life Science) and 1.6 µL of ddH₂O; Mix 2: 1.75 µL of Sequenase Dilution Buffer (10 mM Tris-HCl, pH7.5, 5 mM DTT), 0.25 µL (3.25 U) of T7 Sequenase DNA Polymerase. Next, 5.5 µL of Mix 1 and 2 µL of Mix 2 were placed on the side of each annealing reaction tube and the tubes were briefly spun in a benchtop centrifuge. A 3.5 µL aliquot from each single reaction was transferred to each of the ddNTP-containing slots of the microtiter plate, mixed by gentle
pipetting, and the reactions were allowed to proceed for 5-10 min depending on the intended
run time of the sequencing gel (10 min for runs longer than 4hrs). The reactions were stopped
by addition of 4 μL of Sequenase Stop Solution (0.02 M EDTA, 50 % xylene cyanol (w/v),
50 % bromophenol blue (w/v) in formamide), heated to 75 °C for 3 min to denature the
products and stored at -20 °C until used.

The sequencing gel was prepared by mixing 70 mL of Pre-Fab solution (8 %
acrylamide:bisacrylamide (19:1), 1 X TBE, 8.3 M urea), 280 μL of 10 % ammonium
persulphate (w/v) and 35 μL of TEMED and after stirring it was cast into a glass-plate
assembly consisting of glass plates (31 x 38.5 cm), 0.4 mM spacer set and 28 cm sharktooth
comb (Model S2, BRL Life Technologies). Once polymerization was complete, the bottom
spacer and the comb were removed and top/bottom gel boundaries were washed with ddH₂O
and then with 1 X TBE to remove unpolymerized acrylamide and air bubbles. The comb was
subsequently re-inserted and the gel was pre-heated by running in 1 X TBE at 60 W for 30
min (Model EC 600 power pack, E-C Apparatus Corporation, St. Petersburg, FA) and at the
end of this run the loading slots were washed with 1 X TBE buffer to remove urea deposits.
After thawing, 2.5 μL of the sequencing reactions were loaded onto the gel, and the gel was
run at 60 W in 1 X TBE for 2 - 4.5 hrs as desired and indicated by the dye fronts. At the end
of the run, the buffer reservoirs were drained and the gel was allowed to cool down at room
temperature for 10-15 min followed by additional cooling with a tap water stream for 1-2
min. After the plates were separated, the gel was picked up with a sheet of 3MM
chromatography paper, covered with plastic wrap and dried under suction for 2 hrs (Model
583 Gel Drier, BioRad). BioMax MR film (Eastman Kodak Co., Rochester, NY) was
exposed to the dried gel with the plastic wrapping removed for 16-24 hrs; the film was then developed, fixed and read.

2.3 Results

2.3.1 Genetic analysis of nitrofuran-resistant mutants

DNA sequence analysis was performed on the PCR-amplified products of the *nfsA* gene, including its putative regulatory regions, obtained from both first- and second-step furazolidone-resistant mutants isolated in this study as well as from the nitrofurazone-resistant mutants NFR402, NFR502 and SIL41 isolated previously by McCalla *et al.* (1978). Mutants with designations JVx1 (ex. JVA1) were first-step furazolidone-resistant mutants derived from parent strain AB1157, while mutants named JVx with numerical characters greater than 1 were second-step furazolidone-resistant mutants obtained from a corresponding first-step mutant (ex. JVA2, JVA3). All JV mutant series were isolated recently in our laboratory (Whiteway *et al.*, 1998). Similarly, mutant NFR502 is a second-step nitrofurazone resistant mutant obtained from a first-step predecessor NFR402 by McCalla *et al.* (1978), while SIL41 is a mutant of putative genotype *nfsA*−*nfsB* that exhibits a wild-type level of sensitivity to nitrofurazone; SIL41 was obtained by crossing an Hfr *nfsA* *nfsB* double mutant and AB1157.

DNA sequence analysis of the *nfsA* gene region in the collection of first-step mutants (n=24) revealed the presence of a relatively high number of insertional events (17 cases) within, and upstream of, the *nfsA* gene coding region (Figure 2.1). In each of 17 cases, a single bacterial insertion element was found to be integrated; the participating elements were
FIGURE 2.1. Summary of mutations recovered from the \textit{nfsA} gene. Gene coding and upstream regulatory sequences as well as the amino acid sequence of \textit{NfsA} are shown. Base pair substitutions are indicated in bold and underlined with the change noted above each relevant base. Sites of IS element insertions are shown with arrows including the number of events observed at each site. A frameshift and deletion are also marked with arrows. ‘-35’ and ‘-10’ denote putative promoter sequences; ‘SD’ represents a putative Shine-Dalgarno ribosome-binding sequence. ‘Nfs0’, ‘Nfs12’, and ‘Nfs3’ are primers used in gene amplification and construction of pTAnfsA expression vector.
identified to be IS1, IS30 and IS186 using the GenBank database. The IS1 element was the most abundant of the three with 8 observed cases and its distribution of insertional sites along the amplified region was quite balanced as depicted in Figure 2.1 with some preference for the termini of nfsA gene with 1 event after A\textsuperscript{246} (JVN1), 1 event after G\textsuperscript{236} (JWW1), 2 events after C\textsuperscript{92} (JVA1, JVV1), 1 event after C\textsuperscript{38} (JVA1) 1 event after C\textsuperscript{119} (JWH1), 1 event after G\textsuperscript{560} (JVP1) and 1 event after G\textsuperscript{644} (JVI1). Four of these events fall within the putative regulatory/promoter region. Insertions of the IS1 element were of two types; type I was the integration of the element in its 5'→3' orientation present in mutants JWW1, JVA1, JVV1, JWH1 and JVP1 whereas type II insertion occurred in the 3'→5' direction of the IS1 element and was observed in mutants JVN1, JVA1 and JVI1. All IS1 integrations were accompanied by perfect duplications of the target region which were 8 or 9 residues in length (Table 2.1).

Integration of the IS186 element accounted for 6 cases of insertional mutagenesis. All 6 integrations occurred at the exact same location within the amplified region, that is, after G\textsuperscript{388} and produced the same perfect target duplication in all 6 cases. As with IS1, insertions were of two types; type I was observed in 3 cases (NFR402, JVD1 and JVE1) and type II insertion accounted for the remaining 3 cases (JVAE1, JVAG1, JVL1). The sequence of the inserted IS186 element was shorter by 2 bp (CC) at the 5' terminus of the element in relation to the sequence deposited in the GenBank database. IS30 was observed in the remaining 3 cases of insertions and in each case its integration occurred as type II after C\textsuperscript{675}. Only a small 2 bp target duplication was found in each case and was imperfect in mutant JVG1 and perfect in mutants JVB1 and JVX1 (Table 2.1).
TABLE 2.1. Summary of insertion events recovered from the amplified *nfsA* gene region obtained from the collection of first- and second-step furazolidone-resistant mutants. The sequence of the IS element is indicated in brackets in each case. The sequence of the IS element termini, and the lengths of IS termini shown, allowed identification of the insertion type. Type I insertion is defined as one involving 5'→3' orientation of the IS element with respect to the host sequence, whereas Type II proceeds in the 3'→5' direction. The sequence surrounding the bracketed IS sequence is the observed target duplication of the host *nfsA* base residues.

<table>
<thead>
<tr>
<th>Mutants</th>
<th>Insertion Type</th>
<th>Insertion Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>JWV1-JWV3</td>
<td>IS1 Type I</td>
<td>GGC\text{GTGTTG}^{216} [GGT\text{GAT}...\text{ATTAC}] GGC\text{GTGTTG}</td>
</tr>
<tr>
<td>JVA11-JVA2</td>
<td>IS1 Type I</td>
<td>CCG\text{CTGCG}^{92} [GGT\text{GAT}...\text{ATTAC}] CCG\text{CTGCG}</td>
</tr>
<tr>
<td>JVZ1-JVZ2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>JVH1-JVH4</td>
<td>IS1 Type I</td>
<td>GT\text{CCAGTTTC}^{119} [GGT\text{GAT}...\text{ATTAC}] GT\text{CCAGTTTC}</td>
</tr>
<tr>
<td>JVP1-JVP5</td>
<td>IS1 Type I</td>
<td>G\text{ATAAAGG}^{560} [GGT\text{GAT}...\text{ATTAC}] G\text{ATAAAGG}</td>
</tr>
<tr>
<td>JVN1-JVN3</td>
<td>IS1 Type II</td>
<td>A\text{ATTGCTCT}^{246} [G\text{TTAAT}...\text{ATCACC}] A\text{ATTGCTCT}</td>
</tr>
<tr>
<td>JVA1-JVA6</td>
<td>IS1 Type II</td>
<td>C\text{CATCGCTC}^{18} [G\text{TTAAT}...\text{ATCACC}] C\text{CATCGCTC}</td>
</tr>
<tr>
<td>JVI1-JVI6</td>
<td>IS1 Type II</td>
<td>C\text{CTGGAGCG}^{644} [G\text{TTAAT}...\text{ATCACC}] C\text{CTGGAGCG}</td>
</tr>
<tr>
<td>NFR402, NFR502</td>
<td>IS186 Type I</td>
<td>T\text{ATATATCG}^{118} [C\text{ATAAGCG}...\text{TATGGG}] T\text{ATATATCG}</td>
</tr>
<tr>
<td>NFR5021, JVE1-JVE5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>JVD1-JVD3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>JVAE1-JVAE6</td>
<td>IS186 Type II</td>
<td>T\text{ATATATCG}^{118} [C\text{ATAAGCG}...\text{TATGGG}] T\text{ATATATCG}</td>
</tr>
<tr>
<td>JVAG1-JVAG6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>JVL1-JVL6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>JVG1-JVG3</td>
<td>IS30 Type II</td>
<td>G\text{C}^{675} [T\text{GTAGATTCAATCTGT}...\text{ACCAATTGAATCTACA}] \text{GT}^{b}</td>
</tr>
<tr>
<td>JVB1-JVB4</td>
<td>IS30 Type II</td>
<td>G\text{C}^{675} [T\text{GTAGATTCAATCTGT}...\text{ACCAATTGAATCTACA}] \text{GC}</td>
</tr>
<tr>
<td>JVX1-JVX7</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) the first two 5'-end C's are absent from the terminus of the IS element  
\(^b\) imperfect target duplication is noted
Other mutations recovered from the collection of first-step mutants included base substitutions, a frameshift and a deletion (Table 2.2). Base substitutions occurred in 5 cases, of which 4 resulted in a missense mutation at the amino-acid level and 1 was a nonsense change. A single -1 frameshift was observed in the mutant JVR1 involving a -G event at the position G$^{626-628}$ which effectively alters the remaining 31 amino acid in the coding region and adds an additional 15 residues at the carboxyl terminus of the NfsA protein. The deletion was recovered from the mutant JVQ1 and resulted in removal of 176 bp from the positions A$^{637}$ to A$^{813}$; the mutation resulted in an alteration of the amino acid sequence of NfsA downstream from the residue R$^{209}$ and introduced a new stop codon thus reducing the length of the mutated protein to 220 residues.

DNA sequence analysis of the amplified nfsA region obtained from second-step mutants confirmed the presence of the same mutations observed in the corresponding first-step mutants and no other mutations in the nfsA gene were recovered. The sequence of strain SIL41 was found to be unchanged relative to the parental strain AB1157.

2.3.2 Restoration of nitrofuran sensitivity by cloned NfsA protein

Nitrofuran-resistant mutants are devoid of the major nitroreductase activity represented by NfsA as demonstrated by biochemical studies of cell extracts from first- and second-step mutants (McCalla et al., 1978). To corroborate the involvement of NfsA as a necessary and sufficient factor modulating cellular sensitivity to nitrofurans, plasmid pTAnfsA was constructed by cloning the nfsA gene along with its putative regulatory regions into a TA vector template to create a trans-acting source of the NfsA enzyme (Figure 2.1).
Table 2.2. Non-IS element mutations recovered from the collection of furazolidone-resistant mutants.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Base change(s)</th>
<th>Coding change</th>
</tr>
</thead>
<tbody>
<tr>
<td>JVJ1-JVJ6</td>
<td>$G_{16} \rightarrow T$</td>
<td>$\text{GAA} \rightarrow \text{TAA}$</td>
</tr>
<tr>
<td>JVK1</td>
<td>$C_{43} \rightarrow T$</td>
<td>$\text{CGC} \rightarrow \text{TGC}$</td>
</tr>
<tr>
<td>JVO1-JVO3</td>
<td>$T_{266} \rightarrow A$</td>
<td>$\text{ATC} \rightarrow \text{AAC}$</td>
</tr>
<tr>
<td>JVV1-JVV2</td>
<td>$A_{32} \rightarrow T$</td>
<td>$\text{CAT} \rightarrow \text{CTT}$</td>
</tr>
<tr>
<td>JVC1-JVC3</td>
<td>$A_{201} \rightarrow C$</td>
<td>$\text{CAA} \rightarrow \text{CAC}$</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Base change(s)</th>
<th>Coding change</th>
</tr>
</thead>
<tbody>
<tr>
<td>JVR1-JVR4</td>
<td>$625 \text{CGG GAT A} \rightarrow \text{CGG ATA}$</td>
<td>-1 frameshift</td>
</tr>
<tr>
<td>JVQ1-JVQ4</td>
<td>$635 \text{GGA...AAC}^{815} \rightarrow 635 \text{GGAAC}^{639}$</td>
<td>176 bp deletion</td>
</tr>
</tbody>
</table>
The construct was transformed into JVQ1 and JVQ2 cells and its influence on sensitivity to furazolidone was evaluated. JVQ mutants were selected for this purpose since they harbor a 176 bp deletion within the *nfsA* gene, thus minimizing the probability of reversion to wild-type. Sensitivities to furazolidone were measured in CFU/mL units and compared among JVQ1 and JVQ2 strains that were either transformed with control TA vector or transformed with pTAnfsA.

Introduction of the pTAnfsA construct into both JVQ1 and JVQ2 mutant strains resulted in a dramatic increase in sensitivity to furazolidone in both strains (Table 2.3). This increase was reflected by reduction in viability of strains harboring pTAnfsA construct on selective media as compared to strains devoid of the construct and amounted to 7 and 9 orders of magnitude for JVQ1 and JVQ2, respectively. The survival of the strains harboring the pTAnfsA construct on selective media could not be accurately determined as only residual zones of confluent growth were noted at the lowest dilution plated (10^6) which has been a frequent finding whenever cells sensitive to nitrofurans such as AB1157 have been plated on furazolidone at a maximal cell density i.e. without dilution of the culture. In such cases, it is conceivable that the presence of a high population of cells could be capable of reducing the concentration of the growth inhibitor below the minimum concentration required for inhibition of bacterial growth, thus allowing for persistence of some survivors. Taking this into account, the growth viability in this case was assumed to be near-zero. The control vector pTA only slightly affected viability of the strains on both types of media, and since the presence of pTAnfsA also had a slight reductive effect on viability of JVQ1 and JVQ2 on control media, this reduction was presumably a result of slower cellular growth in
TABLE 2.3. Effect of introduction of vector-encoded NfsA into furazolidone-resistant mutants on sensitivity to furazolidone. Viable counts are measured as CFU/mL. Media used were LB agar; selective media contained 1.5 μg/mL of furazolidone.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Control Media (- furazolidone)</th>
<th>Selective media (+ furazolidone)</th>
</tr>
</thead>
<tbody>
<tr>
<td>JVQ1</td>
<td>$3.2 \times 10^9$</td>
<td>$2.5 \times 10^7$</td>
</tr>
<tr>
<td>JVQ2</td>
<td>$2.7 \times 10^9$</td>
<td>$3.1 \times 10^9$</td>
</tr>
<tr>
<td>JVQ1 (pTA)</td>
<td>$3.7 \times 10^8$</td>
<td>$4.3 \times 10^7$</td>
</tr>
<tr>
<td>JVQ2 (pTA)</td>
<td>$7.0 \times 10^8$</td>
<td>$3.5 \times 10^9$</td>
</tr>
<tr>
<td>JVQ1 (pTAnfsA)</td>
<td>$3.2 \times 10^8$</td>
<td>$\sim 0^a$</td>
</tr>
<tr>
<td>JVQ2 (pTAnfsA)</td>
<td>$1.0 \times 10^9$</td>
<td>$\sim 0^a$</td>
</tr>
</tbody>
</table>

$^a$ Small zones of partial confluent growth were observed on the plates plated at maximal cell density ($10^9$).
the presence of a high-copy vector. An approximately 100-fold difference between the viabilities of untransformed JVQ1 and JVQ2 as well as between JVQ1 (pTA) and JVQ2 (pTA) was evident on selective media, reflecting the difference in sensitivity to nitrofurans between first- and second-step mutants.

2.3.3 Mutation frequency

Mutation frequencies of nfsA and lacI genes were determined in the NR3835 strain of *E. coli* to provide a measure of comparison between nfsA and a well-characterized gene such as lacI. The frequencies were determined as ratios of viable counts obtained on selective media to those observed on control media with furazolidone and P-Gal used as selective agents for nfsA and lacI mutants, respectively. In three independent trials, mutation frequencies were determined to be $6.3 \times 10^{-6}$ ($\pm 0.6 \times 10^{-6}$) and $3.2 \times 10^{-6}$ ($\pm 0.8 \times 10^{-6}$) for nfsA and lacI, respectively. In addition, to compare the mutation variation with respect to gene size between the obtained nfsA and lacI mutants, 15 colonies of each type of mutant were selected, denatured and used as a source of DNA for PCR amplification of the relevant genes. As depicted in Figure 2.2, the nfsA gene was increased in size in 7 of the 15 selected nfsA mutants, and most likely harbor insertion element IS1 in 4 cases (HB1, HC1, HD1 and HK1) and IS186 in 3 cases (LA1, LD1 and HJ1) as predicted from the size of the amplified fragments. In contrast, all of the amplified lacI genes from the colonies of the lacI mutants retained their wild-type size (three colonies did not amplify any DNA).

Mutation frequency in nfsA was also determined using the cloned nfsA gene residing on the construct pTAnfsA. The fact that pTAnfsA is present in multiple copies within the
FIGURE 2.2. Comparison of \( nfsA \) and \( lacI \) gene size variation between furazolidone-resistant and P-Gal-utilizing mutant NR3835 cells. Furazolidone-resistant colonies were selected on a medium containing furazolidone, whereas P-Gal-utilizing mutants were selected on a medium containing P-Gal as the sole nutrient source. \( nfsA \) and \( lacI \) genes were subsequently amplified from furazolidone-resistant colonies (LA1-HL1) and P-Gal-utilizing colonies (PG1-PG15), respectively, using primer pairs Nfs12-Nfs3 for \( nfsA \) (Figure 2.1) and LacIS-PL for \( lacI \) (Appendix I), and the products were separated on a 0.7% agarose gel containing ethidium bromide. Fragment 2.2 kb in size corresponds to the \( nfsA \) gene harboring a putative \( IS186 \) element; fragment 1.6 kb indicates \( nfsA \) gene containing a putative \( IS1 \) element. Fragments 0.8 and 1.2 kb correspond to predicted wild-type sizes for \( nfsA \) and \( lacI \), respectively. No product was observed for PG11-PG13. (+) - positive PCR control consisting of \( nfsA \) product amplified from wild-type NR3835; (-) - negative PCR control using no DNA source for amplification; L - linear DNA size standard composed of the following fragments: 0.5 kb (marked), 1.0, 1.6, 2.0, 3.0, 4.1, 5.1, 6.1, 7.1 kb and higher (not resolved).
host cell, raises the problem of genetic differences between the copies within the same cell. Wild type nfsA gene would exert a dominant effect in such circumstances and therefore it was necessary to harvest the plasmid and retransform the host to ensure the presence of a single type of vector progeny which could be analyzed. For this purpose, pTAnfsA was isolated from JVQ1 cells and transformed back into JVQ1 competent cells which were then applied onto selective media. Very few resistant colonies (80) were recovered in the selection resulting in a calculated mutation rate of plasmid-resident nfsA of $1.5 \times 10^{-6}$. Size analysis of the nfsA PCR product amplified from 21 of the mutants indicated that only 1 of them harbored an insertion (isolate 6), one (isolate 9) carried a putative deletion, whereas the remaining 19 retained their wild type size (Figure 2.3).

2.4 Discussion

In this study, the role of the nfsA gene in development of resistance to nitrofurans was investigated through sequence analysis of the gene in a collection of nitrofuran-resistant mutants. It was observed that all of the 24 first-step isolates harbored mutations within the nfsA gene which persisted in the corresponding second-step progeny. This is in accordance with the proposed mechanism for development of nitrofuran resistance in which a mutation in nfsA is an obligatory event producing first-step nitrofuran-resistant mutants, and mutation in nfsB yields mutants exhibiting second-step levels of resistance. In a parallel study concerned with the nfsB gene, mutations within the gene were analyzed in the same collection of first- and second-step mutants and revealed the absence of any mutations within nfsB in first-step mutants and the presence of nfsB mutations in 52 of 55 second-step isolates.
FIGURE 2.3. PCR-amplification of the $nfsA$ gene from 21 furazolidone-resistant JVQ1(pTAnfsA) mutants. Primers Nfs0 and Nfs3 were used to amplify plasmid-borne $nfsA$, since the Nfs3 primer site is deleted on the chromosome of JVQ1, and the products were separated on a 0.7% agarose gel containing ethidium bromide. Fragment 1.1 kb in size corresponds to the wild-type $nfsA$ product; fragment 2.6 kb in size indicates $nfsA$ carrying a putative insertion element. (+) - positive PCR control consisting of $nfsA$ product amplified from wild-type NR3835; (-) - negative PCR control using no DNA source for amplification; L - linear DNA size standard composed of the following fragments: 0.5 kb (marked), 1.0, 1.6, 2.0, 3.0, 4.1, 5.1, 6.1, 7.1 kb and higher (not resolved).
(Whiteway et al., 1998). In three second-step mutants no mutations were recovered from either the \textit{nfsB} gene, or the suspected \textit{nfsB} regulatory sequences, suggesting that additional factors are involved in modulation of resistance to nitrofurans. These factors may include involvement of enzymes other than NfsA and NfsB, changes in putative trans acting elements responsible for full nitroreductase activities, or alteration in cellular permeability to exogenous nitrofurans. These studies provide convincing evidence in support of the mechanism for development of resistance to nitrofurans proposed by McCalla et al. (1978). Further support for the role of NfsA and NfsB enzymes in mediation of sensitivity to nitrofurans is gained by restoration of nitrofuran-sensitivity in first- and second-step mutants by cloned \textit{nfsA} and \textit{nfsB} genes. As demonstrated in this study, introduction of a high-copy construct harboring cloned \textit{nfsA} into JVQ1 and JVQ2 strains abolished furazolidone-resistance of the mutant strains indicating that the extrachromosomal source of NfsA is sufficient to generate toxic products with lethal consequences to the cell. In a similar experiment involving comparison of diameters of bacterial growth zones around a nitrofurazone impregnated filter paper disc, JVQ1 and JVQ2 and AB1157 strains were transformed with constructs carrying either \textit{nfsA} or \textit{nfsB} genes. It was noted that similar levels of sensitivity were obtained in the strains transformed with either construct, yet in the cases of JVQ1 and JVQ2 harboring the constructs sensitivity levels to nitrofurazone were substantially higher than those observed with the control vector devoid of either gene (Whiteway et al., 1998). The lack of significant difference between \textit{nfsA} and \textit{nfsB} constructs is somewhat unexpected in this case, given that nitroreductase activity due to NfsA protein represents roughly 80\% of the total cellular type I nitroreductase activity, in contrast to that
due to NfsB alone amounting to about 20% (Bryant et al., 1981). However, the presence of multiple gene copies might result in a maximum level of nitroreductase activity attainable and thus maximum sensitivity to the drug. Also, since radial diffusion of substances produces a non-linear concentration gradient, the activity difference between NfsA and NfsB might not be resolvable in this assay.

The original nitrofurazone-resistant mutants, NFR402, NFR502, NFR5021 isolated and described previously (McCalla et al., 1978; Bryant et al., 1981) were also included in this study and were shown to have the same pattern of genetic changes with respect to the nfsA and nfsB genes as other first- and second-step mutants analyzed (Whiteway et al., 1998). SIL41 is a strain whose response to nitrofurazone is distinct from all the other mutants described, since it exhibits wild-type levels of resistance, but can attain levels of resistance to nitrofurazone characteristic of second-step mutants in a single step. Sequencing analysis of nfsA in this mutant revealed no mutations within the nfsA; however, an amber mutation was observed in nfsB (Whiteway et al., 1998) indicating that mutations in nfsB without changes in nfsA are phenotypically silent and confirm the obligatory nature of mutations in nfsA as the first step in generation of nitrofuran resistance.

The general phenomenon of nitrofuran resistance is not of uniform nature among the members of this drug class. Nitrofurazone-resistant mutants attain resistance to a maximum of sevenfold in two steps, with third-step mutants spontaneously reverting to second-step levels of resistance, whereas nitrofurantoin-resistant mutants can achieve a much higher degree of resistance to nitrofurantoin up to 25-fold in four steps (Breeze and Obaseiki-Ebor, 1983). Cross-resistance between nitrofurantoin- and nitrofurazone-resistant mutants is also
observed, yet the resistance levels to nitrofurantoin tend to be higher than those to nitrofurazone. It is therefore likely that nitroreductases other than NfsA and NfsB may participate in metabolizing different classes of nitrofurans and the observed phenomenon of sensitivity/resistance might be a function of differential involvement of enzymes capable of nitroreduction, depending on the type of nitrofuran and the enzyme's specificity for it, with distinct metabolic pathways leading to toxic products and deleterious effects in each case. The cell's ability to counteract this damage through, for example, DNA repair pathways may also be an important determinant of cellular drug resistance or tolerance. NfsA and NfsB belong to a family of NAD(P)H-flavin oxidoreductases based on sequence similarities to other enzymes (Zenno et al., 1996a, Zenno et al., 1996b). Both enzymes have a wide substrate specificity that includes chemicals other than nitrosubstituted ones; other enzymes of this type might also be able to mediate the reductive metabolism of the nitro group given that it is a facile electron acceptor. At the present, no other NAD(P)H-flavin oxidoreductases capable of nitroreduction have been identified in E. coli.

Interestingly, nfsA gene had been cloned and partially described before it was identified as a nitroreductase. The mdaA gene was identified with another gene, mdaB, from an E. coli library as both genes imparted resistance to a tumoricidal agent DMP 840 when overexpressed separately in an E. coli host (Chatterjee and Sternberg, 1995). Moreover, cloned mdaA was able to confer adriamycin resistance on the E. coli host, while cloned mdaB imparted resistance to adriamycin and etoposide, both of which are known topoisomerase inhibitors. The fact that nfsB was not identified from the clonal library to have effects similar to nfsA/mdaA is supportive of the above claim concerning differential involvement of
enzymes depending on their variable specificities for a given substrate. In other terms, although enzymatic activities of NfsA and NfsB converge with respect to reduction of a variety of compounds including nitrofurazone, this similarity in substrate preference might not extend to metabolism of other substrates. The authors of the study suggested that the proximity of mdaA to the grx gene, encoding glutaredoxin, which is suspected of being involved in drug detoxification pathways, might implicate mdaA in a similar pathway (Chatterjee and Sternberg, 1995). The mdaB gene, on the other hand, was proposed to be associated with a modulating action of topoisomerase IV, rather than metabolic detoxification, based on its genetic location. MdaB does demonstrate some sequence similarity to several known mammalian and bacterial NAD(P)H-oxidoreductases/dehydrogenases. This similarity, however, is not sufficient to allow for the placement of MdaB in this enzyme class, and therefore its physiological function remains speculative at this time.

NfsA/MdaA has been recently implicated to participate in a multifaceted cellular response to oxidative stress that is regulated by the soxRS regulon in E. coli (Liochev et al., 1999a). Exposure to paraquat, a redox cycling agent capable of mediating univalent reduction of dioxygen, resulted in a threefold induction of nitroreductase activity in AB1157, but this increase was absent from strains JVQ1 and JVQ2 which contain a deletion within nfsA indicating that the induced activity originated from nfsA. The oxidative stress has been shown to be sensed by the soxRS regulon indirectly or directly through an elevated concentration of O$_2^\cdot$ free radical, which is a common product of oxidative metabolism (Liochev et al., 1999b). A possible role for nfsA in the cellular defence mechanism against
$O_2^-$ is divalent reduction of nitro and perhaps other compounds to make them unavailable for redox cycling and production of damaging $O_2^-$ radicals. Other inducible members of the soxRS regulon include MnSOD to scavenge $O_2^-$ radicals, glucose-6-phosphate dehydrogenase to supply NADPH, fumarase C to replace $O_2^-$ sensitive fumarases A and B, endonuclease IV to participate in the repair of oxidized DNA and at least three other identified gene products. Functional association of nfsA with the other members of the soxRS regulon response is a first documented clue providing insight into physiological function of nfsA.

The mutation spectrum obtained from first-step furazolidone- and nitrofurazone-resistant mutants contains a rather large proportion (71%) of insertion events in which bacterial elements IS1, IS30 and IS186 were found to participate. IS1 was found in 8 distinct sites along nfsA, 4 of which fell within the putative regulatory region. As shown in Figure 2.1, the putative -35 and -10 promoter boxes identified on the basis of sequence consensus (Zenno et al., 1996b) are separated by an unusually long leader sequence from the beginning of the coding region. The significance of this leader region is unknown at present, but in light of demonstrated inducibility of nfsA mediated by soxRS it might contain binding sites for regulatory elements required for participation of nfsA in an oxidative stress response. It is also necessary for proper expression of nfsA, since disruption of this region by IS elements produced first-step mutants confirming loss of functional NfsA. In addition, there does not appear to be any cryptic promoter sites downstream from the putative -10 box sufficient for wild-type levels of expression of the gene as demonstrated by two cases of IS1 insertion between the -35 and -10 boxes (JVN1, JVV1). The distribution of IS1 insertion sites was observed to be quite balanced within the gene and its regulatory region, although a preference
for nfsA termini may be argued since no insertions were recovered within the middle of the coding region. The sequences within and surrounding the IS1 insertion sites were unremarkable in context which supports previous observations that IS1 lacks a specific target sequence (Mahillon and Chandler, 1998). In addition, the insertion sites did not exhibit base content preference; some preference for AT-rich sequences has been observed in other studies (Mahillon and Chandler, 1998). There was also no clear sequence preference for directionality of insertion or the size of target duplication which is also in agreement with previous findings suggesting that the latter two events may occur at random.

In contrast to IS1, IS186 demonstrated clear preference for insertion at a single site, following G$_{388}$, within the nfsA coding region. The region surrounding this hot-spot is relatively GC rich which supports the observations that IS186 integrates preferentially into G+C-rich loci (Sengstag et al., 1986). Insertions of IS186 were found in a 1:1 ratio with respect to orientation of the element and produced identical target duplications in both cases, suggesting that orientation of the insertion is independent of the target sequence.

The last IS element encountered in the mutation spectrum, IS30, accounted for 3 cases of insertional mutagenesis and occurred at the same site following C$_{675}$ in a 3' to 5' orientation, producing a short target duplication (GC) which was found to be imperfect in one case. This target duplication is an interesting characteristic of IS30 as it has been found in all characterized IS30 insertions, and has been shown to occur with high fidelity with respect to the length and sequence identity of the duplication (Olasz et al., 1993). In one IS30 insertion recovered in this study, the target has not been perfectly repeated and a change from GC to GT was observed. Such infidelity in replication of the direct repeat is quite infrequent,
being observed in <5 % of IS30 insertions studied and the imperfect repeat may possibly originate from the transposition intermediate (Olasz et al., 1998). Orientation of the IS30 insertions has been reported to be sequence-independent, since remarkable bias with respect to direction exists between multiple insertions characterized at the same perfectly symmetric target site which may be the case for other insertion elements, as suggested above for IS/ and IS/86.

IS30 shows a pronounced specificity for the insertion site at an E. coli consensus sequence corresponding to TAAAAAWGGCn(RY)CGCnWTITTTA, where (RY) marks the insertion site and the identity of the target duplication, R is A or G, Y is T or C, W is A or T and n is undetermined (Olasz et al., 1998). The site of insertion of IS30 obtained in this study TAAAAGAAAGCC(GC)CCATTTATTCT contains 7 mismatches from the 24 bp long nearly-palindromic consensus sequence. As Olasz and colleagues remark, deviations from the consensus sequence occur, since a highly conserved 24 bp-long sequence would be a rare occurrence in the bacterial genome, but changes to certain bases within the consensus sequence are “prohibited” and greatly decrease the chance for transposition (Olasz et al., 1998). In the IS30 insertions recovered in this study, none of the mismatched bases within the 24 bp-long insertion locus correspond to “prohibited” bases in the IS30 consensus insertion sequence providing sound support for the nature of this site.

The mutation spectrum of nfsB gene obtained from the same second-step mutants used in this study contains an equally impressive proportion of cases of insertional mutagenesis (52 %) as the one obtained for nfsA (Whiteway et al., 1998). The elements identified within nfsB were IS1, IS2 and IS5. A hot-spot for integration of IS5 was clearly
evident in the sequence of \( nfsB \) as 10 insertion events at this locus were noted. \( IS I \) also displayed preference for insertion sites closer to the termini of the \( nfsB \) gene, as was observed with \( nfsA \). This preference might be due to the selective nature of the \( IS I \) insertion process for transcriptional state of target genes in which chromosomal changes such as unwinding and annealing may temporally and/or structurally enhance the attractiveness of the gene termini as targets for transposition. This hypothesis is supported by observations that local DNA structures such as the degree of supercoiling, bent DNA, replication and transcription as well as protein-mediated targeting have all been proposed as parameters which influence target choice (Mahillon and Chandler, 1998). The extent to which insertion sequences populated both \( nfsA \) and \( nfsB \) genes in these studies is unexpectedly high; this high transposition frequency observed might be an intrinsic characteristic of the parental strain AB1157. To test this hypothesis, several furazolidone- and P-Gal-utilizing mutants were selected from another \( E. coli \) K12 strain, NR3835, in which the contribution of insertional events to mutagenesis in the mutation spectrum of \( lacI \) gene was known to be much less significant, amounting to less than 10 % (Schaaper et al., 1986; Halliday and Glickman, 1991) and the sizes of \( nfsA \) and \( lacI \) amplified genes were analyzed. A proportion of insertions recovered from amplified \( nfsA \) products (47 %) was somewhat lower than that obtained for AB1157, yet sufficiently high to demonstrate that this phenomenon was independent of the bacterial strain used. Although no insertions were evident in amplified \( lacI \) gene from NR3835, the attractiveness of \( nfsA \) and \( lacI \) genes as targets for transposition were not necessarily comparable, since the \( lacI \) gene in this strain is resident on an F' episome while \( nfsA \) is chromosomal. To examine the influence of chromosomal location,
nfsA mutants were selected on a plasmid. The rather interesting observation that the proportion of insertions recovered from a cloned copy of nfsA decreases to less than 5% places cloned nfsA in rank with lacI in insertion frequency and is perhaps not surprising given the influence of local chromosomal factors in promoting transposition.

Another factor potentially contributing to increased frequency of transposition within nfsA and nfsB genes may be proximity to loci naturally occupied by insertion elements, since their distribution is often nonrandom as shown for IS1 (Boyd and Hartl, 1997) and is believed to result from local hopping of replicated progeny to proximal locations. This effect is usually observed within approximately 10 kb (0.2 min) of parental elements and results in local clustering of IS elements. It may be noted, however, that no IS elements have been mapped within 10 kb of nfsA (Blattner et al., 1997). The influence of the local DNA sequence context and structure is therefore more likely to be responsible for the high rate of transposition observed for chromosomal nfsA and nfsB. This high transposition rate is most likely the reason for an increase in mutation frequency of nfsA ($6.3 \times 10^{-6}$) relative to that observed for lacI ($3.2 \times 10^{-6}$) with the latter remaining in good agreement with an independent determination of $3.4 \times 10^{-6}$ (Schaaper and Dunn, 1987). A 4-fold reduction in mutation rate observed for cloned nfsA ($1.5 \times 10^{-6}$) relative to the chromosomal locus underscores the importance of target location for transposition. Alternatively, this value may be underestimated as a result of the low number of colonies obtained which was due to a limitation in transformation efficiency.

Several missense mutations within the nfsA gene were recovered from the collection of nitrofurazone-resistant mutants which are of value in investigation of the NfsA catalytic
cycle. These mutations will be discussed in the context of a molecular model of NfsA in the subsequent chapter.
Chapter 3
3.1 Introduction

The major nitroreductase from *E. coli*, NfsA, shares significant sequence similarity with several identified NAD(P)H oxidoreductases (Figure 1.2). NfsA also shares some of the biochemical properties exhibited by members of this family, suggesting that it may also function in a broader sense as NAD(P)H oxidoreductase. In particular, the size, kinetic profile, cofactor characteristics as well as electron acceptor specificity of NfsA correspond closely to that of Frp, the major NADPH oxidoreductase from *Vibrio harveyi*, Frp (Zenno *et al.*, 1996 b). One of the more notable differences between Frp and NfsA is their flavin reductase activity which is 10 times higher in the former. This activity can be artificially engineered, however, in NfsA to levels even higher than those observed in Frp by a single amino acid substitution involving the change of Glu 99→Gly which is thought to relax the active site of NfsA allowing access to larger molecules like FMN (Zenno *et al.*, 1998). Such modification of functionality through relatively minor changes underscores the closeness of evolutionary relationships between the two enzymes and provides a rational basis for their comparison at the molecular level.

Examination of the sequence alignments of NfsA, Frp and a third well-characterized enzyme which shares significant sequence similarity to NfsA and Frp, NADH oxidase from *Thermus thermophilus* (Nox), indicates conservation of several regions, as well as single amino acid residues in the alignment (Figure 1.2). A conserved motif near the N-terminus of the proteins [(H)RSIR] merits particular attention as it has been found to be modified in several spontaneous NfsA mutants recovered in the previous part of this study as outlined in Chapter 2. Crystal structures of both Frp and Nox have been solved (Tanner *et al.*, 1996;
Hecht et al., 1995); in addition, a co-crystal structure of Frp containing its inhibitor, NAD, is available (Tanner et al., 1998). These structures provide for a conceptual framework for analysis of NfsA enzyme at the molecular level and make examination of NfsA mutations possible in a structural context as well as inferences to functionality to be made. Indeed, the importance of the motif (H)RSIR is reflected in the structures of both Frp and Nox as some of the residues present in the motif participate in binding the flavin cofactor through hydrogen bonding. Since the crystal structure of NfsA has not yet been solved, similar analysis of structural features between it and other NAD(P)H oxidoreductases can only be addressed through a model of NfsA.

The objective of this chapter was to construct an expression system of NfsA which would allow for overexpression of the enzyme and its purification in amounts sufficient for biochemical analysis of the nitroreductase activity, and determination of cofactor characteristics. Random mutagenesis was used to generate NfsA mutants in an attempt to identify critical residues in NfsA function and analyze them in a structural context. As an aid in interpretation of the results, a computer model of NfsA was constructed using currently available structural templates.
3.2 Methods And Materials

3.2.1 Bacterial strains, media and growth conditions

M15[pREP4] (Nαβ Strr rifr lac' ara' gal' met' F' recA' uvr' pREP4) is a derivative of E. coli K12 harboring a repressor plasmid pREP4 which provides for high levels of lacI repressor and was provided as part of QIAexpress System (QIAGEN Inc., USA). JM103 (F' traD36 lacIΔ(lacZ)M15 proA' B' endA1 supE sbcBC thi-1 rpsL (Str') Δ(lac-pro) (P1) (rK- mK- rPl- mPl-)) is a strain commonly used for expression of lacI-controlled systems as it carries a mutated lacI promoter (upregulated) and attains levels of lacI repressor up to 10-fold higher than the wild-type. Bacterial strains were routinely grown at 37 °C with shaking at 225 rpm in Luria-Bertani (LB) broth (1 % tryptone, 0.5 % yeast extract, 10 g/L NaCl, pH 7.0) or on LB agar (LB medium containing 1.5 % agar) obtained as premixed powder from Canadian Life Technologies (Burlington, Ontario, Canada). The media were supplemented with the following where indicated: kanamycin (25 μg/mL) to maintain pREP4, ampicillin (100 μg/mL) to maintain pQE-derived nfsA construct, furazolidone (4 μg/mL) to select for nfsA mutants and IPTG (10 μM in solid and 2 mM in liquid media) to induce expression of NfsA.

3.2.2 Preparation of His-tag nfsA construct

An expression vector harboring nfsA was constructed using the QIAexpress System (QIAGEN Inc., USA) to allow for modification and subsequent expression and purification of recombinant NfsA. The nfsA gene was prepared by PCR amplification from the strain AB1157 with the primer pair Nfs13-Nfs3, containing restriction enzyme sites for KpnI; the
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concentration of 100 U/mL). The tube was incubated in a 37 °C water bath for 14 hrs and at the end of this period, the labeling reaction was stopped by addition of 2 μL of EDTA.

To purify the probe, 1 μL of 20 μg/μL glycogen solution was added into the labeling reaction, followed by 2 μL of 4 M LiCl and 70 μL of ice-cold ethanol. The contents were mixed by inversion, precipitated at -20 °C for 20 hrs, collected by centrifugation and dried as outlined in section 2.2.8. The final pellet was resuspended in 50 μL of TE (pH 7.4) and stored at -20 °C. The amount of labeled probe in the labeling reaction was quantified by comparing the signal from serial dilutions of the probe against that of a control of known concentration in the following manner. A DIG-labeled control DNA provided with the System was prediluted by mixing 5 μL of the control with 20 μL of DNA dilution buffer to a final concentration of 1 ng/μL. A series of dilutions (1:10 to 1:100,000), containing 100 pg/μL, 10 pg/μL, 1 pg/μL, 0.1 pg/μL and 0.01 pg/μL, respectively, was then prepared for the prediluted control DNA along with 1:10, 1:100, 1:1000, 1:10,000 and 1:100,000 serial dilutions of the labeled probe. A 1 μL aliquot of each dilution of the control DNA and DIG-labeled probe was subsequently applied to a nitrocellulose membrane in separate rows and the membrane was fixed by exposure to UV light for 15 min on each side.

To detect the signal from DIG-labeled DNA, the membrane was washed briefly (1 min) in washing buffer (100 mM maleic acid, 150 mM NaCl (pH 7.5), 0.3% (v/v) Tween ® 20) and incubated at room temperature in blocking solution (1% (w/v) Blocking Reagent in 100 mM maleic acid, 150 mM NaCl; pH 7.5) for 45 min with occasional agitation. The membrane was subsequently transferred into blocking solution containing diluted Anti-DIG-Alkaline Phosphatase 1:5000 and allowed to incubate for 30 min at room temperature with
occasional swirling. After the antibody solution was poured off, the membrane was washed twice in washing buffer (15 min per wash) and allowed to equilibrate for 2 min in detection buffer (100 mM Tris-HCl, 100 mM NaCl; pH 9.5). To apply the chemiluminescent substrate, the membrane was placed between two sheets of acetate, and 0.5 mL of CSPD® (Boehringer Mannheim, Germany) diluted 1:100 in detection buffer was added on top of the membrane. The upper sheet was lowered and any excess liquid was removed by slightly wiping the top sheet with a damp tissue. After 5 min of incubation at room temperature, the sheets were sealed around the membrane and the membrane was transferred into a 37 °C oven for 15 min to speed up the chemiluminescent reaction. The signal was visualized by exposing the membrane to BioMax MR film (Eastman Kodak Co., Rochester, NY) for amounts of time sufficient to produce satisfactory signal (1 min to 2 hrs). The amount of the DIG-labeled probe obtained in the labeling reaction was quantified by comparing signal intensities from the serial dilutions of the probe against those of the control DNA. Prior to use, the probe was denatured by boiling for 5 min and quick-chilled on ice.

3.2.3.2 Transformant screening

The screening of His-Tag nfxA transformants was accomplished by colony hybridization using the DIG-labeled nfxA probe prepared as outlined in the previous section. Transformant colonies were picked from the transformation plates and transferred using sterile toothpicks onto duplicate gridded media supplemented with ampicillin (50 colonies/plate). The plates were incubated at 37 °C for 20 hrs and subsequently cooled by placing in a 4 °C chamber for 30 min. Colonies were lifted from the plates onto
nitrocellulose membranes by placing the membrane on top of the colony plate for 1 min and gently removing it. The membrane discs were subsequently blotted briefly on dry Whatman 3MM paper and placed sequentially on filters soaked with denaturation solution (0.5 M NaOH, 1.5 M NaCl) for 15 min, neutralization solution (1.0 M Tris-HCl, pH 7.5; 1.5 M NaCl) for 15 min and 2 X SSC (0.3 mM NaCl, 30 mM sodium citrate, pH 7.0) for 10 min with brief blotting on Whatman 3MM paper between the soakings. The membranes were then dried in a 37 °C oven for 2-5 min and the transferred DNA was crosslinked to the membrane by exposing it to UV light for 15 min on each side. To remove the cellular debris after crosslinking, 0.5 mL of 2 mg/mL of proteinase K solution (Boheringer Mannheim, Germany) was distributed evenly on top of each membrane, the membranes were placed in a 37 °C oven for 1 hr and subsequently blotted repeatedly between two layers of Whatman 3MM paper soaked with ddH₂O. Pre-hybridization was carried out in plastic Corex tubes (1 per membrane) in 15 mL of prehybridization solution (5 X SSC, 0.1 % N-lauroylsarcosine, 0.02 % SDS and 1 % Blocking Reagent) in a 68 °C oven (VWR Scientific, Model 2710) for 1 hr. Afterwards, prehybridization solution was replaced with 15 mL of hybridization solution (prehybridization solution containing 15 ng/mL denatured DIG-labeled nfxA probe) prewarmed to 68 °C, and the membranes were allowed to hybridize overnight at 68 °C. The hybridization solution was stored at -20 °C and heated to 95 °C for 10 min before reuse. Prior to chemiluminescent detection, the membranes were washed twice in 2 X SSC, 0.1 % SDS for 5 min at room temperature, and twice in 0.5 X SSC, 0.1 % SDS for 15 min at 68 °C with gentle agitation. Detection was performed as outlined in the previous section with the exception that Anti-DIG-Alkaline Phosphatase was used at 1:10,000 dilution.
Putative His-Tag nfsA construct-bearing clones identified from the X-ray film were located on the starting gridded master plates and plasmid preparations were made as outlined in section 2.2.6. Size analysis of the isolates on a 0.7 % agarose gel followed by DNA sequencing analysis of the nfsA region were performed as described in sections 2.2.8-2.2.9.

3.2.4.1 Expression of NfsA

A 50 mL aliquot of LB broth supplemented with 100 μg/mL of ampicillin, and 25 μg/mL of kanamycin to maintain pREP4 as necessary, was inoculated 1:50 (1 mL) with an overnight culture of the strain harboring nfsA expression construct. The culture was grown at 37 °C with shaking at 225 RPM to an O.D.₆₀₀ of 0.5-0.6, at which time IPTG was added to a final concentration of 2 mM, and the growth was continued for an additional 4 hrs under the same conditions. When desired, an uninduced control was prepared in the similar manner without IPTG and was subjected to the same downstream manipulations as the induced culture. At the end of this growth period, the culture was spun at 8000 X g at 4 °C to collect the cells (Sorvall Refrigerated Centrifuge Model RC-5B, DuPont Instruments), and the supernatant was removed by decanting and suction. The pellet was resuspended in 2-3 X volumes of Sonication Buffer (20 mM Tris-HCl, 10 mM imidazole, 300 mM NaCl, 20 % glycerol) per volume of the pellet, transferred to a 1.5 mL Eppendorf tube and placed on ice. The cells were sonicated in 5-6 cycles consisting of 30 sec bursts and 30 sec coolings at 200-300 Watt using a Micro-Ultrasonic Cell Disrupter (Kontes). A small aliquot of the produced lysate was transferred into a fresh 1.5 mL Eppendorf tube and retained for analysis (crude cell extract). The remaining lysate was subsequently centrifuged at 15,000 X g for 20 min
at 4 °C (Eppendorf Centrifuge 5403) to separate soluble and insoluble fractions and the supernatant was transferred into a fresh 1.5 mL tube and placed on ice. The pellet remaining in the tube was resuspended in 100-200 μL of Sonication Buffer and was optionally analyzed as “insoluble” fraction.

3.2.4.2. Purification of NfsA on Ni-NTA resin

A 200 μL aliquot of a 50 % slurry of Ni-NTA resin in ethanol (QIAGEN Inc., USA) per sample was equilibrated in Sonication Buffer in three cycles of resuspension and quick-spinning in a standard benchtop centrifuge (10,000 X g; Eppendorf Centrifuge 5412) and the final 200 μL were combined with the supernatant from the soluble fraction obtained in the previous section and placed on ice. The contents were inverted and gently rolled over a period of 40 min to allow for binding of the resin to the protein. The purification of NfsA was carried out in a 4 °C cold-room using solutions pre-chilled to ambient temperature and was initiated by loading the resin-supernatant mix onto a PAGE purification column (BioRad, CA) previously equilibrated with the Sonication Buffer. The eluate was allowed to pass through the column by gravity and the column containing the resin was washed twice with 4 mL portions of Wash Buffer (20 mM Tris-HCl, 20 mM imidazole, 300 mM NaCl, 20 % glycerol). Elution of purified recombinant NfsA was effected by washing the column with four 0.5 mL aliquots of Elution Buffer (20 mM Tris-HCl, 250 mM imidazole, 300 mM NaCl, 20 % glycerol); the fractions were collected and analyzed immediately or stored at -20 °C until used.
3.2.5 SDS-PAGE analysis

The extracts were analyzed on a 12.5 % acrylamide gel using Mini-Protean II protein electrophoresis system. Resolving gel mix consisting of 4.15 mL of acrylamide : bisacrylamide (30:0.8), 1.25 mL of resolving gel buffer (3.0 M Tris-HCl (pH 8.8)), 0.1 mL of 10 % SDS and 4 mL of water was combined with 0.5 mL of 1.5 % ammonium persulphate and 5 μL of TEMED to initiate polymerization, and after swirling the mixture was poured between glass plates (10.2 x 7.3 cm and 10.2 x 8.3 cm) mounted on a casting stand. After 45 min, a stacking gel consisting of 0.625 mL of acrylamide : bisacrylamide (30 : 0.8), 1.25 mL of stacking gel buffer (0.5 M Tris-HCl (pH 6.8)), 50 μL of 10 % SDS, 2.85 mL of water, 0.25 mL of 1.5 % ammonium persulphate and 5 μL of TEMED was poured on top of the resolving gel. A 10-well 0.75 mm comb was inserted into each glass plate assembly, and the gels were allowed to polymerize for another 45 min. Afterwards, the combs were removed and each glass plate assembly was unmounted from the casting stand and attached to an electrode assembly unit. The whole assembly was lowered into a lower buffer chamber and the internal space was filled with a reservoir buffer (0.25 M Tris (pH 8.3), 1.92 M glycine, 1 % SDS) to the level slightly above that of the stacking gel. Cell extract/protein samples were prepared by mixing 1-5 μL of the sample with 10 μL of 1 X loading buffer (50 mM Tris-HCl (pH 6.8), 100 mM dithiothreitol, 2 % SDS, 0.1 % bromophenol blue, 10 % glycerol) and boiling for 5 min to denature the contents. Following a quick-spin, the samples were loaded into wells and the gels were run at 100 V (Electrophoresis Power Supply EPS 500/400) until the dye front reached the bottom of the resolving gel (approximately 2 hrs).

At the end of the run, the gels were removed from the electrode assembly unit and
separated from their glass plates. Following a quick rinse with distilled water, the gels were lowered into a dying tank filled with staining solution (0.25 mg of Coomassie Brilliant Blue R250 dissolved in 100 mL of 90 % methanol (v/v) and 10 % glacial acetic acid (v/v)). The tank was swirled for 30 min after which time the gels were transferred to a destaining tank filled with an ample amount of destaining solution (10% acetic acid (v/v), 12.5% isopropanol (v/v)). The destaining solution was swirled continuously and replaced with fresh solution after 5, 30, 60 min and 2 hrs, and finally with distilled water the next day. Gels were routinely preserved by sealing between BioGel Wrap Drying System membranes (BioDesign Inc., New York).

3.2.6 Concentration assay

Protein concentration was determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories) as follows. Solutions of bovine serum albumin in water containing 1.0 to 10.0 µg/mL of protein (in increments of 1.0 µg/mL) were freshly prepared in the amount of 1 mL each and 800 µL of each standard was transferred into a 1.5 mL Eppendorf tube. A 200 µL aliquot of Dye Reagent Concentrate was added into each tube, the contents were vortexed and allowed to incubate at room temperature for 6 min. Absorbance of each standard was measured at 595 nm (Ultrospec 3000, Pharmacia-Biotech) and recorded to construct a BSA standard curve. Typically, three or four dilutions of a sample of unknown concentration were assayed in the same manner in triplicate and the concentration was determined from the BSA standard curve.
3.2.7 Activity assays

NADPH-nitroreductase activity of NfsA enzyme was analyzed as follows. Standard assay mixtures containing 50 mM Tris-HCl (pH 7.0) and 0.100 mM of nitrofurazone (from a 100 mM stock in DMSO) were prepared in water up to a projected final volume of 1 mL and transferred into 1 mL disposable plastic cuvettes (DiaMed, Canada). Six cuvettes at a time were placed in a spectrophotometer sample holder (Ultrospec 3000, Pharmacia-Biotech) connected to a 37 °C water bath and were allowed to equilibrate for 3 min. At the end of this period, a desired amount of purified NfsA enzyme held on ice was added to each cuvette and the reactions were initiated by addition of 41.6 μL of freshly prepared 2.4 mM NADPH solution to each cuvette (final NADPH concentration of 0.100 mM). Progress of reactions was monitored as extinction of nitrofurazone at 400 nm over a period of 3 min. Reaction velocity (μmol/min/mg of enzyme) was calculated from the slope of the linear region of the obtained curve (dA/dt) in each case using the equation \( V = (dA/dt)/(E \cdot \text{enzyme amount}) \) where the extinction coefficient for nitrofurazone was \( E = 12,960 \text{ M}^{-1} \). To calculate \( V'_{\text{max}} \) and \( K'_{\text{M}} \) parameters for the reaction, the assays were carried out with 10, 25, 50, 75, 100 and 125 μM nitrofurazone and 100 μM NADPH as substrates and a double-reciprocal Lineweaver-Burk plot of 1/v vs. 1/[nitrofurazone] was constructed. Apparent \( V_{\text{max}} \) (\( V'_{\text{max}} \)) was obtained from the Y-intercept of the plot (1/\( V'_{\text{max}} \)) whereas \( K'_{\text{M}} \) was calculated from the X-intercept (-1/\( K'_{\text{M}} \)). True \( V_{\text{max}} \) and \( K_{\text{M}} \) parameters were obtained by repeating the above assays with concentration of NADPH of 50, 75 and 100 μM for each single series of assays and constructing double-reciprocal Lineweaver-Burk plots of 1/V vs.1/[nitrofurazone]. The Y-intercepts obtained from the graph were replotted against 1/[NADPH] and \( K_{M1} \)
(nitrofurazone), $K_M$ (NADPH) and $V_{max}$ parameters were calculated from the graph. Regression analysis, calculation of X- and Y-intercepts were performed using Graph Pad Prism software package.

### 3.2.8 FMN enrichment

A 1 mL aliquot of purified enzyme preparation in elution buffer (approx. 1 mg/mL) was injected into a Slide-A-lyzer dialysis cassette (MW exclusion limit of 12,000), (Pierce Chem. Co., USA) and dialyzed against 2 L of 50 mM Tris-HCl (pH 7.0) for 24 hrs with buffer changes every 6 hrs to remove the elution buffer. The enzyme was then removed from the cassette and transferred into a 1.5 mL Eppendorf tube into which FMN crystals were added to a final concentration of 1 mM. The contents were incubated at 4 °C for 48 hrs with occasional mixing by inversion and subsequently dialyzed again as above to remove any residual FMN from the solution.

### 3.2.9.1 Mutagenesis

Random mutagenesis of the His-Tag $nfsA$ construct was performed using hydroxylamine as a mutagenic agent as follows. Approximately 5 μg of plasmid was added into each of four glass test tubes containing a solution of 0.4 mL of 0.5 M potassium phosphate buffer (pH 6.0), 0.5 mL of 0.5 M hydroxylamine (pH 6.0) (replaced with 0.5 mL of ddH$_2$O in control), 20 μL of 0.5 M EDTA (pH 8.0) and 1.08 mL of ddH$_2$O. After brief mixing, the tubes were incubated in a 37 °C water bath with gentle shaking for 0 (control), 4, 8 and 16 hrs. Plasmid DNA was precipitated, collected and dried as described in section
2.2.7; 100 ng was used for electroporation into JVQ2(pREP4) host. Transformants were plated on non-selective media (LB agar supplemented with ampicillin and kanamycin) as well as selective induced media (LB agar supplemented with kanamycin, ampicillin, furazolidone and IPTG) and on selective non-induced media (the latter without IPTG). Mutant colonies were picked from selective plates for characterization the next day.

3.2.9.2 Testing for sensitivity to nitrofurazone

Gradient media containing nitrofurazone were prepared in wide (D=14 cm) Petri dishes by pouring a layer of LB agar supplemented with 100 μg/mL of ampicillin, 25 μg/mL of kanamycin and 50 μg/mL of nitrofurazone, placed on a slanted surface to produce a linear concentration gradient of nitrofurazone across the plate starting 1.5 cm to the right of a labeled pole of the plate (origin) and allowed to solidify. Afterwards, a layer of LB agar (100 μg/mL ampicillin, 25 μg/mL kanamycin) of equal volume was poured atop the layer containing nitrofurazone and the plates were placed on an even surface and left for 16 hrs to allow for diffusion of nitrofurazone through the medium. Cultures of JVQ2(pREP4) harboring pQEnfsA mutants were streaked across the gradient starting at the origin using sterile cottons swabs dipped in 10 X and 100 X dilutions of the appropriate culture. A control strain JVQ2(pREP4)(pQEnfsA) was also streaked across each gradient plate (10 X dilution). Three mutants plus the control were accommodated on a single gradient plate in this manner (7 streaks/plate). The plates were subsequently placed in a 37 °C oven and incubated overnight. The level of resistance was calculated by noting the distance from the origin to the margin of growth cessation (x cm) and using the equation (x/14)(50 μg/mL). The error on
3.2.9.3. Characterization of NfsA mutants

NfsA mutants generated by NH₂OH treatment as outlined in section 3.2.9.1 were prepared for characterization as described in section 3.2.4.1 and analyzed by SDS-PAGE (section 3.2.5). Testing for nitrofurazone sensitivity was also performed for each isolate as outlined in the previous section. Mutants in which all of overexpressed protein was confined to the insoluble fraction as demonstrated by SDS-PAGE analysis were characterized further only by DNA sequencing (below). NfsA from soluble fractions of other mutants was purified on Ni-NTA resin (section 3.2.4.2) and its concentration was measured (section 3.2.6). Spectral analysis of pure NfsA preparations was performed on Ultrospec 3000 spectrophotometer (Pharmacia-Biotech) by scanning in the 250-600 nm range in 0.5 nm intervals (slow mode). FMN peaks at approximately 375 nm and 450 nm were recorded and the absorbance value at each peak was divided by enzyme concentration to provide a measure of comparison of FMN contents between isolates. NADPH-nitroreductase activity was assayed in triplicate using 10, 25, 50, 75, 100 and 125 μM nitrofurazone and 100 μM NADPH as substrates (section 3.2.7). For each isolate, DNA sequencing analysis of the pQEnfsA construct in the region of the nfsA gene and its regulatory elements was performed as outlined in section 2.2.9.
3.2.10. Modeling of His-tag NfsA

Modeling of the NfsA protein was performed online using the Swiss-Model Automated Comparative Protein Modeling Server (Guex and Peitsch, 1999) as follows. The modeling request was submitted to the server by including the amino acid sequence of His-tag NfsA in the First Approach mode. When desired, structural template selection was forced by checking the appropriate fields and including ExPDB codes for accessible templates. The output of the modeling server was visualized using Swiss-PdbViewer software (Guex and Peitsch, 1997) and preliminarily analyzed by aligning with the source structural templates used. Dimer of NfsA was assembled in Swiss PdbViewer by duplicating the modeled polypeptide chain and superimposing both chains onto the physiological structure of the nearest protein sequence homologue, NADPH-oxidoreductase (Frp) from V. harveyi (PDB entry 2BKJ) using the “Magic Fit” function. Coordinates for FMN and NAD heteroatoms were imported into the model from the 2BKJ entry to complete the model.
3.3 Results

3.3.1 Preparation of His-tag nfsA construct

The expression system used in this study was based on incorporation of a 6 X His affinity tag into a protein sequence of interest which allowed for quick and efficient purification of the expressed protein due to a strong and selective affinity of Ni-NTA resin for the 6 X His tag. For this purpose, the coding sequence of nfsA was cloned into a vector containing a sequence encoding the affinity tag upstream from the multicloning site, and the construct was transformed into a bacterial host (DH5α) for propagation. Several cloning attempts were necessary to obtain a functional clone of nfsA in this way as the initial cloning experiments suffered from low recovery of putative clones (0 to 5 per 200 transformants screened), all of which were mutated with respect to nfsA coding sequence or regulatory regions as shown by DNA sequencing. A particularly frequent mutation recovered from the pool of such mutants was a partial deletion of vector regulatory sequences which invariably resulted in loss of the operator and TATA promoter sequences (Figure 3.1, marked with arrows). Other mutations recovered from putative clones were within the coding region of nfsA and included several point mutations. After several such cloning attempts, the transformation host was changed from E. coli strain DH5α to JM103. The latter harbors a lacP promoter and provides levels of repressor up to 10 times higher than wild-type strains such as DH5α, thus allowing for a tighter control of expression. In a transformation experiment employing JM103, 31 putative clones were identified in a pool of 200 transformants subjected to screening, 21 of which were found to contain the cloned fragment as shown by size analysis of the vector DNA. Further restriction analysis revealed that only
TCGAGAAATCATAAAAATTTATTGTGTGAGCGGATACCAAATTTAATAGATTCA

Operator 1

Operator 2

TATA-box

RBS/SD

ATTTGAGACGGATAACAATTTACACACGAATTCAATTAAGAGGAATTAATAGAGA

6

+1 start mRNA

MR

2

6x His

KpnI → Native nfsA

GGATCGCATTACCATCATACCATACCGCACTCGGATACCGAGCTCGGTAGACGCCAACCGATT

GS

HHHHHGGGSGACELGTTPT

GAACGTATTTTGGGCTTCCATCGGATTTTCACTGATGAAACCTTCCCGAAG

ELICRGHSRFTDEPISEA

CAGCGTGGGCGGATTATAACACCGCCGCTCGGCCTCGACATTCTTTTTTTACAGTGCC

QREAIINSARATSSSSFLQC

AGTACCAATTCGATATACCCGACCAACAGCCTATCAGTGAGCTGACCGTACCGGC

SSIRITDKALKRELVTLTG

GGGCNAAAAACACGTAGCCGAAGCCGCCGAGTTCTGGGTGTTCTGGTGGGAGCTG

GQKHKVAEAFWVFCADEFN

CATTTACAGATCTGGCGATGTCCATCGCTGCTGGCGGTGTACAGCTTTTCTGGTG

HLQICPDAQLQLGAEQLLLGV

GTTGATCGCAGATCTGGCGAATATCAGATTATCAGCGGTAACCTGCTGGATTTGGC

VDTAMAMQNALIAAESLGLG

GGGGTTATATATCGGGCTCGCCAAATAATTGAAAGCGGTACGAAAACCTGTCTTAATTA

GVYIGGLRNNIEMVTLLKL

CCCGACGATGTCTGGCGCTGTGGTGCTGGCTGGCTGGCTGGGATATACCGGGAT

PQHPVLFLGLCLGWPADNP

CTTAAACCGGCGTTYACCACCCTCATTGGATGAAGAAGACGCTATCAACCGGCTGA

LKPRLPASILVHENVSTYQPLD

AAAGGCACGGCTGGCCGGATATGACGAGCACTGGCGGAATATTACCTCACCCGCTGGGACC

KGALAQYDEQLAEYYLRTGS

AATAATCGCAGGATACCTGAGGACGCTACATCAGCCGAAACATGATTAAGAGGACGCG

NNRRDTWSDHRRTIIKESR

CCATTATTTCTGTGATATTGCGAACAACAGGGTTGGCGAGCGGCTACAAACCGCGACGT

PFILDLHKLQKGWATR

Stop

GATGTATGATACCGGGGCTTCTTGACCAGTCTGACAGAGGAGGAGTCGGGTTGAAAATTTGCC

Transcription Terminator

ATATTTGAGACGGATAACAGCCTATTTGAGACGCTACCCGAGGTTCGACCTGCAGCAGGCTTA

KpnI

Figure 3.1 Regulatory and coding regions of His-tag nfsA (pQEnfsA) construct and amino acid sequence of the enzyme. The nfsA gene was amplified by PCR with its original Stop codon and putative transcription terminator using primers incorporating artificial KpnI restriction enzyme sites, and was cloned into the KpnI site of the pQE30 template as described in Methods and Materials. Regulatory regions for transcription are provided by the vector template and are labeled: Operator 1 and 2 for binding of lacI repressor, RBS/SD (ribosome binding site/ Shine-Dalgarno) as well as TATA-box. Also indicated are 6x His tag, Start and Stop codons for transcription (bold), first codon corresponding to native nfsA gene (bold), putative transcription terminator sequence as well as KpnI sites used in cloning. Two sets of arrows (↑ and ↓) denote a commonly occurring deletion in recovered potential clones (see text).
4 of the above constructs contained the cloned fragment in the correct orientation. DNA sequencing indicated that one of the constructs (L22) contained a deletion of the vector operator sequence as noted above, one harbored a missense mutation within the \textit{nfsA} gene coding region (L3) and two were correct with respect to the desired sequence (L21 and L29).

The map and the control region of the final construct, pQEnfsA are shown in Figure 3.2 and Figure 3.1, respectively. The construct is 4288 bp in size and gives rise to a NfsA protein complexed with 6 X His tag (referred to simply as NfsA) and leader sequences totaling 257 amino acid residues (M.W. 28,643 Da). This represents an increase of 17 residues over native NfsA (M.W. 26,801). Expression of NfsA is under control of LacI repressor coded on a co-expressed plasmid, pREP4 (Figure 3.2), which binds to a double operator sequence on pQEnfsA upstream of the cloned \textit{nfsA} gene (Figure 3.1). Transcription of the repressor from pREP4 is constitutive from the \textit{lacP} promoter and induction by exogenous IPTG is required for derepression and overexpression of NfsA from pQEnfsA to occur.

\textbf{3.3.2 Purification and analysis of NfsA protein}

For the purpose of expression and purification, pQEnfsA was routinely propagated in the strain JVQ2(pREP4) since it contains deletions within chromosomal \textit{nfsA} and \textit{nfsB} genes thus allowing us to selectively analyze nitroreductase activity originating from the plasmid copy of \textit{nfsA} in both crude and purified extract preparations. As demonstrated in Figure 3.3, induction of expression by IPTG brought about an enormous increase in the amount of expressed NfsA (induced lane I vs. uninduced lane U) although expression did
FIGURE 3.2. Maps of pQEnfsA and pREP4 plasmids used in the study. pQEnfsA is a construct obtained by cloning the coding sequence of the \( nfsA \) gene along with its original Stop codon and transcription terminator into the \( KpnI \) restriction site of the parent pQE-30. pREP4 is a co-expressed plasmid used in the expression system, providing for tight control of NfsA expression from pQEnfsA.
FIGURE 3.3. Purification of overexpressed NfsA with Ni-NTA resin. JVQ2(pREP4)(pQEnfsA) cells were grown in appropriate media and induced with IPTG as described in Methods and Materials. Cells were lysed by sonication to produce crude cell extracts which were separated further into soluble and insoluble fractions by centrifugation. NfsA was purified from the soluble fraction with Ni-NTA resin as described. Aliquots of fractions were separated by SDS-PAGE and visualized by staining. Left to right: (S) Broad range protein molecular weight standard (Bio-Rad, CA) containing the bands of indicated sizes; (U) uninduced crude cell extract; (I) induced crude cell extract; (Ins), (Sol) insoluble and soluble fractions of (I), respectively; (P) purified NfsA represented by 29 kDa band.
occur to an appreciable extent under uninduced conditions giving rise to a low intensity band, approximately 30 kDa in size, corresponding to NfsA. A majority of expressed NfsA was observed in the soluble fraction with a small amount of product visible in the insoluble fraction, probably representing residuum trapped within the cellular debris. Purification of NfsA with Ni-NTA resin was effected to near homogeneity as demonstrated in Figure 3.3 (lane P) and usually yielded amounts of pure enzyme sufficient for downstream manipulations (typically 2-3 mg from 50 mL culture). Several contaminating species were evident in the purified fraction, particularly a 25 kDa band and several higher molecular weight (>66 kDa) bands which persisted in most preparations utilizing this purification protocol. Purification factor (specific nitroreductase activity in crude lysate / purified fraction) was calculated to be 6.0 (Table 3.1). The purified preparation of NfsA was yellow in color, and was stable on ice for several hours and at least 2 weeks when stored at -20 °C in the elution buffer, as demonstrated by repetitive activity measurements.

Reduction of nitrofurazone by purified NfsA was linear in the range of 0.25 to approximately 3.0 µg of enzyme as tested under standard assay conditions (Figure 3.4). A series of parallel lines obtained on a Lineweaver-Burk plot (Figure 3.5A) indicates that the mechanism of NADPH-nitroreductase activity of NfsA obeys the ping-pong Bi-Bi mode of catalysis. $V_{\text{max}}$ for the activity was estimated as 43 µmol·min$^{-1}$·mg enzyme$^{-1}$, whereas $K_m$ constants for nitrofurazone and NADPH were calculated to be 72 and 22 µM, respectively (Appendix II).

Spectral analysis of NfsA in solution revealed two distinct peaks in the visible region at 376.5 and 451.5 nm (Figure 3.6). The peaks are red-shifted by approximately 2 and 4 nm
TABLE 3.1. Purification factor for NfsA enzyme purified using the Ni-NTA resin purification protocol. All fractions were collected during a single purification routine and were assayed in triplicate as described.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Specific Activity (μmol/min/mg enzyme)</th>
<th>Average Specific Activity (μmol/min/mg enzyme)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude Cell Extract (Induced)</td>
<td>3.83</td>
<td>3.35 3.21</td>
</tr>
<tr>
<td>Soluble Fraction</td>
<td>6.45</td>
<td>5.67 6.34</td>
</tr>
<tr>
<td>Insoluble Fraction</td>
<td>0.86</td>
<td>1.15 1.12</td>
</tr>
<tr>
<td>Purified Enzyme</td>
<td>20.70</td>
<td>22.26 19.10</td>
</tr>
</tbody>
</table>

Purification Factor = 20.69/3.46 = 6.0
FIGURE 3.4. Reduction of nitrofurazone by NfsA. The enzyme was purified as described in Methods and Materials and variable amounts of enzyme were assayed in triplicate in a standard reaction mixture containing 100 μM nitrofurazone and 100 μM NADPH as substrates.
FIGURE 3.5. (A) Lineweaver-Burk double-reciprocal plots for NfsA enzyme. The assays were performed in triplicate for three given concentrations of NADPH which were kept constant in each case. (B) Plot of Y-intercepts \(1/V'_{\text{max}}\) vs. \(1/[S]\) used to obtain \(V_{\text{max}}\) and \(K_M\) values (see Appendix II)
FIGURE 3.6. Absorption spectra of purified NfsA enzyme at 0.80 mg/mL (28 µM) and FMN (30 µM) in elution buffer. The spectra were taken at a resolution of 0.5 nm in a single scan from 250 to 600 nm. The peaks (nm) are indicated on each spectrum.
in relation to corresponding peaks identified in the spectrum of FMN of 374.5 and 447.5 nm, respectively, in concordance with the protein-bound nature of FMN cofactor. Attempts at enriching the purified enzyme preparation with exogenous 1 mM FMN and subsequent dialysis did not increase the magnitude of FMN peaks in the spectrum of NfsA or its NADPH-nitrofurazone activity. This step was therefore not used in further preparations of the enzyme for analysis.

3.3.3 Mutagenesis of pQEnfsA construct

In order to generate point substitutions in the NfsA protein, a random mutagenesis approach was employed using hydroxylamine as the mutagenic agent (NH₂OH). Hydroxylamine is commonly used to generate base pair changes in DNA and the resulting amino acid substitutions in the protein. Since this study attempted to identify the amino acid residues critical for the function of NfsA, this approach was expected to yield mutants with modified nitroreductase activity which would be useful in this regard. As shown in Table 3.2, the treatment of pQEnfsA with hydroxylamine increased the recovery of mutants resistant to furazolidone which increased with the time of treatment. Both IPTG-induced and non-induced media were used for selection of mutants in an attempt to optimize the selection protocol. Slightly higher numbers of resistant colonies were recovered from non-induced media than the induced media, presumably due to higher levels of overexpression and resulting toxicity of the NfsA enzyme in the latter case. Of the 58 colonies chosen from the pool of mutants and tested for resistance to nitrofurazone under non-inducing conditions, a wide range of resistance levels was evident, ranging from 3.9 μg/mL to 26.1 μg/mL (Figure
TABLE 3.2. Summary of the hydroxylamine mutagenesis experiment. pQEnfsA DNA was treated with hydroxylamine for the period of time indicated in each case and transformed into a JVQ2(pREP4) host. Transformants and mutants were scored on non-selective and selective media, respectively. Induced media were supplemented with 10 μM IPTG. Mutation frequency is expressed in relation to counts obtained from non-induced media. The experiment was performed once.

<table>
<thead>
<tr>
<th>Treatment Time (h)</th>
<th>Transformants (CFU $\cdot 10^7$/mL)</th>
<th>Mutants (CFU $\cdot 10^4$/mL)</th>
<th>Mutation frequency ($\times 10^3$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-induced</td>
<td>Induced</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>1.04</td>
<td>1.02</td>
<td>0.77</td>
</tr>
<tr>
<td>4</td>
<td>1.09</td>
<td>2.80</td>
<td>1.65</td>
</tr>
<tr>
<td>8</td>
<td>1.36</td>
<td>7.33</td>
<td>5.40</td>
</tr>
<tr>
<td>16</td>
<td>2.10</td>
<td>23.5</td>
<td>18.9</td>
</tr>
</tbody>
</table>
3.7). In comparison, the control strain harboring wild-type pQEnfsA did not exhibit any resistance under the same conditions.

The size of the overexpressed protein also varied among the mutants; 19 were found not to overexpress any protein as observed in crude and purified extracts, whereas in the remaining 39 cases proteins of varying molecular weights were obtained. Commonly, five variants of approximately 27, 23, 20, 16 and 14 kDa in size were observed, in addition to species having wild-type size of 29 kDa (Figure 3.8). There was no apparent correlation between the level of nitrofurazon resistance and the observed size of the overexpressed protein. In addition, the selection conditions of the mutants with regards to induction of expression as well as plasmid DNA NH₂OH treatment time did not play a role in the overall resistance and expression profiles.

3.3.4 Characterization of NfsA mutants

Of the 58 furazolidone-resistant JVQ2(pREP4)(pQEnfsA) mutants analyzed above, 19 were selected for further analysis involving sequencing of the cloned nfsA gene and its regulatory regions as well as spectral analysis and NADPH-nitrofurazon activity measurements of the overexpressed protein. Ten of the mutants were chosen from the group expressing NfsA of wild-type size, whereas the remaining 9 expressed truncated variants. Only 8 of the 19 mutants were found to produce soluble extracts of the overexpressed protein in amounts sufficient for purification and downstream analysis. In the remaining 11 cases, all of the overexpressed protein was confined to the insoluble fraction; attempts at solubilization by induction of expression at a lower growth temperature (23 °C) and
FIGURE 3.7. Nitrofurazone resistance levels and protein expression characteristics of 58 mutants selected from the pool of furazolidone-resistant mutants. Mutants were obtained by treating pQEnfsA vector with hydroxylamine for a specified period of time and transforming into JVQ2(pREP4) host. Bars represent the level of nitrofurazone resistance of any particular mutant, whereas the category on the Y-axis refers to the size of overexpressed protein observed. The time of hydroxylamine treatment for each mutant is shown in the legend in the top right corner. The conditions for selection of each mutant (inducing vs. uninducing) are not included in the Figure.
Figure 3.8. NfsA size variants observed in a collection of mutants produced in the NH$_3$OH mutagenesis experiment. pQE4nfsA vector was treated for variable amounts of time with hydroxylamine and transformed into JVQ2(pREP4) host for subsequent protein expression and purification. (S) Broad range protein molecular weight standard (Bio-Rad, CA) consisting of the bands of indicated sizes; (C) Purified wild-type size NfsA (29 kDa); lanes 29-14 - crude cell extracts of several mutants from the collection showing each size variant observed.
inclusion of 0.1 % Triton-X detergent during cell disruption stage did not increase the yield of soluble material to any appreciable extent.

DNA sequencing analysis of the 19 JVQ2(pREP4)(pQEnfsA) mutant isolates revealed the presence of 13 discreet mutational events within the nfsA gene coding region. As shown in Table 3.3, 7 G:C→A:T and 3 C:G→T:A transitions as well as 1 C:G→G:C and 1 T:A→G:C transversions were observed; a single 85 bp deletion was also recovered. Eight of the 12 point mutations were nonsense in character resulting in a premature termination of the transcript; the remaining four resulted in amino acid substitutions. In 5 cases a single mutation was observed in more than one isolate. Mutants M416 and M417 were derived from the same treatment group and may be of common origin; in all other cases the identical mutations arose independently as they originate from mutants derived from different NH2OH treatment time groups and under different selection conditions (Table 3.4). The calculated size of the transcript in most cases corresponded closely to those observed on SDS-PAGE analysis of crude cell extracts; in one case (M009) where no overexpressed product was evident on SDS-PAGE, the transcript of calculated size of 8 kDa would be expected to be present as shown by DNA sequencing analysis of the pQEnfsA construct. In this and 19 other cases mentioned above in which no product was visible on SDS-PAGE, the NfsA protein may, in fact, have been present as a truncated transcript of size sufficiently small to be eluted off the gel in routine SDS-PAGE runs or, alternatively, subject to proteolytic digestion during expression.

NADPH-nitroreductase activity assays were carried out with pure NfsA preparations obtained from 8 mutants in which the overexpressed product was soluble. As shown in Table
TABLE 3.3. Summary of mutations identified in the *nfsA* gene coding region in a collection of furazolidone-resistant JVQ2(pREP4)(pQEnfsA) mutants.

<table>
<thead>
<tr>
<th>Mutant(s)</th>
<th>Base change</th>
<th>Codon change</th>
<th>Amino acid change</th>
</tr>
</thead>
<tbody>
<tr>
<td>M402, M409</td>
<td>G&lt;sub&gt;687&lt;/sub&gt;→A</td>
<td>TGG→TGA</td>
<td>W&lt;sub&gt;229&lt;/sub&gt;→Stop</td>
</tr>
<tr>
<td>M403, M424, M1608</td>
<td>G&lt;sub&gt;511&lt;/sub&gt;→A</td>
<td>GGG→AGG</td>
<td>G&lt;sub&gt;171&lt;/sub&gt;→R</td>
</tr>
<tr>
<td>M423</td>
<td>G&lt;sub&gt;762&lt;/sub&gt;→A</td>
<td>TGG→TGA</td>
<td>W&lt;sub&gt;254&lt;/sub&gt;→Stop</td>
</tr>
<tr>
<td>M427</td>
<td>G&lt;sub&gt;761&lt;/sub&gt;→A</td>
<td>TGG→TAG</td>
<td>W&lt;sub&gt;254&lt;/sub&gt;→Stop</td>
</tr>
<tr>
<td>M428</td>
<td>G&lt;sub&gt;427&lt;/sub&gt;→A</td>
<td>GGG→AGG</td>
<td>G&lt;sub&gt;143&lt;/sub&gt;→R</td>
</tr>
<tr>
<td>M430</td>
<td>G&lt;sub&gt;428&lt;/sub&gt;→A</td>
<td>GGG→GAG</td>
<td>G&lt;sub&gt;143&lt;/sub&gt;→E</td>
</tr>
<tr>
<td>M805, M1605</td>
<td>G&lt;sub&gt;528&lt;/sub&gt;→A</td>
<td>TGG→TGA</td>
<td>W&lt;sub&gt;176&lt;/sub&gt;→Stop</td>
</tr>
<tr>
<td>M004, M401</td>
<td>C&lt;sub&gt;349&lt;/sub&gt;→T</td>
<td>CAA→TAA</td>
<td>Q&lt;sub&gt;117&lt;/sub&gt;→Stop</td>
</tr>
<tr>
<td>M416, M417</td>
<td>C&lt;sub&gt;634&lt;/sub&gt;→T</td>
<td>CAA→TAA</td>
<td>Q&lt;sub&gt;212&lt;/sub&gt;→Stop</td>
</tr>
<tr>
<td>M1601</td>
<td>C&lt;sub&gt;703&lt;/sub&gt;→T</td>
<td>CGA→TGA</td>
<td>R&lt;sub&gt;235&lt;/sub&gt;→Stop</td>
</tr>
<tr>
<td>M002</td>
<td>C&lt;sub&gt;374&lt;/sub&gt;→G</td>
<td>ACG→AGG</td>
<td>T&lt;sub&gt;125&lt;/sub&gt;→R</td>
</tr>
<tr>
<td>M009</td>
<td>T&lt;sub&gt;218&lt;/sub&gt;→G</td>
<td>TTA→TGA</td>
<td>L&lt;sub&gt;73&lt;/sub&gt;→Stop</td>
</tr>
<tr>
<td>M429</td>
<td>&lt;sup&gt;448&lt;/sup&gt;CGC...GG&lt;sup&gt;536&lt;/sup&gt;→ &lt;sup&gt;448&lt;/sup&gt;CGGA&lt;sup&gt;551&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
TABLE 3.4. Summary of characteristics of furazolidone-resistant JVQ2(pREP4)(pQEfnfsA) mutants and the properties of the overexpressed product. All species are His-tag derivatives. JVQ2(pREP4)(pQEfnfsA) mutants were generated by NH$_2$OH treatment of the pQEfnfsA, transformation into JVQ2(pREP4) cells and subsequent selection on furazolidone media. The first digit in mutant name denotes NH$_2$OH treatment time (0, 4, 8 or 16 hrs). Induction refers to selection conditions which contained IPTG in growth media (+) or did not (-). Observed size of the protein refers to the band observed on SDS-PAGE, whereas the calculated value was obtained from the actual amino acid sequence of each mutant. Solubility of the overexpressed product as observed in the soluble fraction following cell disruption is indicated as '+' or '-'. FMN peaks were obtained by spectral analysis of pure protein and are expressed as a ratio of absorbance noted at the peaks at approximately 375 and 450 nm to protein concentration in the sample to provide a measure of comparison of flavin content between mutants. The number in brackets next to each value is the actual peak wavelength at which a maximum was noted (absent where not resolved). $V'_{\text{max}}$ is the apparent maximum reaction velocity for NADPH-nitroreductase activity and was measured using 100 $\mu$M NADPH and 10,25,50,75,100 and 125 $\mu$M of nitrofurazone as described in Methods and Materials (section 3.2.7).
<table>
<thead>
<tr>
<th>Mutant</th>
<th>Nitrofurazone Resistance (±1 μg/mL)</th>
<th>Induction</th>
<th>Size of protein (kDa)</th>
<th>Solubility</th>
<th>Amino Acid Change</th>
<th>FMN Peaks</th>
<th>V'_{max}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Observed</td>
<td>Calculated</td>
<td></td>
<td>~450 (μL x μg enzyme(^{-1}) x 10(^2))</td>
<td>~375 (μmol·min(^{-1}) x mg enzyme(^{-1}))</td>
</tr>
<tr>
<td>M402</td>
<td>17.8</td>
<td>-</td>
<td>27</td>
<td>25.2</td>
<td>+</td>
<td>W(_{229}) - Stop</td>
<td>30 (447.0)</td>
</tr>
<tr>
<td>M409</td>
<td>20.0</td>
<td>+</td>
<td>27</td>
<td>25.2</td>
<td>+</td>
<td>W(_{229}) - Stop</td>
<td>28 (446.0)</td>
</tr>
<tr>
<td>M403</td>
<td>23.3</td>
<td>-</td>
<td>29 (wt)</td>
<td>28.6</td>
<td>-</td>
<td>G(_{171}) - R</td>
<td></td>
</tr>
<tr>
<td>M424</td>
<td>21.7</td>
<td>-</td>
<td>29 (wt)</td>
<td>28.6</td>
<td>-</td>
<td>G(_{171}) - R</td>
<td></td>
</tr>
<tr>
<td>M1608</td>
<td>21.1</td>
<td>-</td>
<td>29 (wt)</td>
<td>28.6</td>
<td>-</td>
<td>G(_{171}) - R</td>
<td></td>
</tr>
<tr>
<td>M423</td>
<td>18.9</td>
<td>-</td>
<td>29 (wt)</td>
<td>28.3</td>
<td>+</td>
<td>W(_{254}) - Stop</td>
<td>1 (447.0)</td>
</tr>
<tr>
<td>M427</td>
<td>20.6</td>
<td>+</td>
<td>29 (wt)</td>
<td>28.3</td>
<td>+</td>
<td>W(_{254}) - Stop</td>
<td>1 (446.5)</td>
</tr>
<tr>
<td>M428</td>
<td>20.0</td>
<td>-</td>
<td>29 (wt)</td>
<td>28.6</td>
<td>-</td>
<td>G(_{143}) - R</td>
<td></td>
</tr>
<tr>
<td>M430</td>
<td>16.1</td>
<td>-</td>
<td>29 (wt)</td>
<td>28.6</td>
<td>+</td>
<td>G(_{143}) - E</td>
<td>1 (447.0)</td>
</tr>
<tr>
<td>M805</td>
<td>25.6</td>
<td>+</td>
<td>20</td>
<td>19.1</td>
<td>-</td>
<td>W(_{176}) - Stop</td>
<td></td>
</tr>
<tr>
<td>M1605</td>
<td>21.1</td>
<td>+</td>
<td>20</td>
<td>19.1</td>
<td>-</td>
<td>W(_{176}) - Stop</td>
<td></td>
</tr>
<tr>
<td>M004</td>
<td>24.4</td>
<td>+</td>
<td>14</td>
<td>13.0</td>
<td>-</td>
<td>Q(_{117}) - Stop</td>
<td></td>
</tr>
<tr>
<td>M401</td>
<td>24.4</td>
<td>+</td>
<td>14</td>
<td>13.0</td>
<td>-</td>
<td>Q(_{117}) - Stop</td>
<td></td>
</tr>
<tr>
<td>M416</td>
<td>24.4</td>
<td>+</td>
<td>23</td>
<td>23.1</td>
<td>+</td>
<td>Q(_{212}) - Stop</td>
<td>74 (445.5)</td>
</tr>
<tr>
<td>M417</td>
<td>23.3</td>
<td>+</td>
<td>23</td>
<td>23.1</td>
<td>+</td>
<td>Q(_{212}) - Stop</td>
<td>72 (446.0)</td>
</tr>
<tr>
<td>M1601</td>
<td>15.0</td>
<td>-</td>
<td>27</td>
<td>26.0</td>
<td>+</td>
<td>R(_{235}) - Stop</td>
<td>79 (448.0)</td>
</tr>
<tr>
<td>M002</td>
<td>25.0</td>
<td>-</td>
<td>29 (wt)</td>
<td>28.6</td>
<td>-</td>
<td>T(_{125}) - R</td>
<td></td>
</tr>
<tr>
<td>M009</td>
<td>25.6</td>
<td>-</td>
<td>None</td>
<td>8.0</td>
<td>-</td>
<td>L(_{79}) - Stop</td>
<td></td>
</tr>
<tr>
<td>M429</td>
<td>24.4</td>
<td>-</td>
<td>29 (wt)</td>
<td>27.0</td>
<td>-</td>
<td>85 bp deletion</td>
<td></td>
</tr>
<tr>
<td>L21 (wild-type)</td>
<td>0</td>
<td></td>
<td>29 (wt)</td>
<td>28.6</td>
<td>+</td>
<td></td>
<td>96 (451.5)</td>
</tr>
</tbody>
</table>
3.4, the enzyme activity represented by apparent $V_{\text{max}}$ (designated as $V'_{\text{max}}$) was drastically reduced in all cases, compared to wild-type His-tagged NfsA preparation which reduced nitrofurazone under the same conditions at 38 $\mu$mol·min$^{-1}$·mg enzyme$^{-1}$. Amino acid substitution $G_{143} \rightarrow E$ in mutant M430 abolished nitroreductase activity to zero as did a nonsense change at $Q_{212}$. Interestingly, nonsense mutations downstream of $Q_{212}$, at $W_{229}$ and $W_{254}$, were associated with non-zero nitroreductase activity with the mutation $R_{235} \rightarrow$ Stop in mutant M1601 resulting in the highest activity noted among the assayed isolates (12.1 $\mu$mol·min$^{-1}$·mg enzyme$^{-1}$). The level of resistance to nitrofurazone was noted to be inversely related to the level of nitroreductase activity; however, in one case zero activity was associated with a relatively low level of resistance (M430).

The flavin content in the purified solutions of NfsA was estimated spectrophotometrically as quotient of absorbance at two FMN peaks, approximately 375 and 450 nm, and protein concentration of the sample to provide a measure of comparison between the samples. Such measurements gave very similar results to $A_{375}/A_{280}$ and $A_{450}/A_{280}$ ratios and correlated well with the intensity of yellow coloration visible in the samples, with higher values being associated with more yellow color present. In all characterized mutants the amount of flavin was reduced relative to wild-type, but was not directly linked to NADPH-nitroreductase activity. For example, as shown in Table 3.4, apparently high flavin content of NfsA enzyme from mutants M416 and M417 was associated with zero activity. Also of note is the fact that the wavelengths at which absorption maxima were recorded varied between mutants suggesting changes in the state, or environment, of flavin.
3.3.5. Modeling of NfsA

The model of NfsA is a homodimer containing 1 FMN molecule per monomer unit and a single NAD molecule. Although the whole NfsA amino acid sequence totaling 257 residues and including His-tag was submitted to the modeling server, the output was reduced to 239 residues with the first 18 residues being absent from the model due to lack of structural homology with available templates. Examination of the server modeling log revealed that a single template of NADPH-oxidoreductase from *V. harveyi*, Frp, was utilized in the modeling process resulting in a high degree of structural similarity between the template and the output (RMS of 229 C backbone atoms of 0.14 Å). Forcing an additional available template, the NADH dehydrogenase from *T. aquaticus*, into modeling the modeling request changed the RMS to 0.83 Å preserving closely both secondary and tertiary structures in the model. The final model was constructed using only the chain modeled on Frp template; the coordinates for FMN and NAD were imported manually from the model of Frp, since the modeling server does not support requests involving heteroatoms. The quaternary structure was also manually adopted from the structure of Frp by fitting two modeled NfsA subunits onto a dimer of Frp with Swiss-PdbViewer software as oligomer modeling with heteroatoms was also not offered by the server at the time.

As seen in Figure 3.9, the modeled dimer of NfsA is an isologous one consisting of two interlocking subunits. The secondary structure layout for the monomer chain is presented in Figure 3.10 indicating that 12 α-helices and 6 β-sheets are formed by each monomer. Each subunit comprises two domains, a sandwich domain and an excursion domain. The sandwich domain is of α-β-α type consisting of four central antiparallel β-sheets (P,Q,R,S
Figure 3.9. A ball-stick model of NfsA showing the interlocking nature of the two subunits. The upper part of the yellow subunit is the excursion domain. The excursion domain of the blue subunit is partly visible in the bottom part of the model. Both active sites are also partly visible in this view. FMN and NAD are indicated in red and blue, respectively.
FIGURE 3.10. Amino acid sequence of the modeled NfsA protein and its secondary structure layout. Helices A-L are indicated by ‘H’, whereas β-sheets O-T are denoted by ‘S’. Dots represent loops in the structure. The naming of secondary structure elements is identical to that seen in Figure 3.11. The numbering of amino acid residues is consistent with the model. Bold amino acids are the sites of observed missense mutations with changes indicated above each residue.
in Figure 3.11) flanked by helices B, F and C,D,G from each side. The excursion domain is composed of subunit residues located on the opposite side of the 2-fold axis relative to the sandwich domain of that subunit and includes helices A, I, J and K as well as β-sheet T. Altogether, three loops from each subunit traverse the axis with the last loop terminating in helix L next to the sandwich domain. The active site is a crevice in the dimer interface composed of residues contributed by both subunits which partly bury the flavin cofactor, especially the dimethylbenzene portion of the isoalloxazine ring (Figure 3.12). Most of NAD molecule, on the other hand, is freely accessible to the solvent. Calculations of the electrostatic potential surface performed with Swiss PdbViewer software revealed that the entire active site crevice is located in a positive potential field, which has also been the case with Frp.

Several residues from both subunits participate in electrostatic stabilization of the flavin cofactor (Figure 3.13). A total of six amino acids are involved with five of them contributed by one subunit. Residues Arg 15, Gly 130 and Gly 131 form hydrogen bonds with isoalloxazine ring of the flavin, whereas His 11, Ser 13, as well as Ser 39 from the other subunit (Ser 39 B), anchor the ribityl chain. Most of the observed interactions involve amino acid side chains. Comparison with similar interactions characterized in a crystal structure of Frp reveals that less hydrogen bonding is observed in NfsA (9 versus 16 in Frp). In particular, several interactions which occur in Frp are not favorably reflected in the modeled structure of NfsA. Hydrogen bonding between Arg 15 NH₂ and O’2 of the flavin ribityl chain as well as between Arg 15 NH and N1 of isoalloxazine ring is not present given distances of 4.17 Å and 3.79 Å, respectively (compared to 3.81 and 3.36 Å for Frp). Also, Gln 67
**Figure 3.11.** NfsA model viewed down the 2-fold axis. Helices and β-sheets are indicated in orange and turquoise, respectively. N-terminals of each subunit are visible in the centre of the image (white); C-terminals are situated in the back and are difficult to visualize. Secondary structure elements are labeled for one subunit and follow the order given on the bottom of image. Two molecules of FMN (red) and one NAD (green) are shown.
FIGURE 3.12. A view down the active site crevice demonstrating buried nature of the flavin cofactor (red). The lowermost visualized part of the cofactor is the isoalloxazine moiety seen down its plane. The active site is formed by residues from both subunits (yellow and green). NAD is shown in blue.
Figure 3.13. Interactions between FMN cofactor and amino acid residues of NfsA. Amino acids from subunits A and B are shown in light blue and red, respectively. Strong hydrogen bonds are represented in green, whereas the weak one is grey.
which stabilizes O4 (3.25 Å) and N3'H (2.71 Å) of the isalloxazine moiety in Frp does not interact similarly in NfsA in which the corresponding distances between atoms are 5.65 Å and 5.37 Å, respectively. Another absent interaction in the model of NfsA is that of Arg 169 guanidyl NH2 groups and OP1 of FMN phosphate seen in Frp with H-bond lengths of 2.70 Å and 2.80 Å. Instead, both of guanidyl NH2 groups in Arg 169 form hydrogen bonds with other amino acid residues in NfsA, namely, O atoms of Ala 36 B (2.33 Å) and Lys 167 (3.22 Å) as seen in Figure 3.13.

NAD is also stabilized by 9 H-bonds contributed by 6 amino acid residues and the FMN cofactor (Figure 3.14). The pyrophosphates hydrogen bond to Arg 225, Gly 131 and Ser 41 B as well as N10 and O2' of the flavin, whereas the adenine moiety is stabilized by a single interaction with Gln 67. Each of the ribose rings is anchored to a single amino acid residue, notably Arg 225 and Gly 65, with a single H-bond being present in each case. Unlike the situation observed in Frp, Asn 164 of NfsA is not involved in hydrogen bonding to NAD due to a relatively large distance between the two (> 4.5 Å) and forms H-bonds with other amino acid residues instead. Examination of the FMN moiety located in the second active site crevice devoid of NAD indicates that the presence of NAD does not alter hydrogen bonding between the FMN cofactor and the dimer chains.

3.3.6 Analysis of missense mutations

The 8 missense mutations in the NfsA protein recovered in this study as well as in the previous experiment (Chapter 2; Table 2.2) have been analyzed in the context of the model of NfsA by manual substitution of relevant residues with the most stable rotamer of
FIGURE 3.14. View of the FMN/NAD complex active site with FMN (yellow), NAD (blue) and amino acid residues from the two subunits (light blue and red). Hydrogen bonds are shown in green.
mutated ones using the Swiss-PdbViewer software. The mutations are summarized in Figure 3.10 and follow the numbering of the model rather than that of the His-tag NfsA transcript. The first three characterized substitutions His 11→Lys, Arg 15→Cys and Gln 67→His are localized to the active site of the dimer and are all predicted to result in weakening of electrostatic stabilization of the FMN cofactor and NAD. Both His 11→Lys and Arg 15→Cys substitutions produce a loss of the single H-bond formed by each residue and FMN (Figure 3.13), whereas the change Gln 67→His weakens the hydrogen bond which exists in the wild-type between Gln 67 and adenine moiety of NAD (Figure 3.14). Three other characterized changes are Ile 89→Asn, Gly 123→Arg as well as Gly 123→Glu which occur near the surface of the dimer giving rise to the following structural predictions. In the first case, change from Ile to Asn results in a loss of one of the two hydrogen bonds Ile 89 forms in the wild-type (Ile 89 : Arg 85) while the other bond (Ile 89 : His 86) remains unchanged. Calculations of solvent accessibility performed using the Swiss-PdbViewer software also indicate that this change slightly buries the side chain of Asn relative to Ile residue in the wild-type. A reverse situation is observed with the Gly 123→Arg substitution which changes the H-bond between Gly 123 and Glu 120 to Leu 122 and results in complete protrusion of the Arg side chain into the solvent. This mutation also results in creation of a positive potential field in an otherwise negatively charged area as shown by calculation of electrostatic potential surface performed with Swiss-PdbViewer. Another mutation observed in this area, Gly 123→Glu, is unremarkable in context besides slightly exposing the side of chain of Glu to the solvent relative to the parental residue. The last two substitutions, Thr 108→Arg and Gly 154→Arg, produce unfavorable changes in the structure by introducing a considerable
amount of steric strain from the arginine side chain which occurs with all tested arginine
rotamers. The strain is localized between the distal portion of β-sheet S and helices C and
D in the former case and between helix F and the initial part of β-sheet S. Both of these
amino acid changes were observed in mutants which did not produce any soluble NfsA
protein, suggesting that they may interfere with protein folding or stability.
3.4 Discussion

In this study, the coding sequence of the *nfsA* gene from *E. coli* was cloned into an expression vector containing an affinity tag which added onto the N-terminal of the protein and allowed for its rapid purification in amounts and of quality sufficient for downstream analysis. To gain insight into the molecular aspects of function of the NfsA enzyme, several random *nfsA* mutants were generated using NH₂OH mutagenesis; the mutants were subjected to biochemical and genetic analysis involving characterization of mutations as well as levels of nitrofurazone resistance of the mutants, nitroreductase activity of purified NfsA enzyme preparations, and determining the FMN cofactor characteristics. As an aid in interpretation of the results obtained in the study, a model of NfsA based on a crystal structure of the nearest sequence homolog of NfsA, NADPH-oxidoreductase from *V. harveyi* (Frp), was constructed using computer modeling.

Cloning of the *nfsA* gene presented significant difficulties when a transformation host typically used for such purposes, DH5α, was employed. The procedure suffered repeatedly from low recovery of transformants and in all cases only mutated clones were recoverable. Success in cloning of the gene obtained upon changing of the host to JM103, which produces higher levels of LacI thus repressing expression of NfsA from the construct, suggests that expression of NfsA in DH5α is toxic to the cell even in the amounts permissible under non-inducing conditions and given the low copy character of the *nfsA*-bearing construct. The selection pressure created in such circumstances would account for the sole presence of transformants harboring mutated *nfsA* constructs in which the mutant population bearing a deletion in the promoter region was particularly emphasized. In other experiments attempting
to further characterize this phenomenon, it was observed that such mutants would back-transform into both DH5α and JM103 host strains with high efficiency as opposed to a wild-type construct for which the recovery of DH5α transformants would be drastically reduced compared to that obtained with the JM103 strain (data not shown). These findings necessitated the use of a high level of regulation for propagation and manipulation of the pQENfsA construct; this was provided by a co-expressed pREP4 construct.

The expression system used in this study allowed for expression and purification of NfsA enzyme with a 6X His tag complexed at the N-terminus of the protein thus increasing its size by 17 amino acid residues. This change may have contributed to alteration of the kinetic parameters of the enzyme, particularly $V_{\text{max}}$ (43 μmol·min$^{-1}$·mg enzyme$^{-1}$) and $K_M$ (72 and 22 μM for nitrofurazone and NADPH, respectively), as compared to native NfsA which reduces nitrofurazone with $V_{\text{max}}$ of 77 μmol·min$^{-1}$·mg enzyme$^{-1}$ and $K_M$ values for nitrofurazone and NADPH of 5.5 and 11.0 μM, respectively (Zenno et al., 1996b). Although the N-terminus of NfsA is exposed to the solvent and is not in direct contact with the active site (Figure 3.11), a flexible loop produced by the additional 17 amino acid residues could possibly interfere with targeting of the substrate to the active site through obstruction. It is conceivable that in such a case, $K_M$ values for the substrates would increase if the loop were effectively functioning as a competitive inhibitor. Other ways in which the added residues could alter enzyme function include subtle folding changes in the structure as well as affecting dimerization of enzyme in solution. In the latter case, it has been well-established that Frp exists in a monomer-dimer equilibrium in solution which is affected by enzyme concentration (Liu et al., 1997). Since many functional features of Frp are shared by NfsA,
extending this observation to NfsA suggests that additional residues present in His-tagged NfsA could possibly interfere with dimerization of NfsA subunits given the proximity of N-termini to the 2-fold axis observed in the model (Figure 3.11). Such an event would shift the monomer-dimer equilibrium towards the monomer form of NfsA which is most likely incapable of catalysis given the interlocking nature of the subunits and participation of both subunits in formation of the active site. It follows therefore that the amount of functional enzyme would be underrepresented in relation to its assayable amount and therefore the $V_{\text{max}}$ of the reaction would be lower. In addition, if NfsA monomers were able to bind nitrofurazone without reducing it, they would be effectively removing it from the pool of substrate available to functional dimers in which case the $K_M$ for nitrofurazone would be increased.

Random mutagenesis of pQEnfsA construct with NH$_2$OH yielded a limited diversity of mutants useful for downstream analysis. Most of the observed base substitutions were G:C $\rightarrow$ A:T transitions which are characteristic for this mutagenic agent (Stolarski et al., 1987). In 12 of the 19 selected mutants 8 discreet nonsense mutations were observed resulting in truncated transcripts. Examination of the $nfsA$ gene indicates the potential for a total of 55 of such distinct events in which a single base-pair substitution could result in a nonsense change. The fact that such changes accounted for over half of observed mutations in the collection may be attributed to the phenotypically silent character of missense mutations which may have escaped selection. In an attempt to enhance the selection of such “weaker” mutations, selection was carried out under noninducing conditions, in addition to inducing conditions, to maximize survival of the mutants. The efficacy of this selection approach is
difficult to judge, since the sample size of the mutants in which nitroreductase activity was assayed is too small. It is interesting to note that in this experiment the need for mutagenesis was dubious given the fact that a satisfactory mutant frequency ($1.0 \times 10^{-3}$) was obtained under noninducing conditions using pQEnfsA constructs not treated with NH$_2$OH. This finding may indicate that expression of NfsA occurs despite the highly repressible environment created by the pREP4 plasmid and that it would be significant enough to allow for selection of spontaneous mutants of NfsA. This argument is only applicable however if the phenomena of reduction of nitrofurans by NfsA to cytotoxic derivatives and ensuing cell death constitute the common and sole denominator for selection of mutants, nitrofurazone sensitivity, as well as nitroreductase activity as assayed in this study. Several pieces of evidence gathered in the study suggest that other factors may be involved. The first is the observation that two mutants truncated to 14 kDa displayed resistance to nitrofurazone low enough to suspect only moderately impaired nitroreductase function as shown in Figure 3.7. In this case it is quite difficult to conceive that a protein truncated to less than half its wild-type size would be able to form a structure capable of catalysis given the complex quaternary interactions required for formation of the active site as observed in Frp and the model of NfsA. Second, in one mutant (M430; Table 3.4) a null nitroreductase activity was associated with a relatively low level of resistance to nitrofurazone which represents a reverse of the previous situation. Therefore, other factors probably played a role in selection of mutants or determination of their level of resistance to nitrofurazone and, as mentioned in the previous chapter, may include modification of drug entry/transport at the cell membrane, detoxification of the drug or neutralization of the cytotoxic effects of its derivatives.
Since most mutants recovered in the study were insoluble, folding of the protein seems to play a predominant role in determining its functionality as a catalyst given the need for a proper quaternary fold to produce an active site as inferred from the structure of Frp and the NfsA model. In support of this, all the mutants which did not yield any soluble NfsA protein displayed high levels of nitrofurazone resistance suggesting low levels of \textit{in vivo} nitroreductase activity most likely attributable to improper folding. Further support in this regard is provided by the fact that two of amino acid substitutions observed in this study, Thr 108→Arg and Gly 154→Arg, occurred in mutants which did not produce any soluble NfsA. These substitutions introduced considerable strain into the structure, as predicted by the model, and therefore may have disrupted the aspects of native folding pathway critical to formation of a functional dimer, thus producing insoluble aggregates. Some of the other mutations associated with complete lack of solubility recovered in this study are quite subtle in character as they are distant from the active side and occur at the dimer surface as predicted by the model. These are particularly difficult to rationalize in the context of the model, given our limited knowledge of processes and forces involved in formation of tertiary and quaternary structures by proteins and resulting lack of plasticity of modeling software in addressing subtle structural alterations. As noted previously, the model of NfsA created in this study is heavily based on the crystal structure of its nearest sequence-homolog, Frp, and may not represent the unique structural aspects of NfsA. Some of these may be evident in the differences between the features of FMN and NAD binding observed in Frp and the NfsA model. Notably, some of the residues which participate in cofactor binding in Frp are involved in hydrogen bonding with other residues in the NfsA model only because of their
often marginally favorable proximity to form the latter interactions. These observations may give rise to false perceptions of existence or absence of structural features when indeed they represent intrinsic limitations of the modeling algorithm and as well as those of a small library of appropriate structural templates available. As an example of such a modeling limitation a defined hydrogen bond detection threshold used in analysis can be cited; a change in this value would predictably result in alteration of several interactions observed and characterized in the model.

An additional factor which may contribute to the observed differences in FMN binding between Frp and the NfsA model is the fact that the structural coordinates of the cofactor were manually imported from Frp. Therefore, the conformation of FMN and the resulting interactions it forms with the side chains are native to Frp and may not fit well in the context of NfsA as visualized in the model. A considerable range of FMN conformations is found among the flavoproteins in which the subunit chains forming the active crevice show significant plasticity. Such adjustments have been shown to influence the redox properties of flavins (Hasford et al., 1997), selectively stabilizing a particular oxidation state of flavin as required by the type of reaction catalyzed. Several interactions have been proposed to be involved in this process including electrostatic forces such as hydrogen bonding, \(\pi\)-stacking and van der Waals forces as well as control of solvation of the flavin (Breinlinger and Rotello, 1997; Niemz and Rotello, 1999). The involvement of different interactions is variable among different flavin systems. Restriction of solvation by burying FMN inside a hydrophobic pocket is generally associated with catalysis involving incremental transfer of electrons to FMN (flavoquinone \(\rightarrow\) flavosemiquinone) as observed
in flavodoxins, as opposed to performing two-electron transfer reactions characteristic of quinones (Niemz et al., 1997). Both electrostatic interactions and an apolar environment have been postulated to operate in thermodynamic stabilization of the flavosemiquinone form of FMN in flavodoxin from Desulfovibrio vulgaris thus allowing for a controlled one-electron redox cycling (Swenson and Krey, 1994; Chung Chang and Swenson, 1997). A single peptide flip was shown to be responsible for similar stabilization of flavosemiquinone in a crystal structure of flavodoxin from Desulfovibrio desulfiticans producing a change in the electrostatic nature of the active site rather than changing the conformation of the flavin isoalloxazine ring itself (Romero et al., 1996). In the latter case, the flavin was observed to be nearly planar in all three oxidation states which is unlike the conformation characterized in Frp in which the isoalloxazine ring is slightly bent in its oxidized state (Tanner et al., 1996). Some controversy still exists as to whether the observed conformations of protein-bound flavins mirror physiological situations, or represent artifacts of crystal packing, since according to molecular orbital theory an oxidized flavin, as exemplified by lumiflavin, should be planar regardless of its protonation state and any hydrogen bonding (Zheng and Ornstein, 1996). Whatever the case may be, the extensive hydrogen-bonding that exists between the flavin and peptide chain of Frp may well afford control of cofactor conformation, and thus modulation of its catalytic properties suited to physiological reactions of the enzyme. The same notion can be extended to NfsA in which the peculiarities of its active site, including cofactor conformation and binding, may reflect upon its catalytic profile. In support of this is the fact that in all NfsA mutants in which the cofactor characteristics were investigated, both the content of the flavin and its spectral features
varied. These however, did not have a predictable outcome on the nitroreductase activity suggesting that both abundance and the state of the cofactor are important determinants of enzymatic functionality.

Based on the model of NfsA used in this study, at least two factors may be implicated to operate in NfsA in control of FMN cofactor conformation and thus its redox properties. The first involves hydrogen bonding interactions which, though predicted by the model to be less extensive than in Frp, nevertheless occurs to an extent sufficient to anchor the cofactor in a fixed position and impose a defined conformation. Three missense mutations observed in this study (His 11→Lys, Arg 15→Cys and Gln 67→His) affected the FMN environment by weakening or disrupting hydrogen bonds between the cofactor and the protein subunits as visualized in the NfsA model. Although these mutants were isolated in the previous part of the study (Chapter 2) and were not characterized with respect to nitroreductase activity or spectral characteristics of the cofactor, it is reasonable to assume that these amino acid changes exerted negative effects on nitroreductase activity through alteration of FMN binding and/or its redox properties. In this respect, the structural predictions of the NfsA model are of satisfactory fidelity in addressing functional implications of the observed amino acid substitutions and support both the accuracy of the model as well as intimate structural relationships between NfsA and Frp.

The second factor that appears to be functioning in controlling the cofactor properties in NfsA is hydrophobic burying of the dimethylbenzene moiety of the isovaloxazine ring as well as its whole si face. This may seem to be of little importance when it is considered that NfsA catalyzes primarily two-electron reductions of the nitro group and one recalls that an
aprotic environment is generally associated with systems that transfer one electron at a time, as it allows for controlled access to protons. It is quite possible, however, that reduction of NfsA substrates other than the nitro group does proceed through a one-electron transfer route. If such a mechanism was operational under physiological conditions, it could constitute the basis for toxicity of NfsA observed when the enzyme was overexpressed in this experiment. A possible mechanism would involve indirect generation of \( \text{O}_2^- \) radicals in one-electron redox cycling reactions with consequential generation of oxidative stress. A NADP(H) flavin oxidoreductase from *E. coli*, flavodoxin (Fre), is indeed known to produce superoxide radicals *in vitro* and may be an important cytosolic site of their generation (Gaudu et al., 1994). Thus, paradoxically, the same elements which participate in cellular defense against oxidative damage from redox cycling may exert such harmful effects when removed from normal cellular control systems.

It is worthwhile to note that despite the extensive hydrogen bonding which anchor FMN cofactor in the active site of Frp, the plasticity of these interactions is much greater than one may infer. It has been generally believed that FMN binding in Frp is tight throughout the catalytic cycle as it is not likely that all 16 hydrogen bonds would be broken during hydride transfer, and it was also observed that protein-bound FMN is generally difficult to remove from solutions of Frp as well as NfsA. Recent studies indicate, however, that FMN may indeed completely dissociate from Frp and bind to luciferase during bacterial bioluminescence reaction in *V. harveyi* (Lei and Tu, 1998). This mechanism is suggested by preferential utilization of FMN cofactor by luciferase rather than FMN substrate reduced by Frp demonstrated using two different approaches. It is quite difficult to visualize how such
a transfer of cofactor would occur given its buried nature, but the monomer-dimer equilibrium of Frp may be a factor important in formation of a proposed Frp-luciferase ternary complex. This finding is a novel and surprising one in flavoprotein chemistry and exemplifies the flexible nature of interactions between the flavin and its cofactor.

The presence of NAD in the model is of a more limited value as it is an inhibitor of Frp due to its bent conformation that is not suited for catalysis (Tanner et al., 1999). It has been therefore proposed that unfolding of NADPH must occur for hydride transfer to FMN to occur as the active site is not large enough to accommodate a bent form of NADPH. In addition, the size limitation of the active site explains the ping-pong bisubstrate-biproduct kinetics of the flavin reduction as it would not be possible to simultaneously accommodate both NADPH and FMN substrate. The details of NADPH specificity characteristic of Frp/NfsA as well as the catalytic mechanism of flavoreduction remain hypothetical at this point.

Biochemical data indicate that NfsA and Frp belong to the same protein family as they share many common characteristics including size, kinetic profile, electron acceptor specificity, and most likely a dimer form as a functional complex. The model of NfsA constructed in this study on the basis of the structure of Frp was quite successful in rationalization of several amino acid substitutions observed in NfsA mutants in the structural context of the enzyme, yet it may fail in addressing some of the structural and functional subtleties of NfsA due to its inherent rigidity. It should be noted that the conformation of amino acid side chains in the modeled structure is based on thermodynamic modeling; in addition, the absence of additional elements, such as water, may be important in both a
functional and structural context. These facts combined with the importance of folding in
generation of a protein structure which can not be directly addressed by the modeling method
used in this study leaves some of the mutations in NfsA unaccounted for in terms of their
impact on the enzyme's functionality. The ultimate proof of whether NfsA is indeed a
structural member of the protein class represented by Frp will come from solving the crystal
structure of NfsA. Preliminary crystallization data of NfsA have not yet conclusively
determined a crucial piece of data as to whether NfsA is indeed present in a dimer form
under physiological conditions (Kobori et al., 1999). Such a finding would effectively
validate many of the parallel structural and functional aspects of Frp and NfsA drawn on the
basis of the model of NfsA in this study. So far, the only confirmed structural homolog of
Frp, based on its crystal structure, is NADH oxidase from *T. thermophilus* (Nox) which
displays some unique functional features not observed in Frp/NfsA, most notable of which
is the ability to use both FMN and FAD as cofactors (Hecht et al., 1995). This observation
illustrates the existence of functional freedom present among members of one structural class
in which changes as little as single amino acid substitutions can have marked effects on
catalytic spectrum. In this regard, several mutations analyzed in this study as well as evidence
of successful augmentation of flavin reductase activity in NfsA to a level exceeding that of
Frp by a single amino acid substitution (Zenno et al., 1998) are in support of existence of a
structural class heralded by Frp, Nox and NfsA.
General Conclusions
General Conclusions

In the first part of this study, the role of the nfsA gene in development of bacterial resistance to nitrofurazone derivatives was studied in a collection of first- (n=24) and second-step (n=57) furazolidone-resistant mutants produced in our laboratory. It was observed that all of the first-step mutants harbored mutations within the nfsA gene with 71% (n=17) of all mutational events resulting from insertional events. Bacterial insertion elements IS1, IS30 and IS186 were found to be responsible for the insertional mutagenesis, with 8, 6 and 3 observed events for each element, respectively. The sequence context for all IS1 insertion sites was found to be unremarkable, whereas all insertions of IS186 occurred at the same G+C rich site. IS30 also targeted a single insertion site within the gene determined to be its natural target sequence. The length of target duplications produced by insertion elements was variable for IS1, but fixed at 9 and 2 residues for IS186 and IS30, respectively. The orientation of all IS element insertions with respect to the nature of host sequence at the insertion site was unremarkable. Other mutations recovered from the collection included 4 missenses, 1 nonsense, 1 deletion and a single frameshift. All mutations characterized in the first-step mutants persisted in the corresponding second-step progeny in each case.

An extrachromosomal source of NfsA protein, provided by the pTAnfsA construct, brought about a dramatic increase in sensitivity to furazolidone when introduced into both first- and second-step furazolidone-resistant mutants. This fact, combined with the finding that mutations in NfsA were found to be necessary and sufficient in yielding first-step nitrofurazone-resistant mutants, provide convincing evidence for the involvement of NfsA in mediating bacterial resistance to nitrofurans. A parallel study conducted in our laboratory,
concerned with the role of NfsB in nitrofuran resistance (Whiteway et al., 1998), found similar relationships between mutations in NfsB and second-step levels of resistance to nitrofurans. These data strongly support the postulates of McCalla et al. (1978) implicating inactivation of the nfsA and nfsB genes in mediating bacterial resistance to nitrofuran derivatives.

In order to further characterize the relatively high contribution of insertional mutagenesis to the mutational spectrum observed in the collection of nfsA mutants, selection of nfsA mutants was performed in a different strain of *E. coli*, NR3835, as well as from an extrachromosomal source of the gene, pTAnfsA construct. In the first case a similar proportion of insertion events was observed in the nfsA gene indicating that the high rate of insertional mutagenesis within the chromosomal nfsA gene is strain-independent. The fact that only approximately 5% of mutants selected from pTAnfsA carried putative insertion elements within the episomal nfsA gene underscores the importance of local chromosomal factors in promoting transposition.

In the second part of the study, an expression construct of His-tagged NfsA, which allowed for rapid expression and purification of the enzyme, was created and was subjected to NH₂OH mutagenesis in an attempt to identify amino acid residues critical for NfsA function. The His-tagged NfsA enzyme was found to display similar characteristics to the native protein, described previously (Zenko et al., 1996b), including a ping-pong Bi-Bi mode of catalysis and the spectral characteristics of a FMN cofactor, but differed from the native enzyme with respect to *Vₘₐₓ* and *Kₐₘ* parameters for the reaction. Lower *Vₘₐₓ* and higher *Kₐₘ* values observed for His-tag NfsA may be attributed to possible interference of the extra
amino acid residues present in His-tag NfsA with dimerization of the monomers and/or substrate binding. NH₂OH mutagenesis of the construct produced a limited number of mutants useful for downstream analysis, as in many cases the mutant protein was insoluble. In mutants which produced soluble extracts of NfsA, the nitroreductase activity was found to be decreased in all cases; FMN content and characteristics also varied. The latter did not have a predictable effect on nitroreductase activity suggesting that both the state, or local environment, of the flavin as well as its abundance are important factors in determining the functionality of the enzyme.

In order to rationalize some of the effects of the mutations in NfsA produced in the NH₂OH experiment in a structural context, a model of NfsA, based on the crystal structure of its best sequence homolog (Frp), was constructed. The structure of NfsA as predicted by its model strongly resembled that of Frp and was not modified to any significant extent by including the only other suitable structural template, Nox. As visualized in the model, three amino acid substitutions in NfsA produced in the experiment were found to affect the flavin environment by disrupting or weakening hydrogen bonds between the flavin and the NfsA subunits; two other mutations introduced steric strain into the structure. These observations support the accuracy of the model as well as the closeness of the structural relationships between NfsA and Frp. Other mutations observed in the study were quite subtle in character and were not successfully addressed by the model, possibly due to their impact on the protein folding pathway.
Appendices
APPENDIX I. Wild-type sequence of the lacI gene in *E. coli* and the primers used in amplification of the gene.

-50 GACACCATCGAATGGCGCAAAAACCTTTTCGGCGGTATGGCATGATAGCGCGCCGGAAGAGAGTGTGACACCATCGAATGGTGCAAAACC

LacIS →

11 CAAATTCAGGTGGAATGTGAAACCCGAGTAAACGTTATACGTGATGCGAGATATGCGGT

71 GTCTCTTATCGACCCTTCGGGCTTGATGAACCAAGCGCCAGCCAGTTCTGACGAAAC

131 CGGGAAAAAGTGGAAGCGCGATGGCCGAGCTGAAATTACATCTCCAAAGCCTGGCGCAAA

191 CAACCTGGCGCGAAACACAGTGTTGCTGATGATGGCGGTGCGCCACTCTCAGTCGCGCCGAC

251 GCGCCGTGGCAAAATTTGTCCGCGCGGAAATTTTTAATCTCGCGCGACATCACGCTGGTGCGTG

311 GTTGTTGCATGTGGTGGAACCGGCGCGGCAATGGGTATATGTTCTGCTGTGCTGACGATGCGATG

371 TCCGCGCAAGCGCGCTAGTGCGATCTGATTACGTATACTCGCGATGGACGCGGCGCGGAGATGCG

431 GCTGGGAAGCTGTCGCTGATGCAATGTGGCCGCTTATTCTTCTTCTGATGCTCTCTGACGAGACA

491 CCAATCAACAGTATTATTATATTCCCGCATGAAAGCGCGGCTGCGGCTGAGACGCGATGCG

551 GTCCGATTTGGTCACCAGCAGGCTGCATGGGTGAGCAGCGGCGGCGCAATAAGTTCTGCTGCTGCGG

611 GCTCTCGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG

671 GAACGGGAAAGCGCACTGGAGTGCCATGTCGGGTTACTCAACAAACATCGAATATGCTGAAAT

731 GAAGGCAATCTCCTCCACATCGGATCTGCTGCTGGCAACAGATCAGATGGCGCTGGCGGCAATG

791 CGGCACCTTTACCGATCCGGGCTGCGCGGTTGCGCGGATCTCGCGGATGCTGCGGCAATGCTGCGG

851 GATACCGGAAACAGCGCTCATGTATATCCGCGCGTCAAACCCATGAAACAGGATTGTGCGCCG

911 GCTGCTGGGCAAAAAGCGCGGCTGCGGCTGCGGCTGCGGCTGCGGCTGCGGCTGCGGCTGCGG

971 GCCAATCGCTGTTGCCGCTTCATTCTCTGGGATGATGATGATGATGATGATGATGATGATGATG

1031 CAAACCGCGCTTCTCCGCGCGTGGCGCCGATCTTATGCGACTGCGCGCGCGCGATGCG

1091 CGACTGAAAAGCGGCGATGACGGCGCAACCGCAATTATATGTGATTGACTACTCATTAGCG

1151 ACCCAGGCTTATACCTTTATGTGCCTCGCTGATGTGGTGGATATGTGAGCGGATA

1211 ACAATTTTCACACAGGAACAGCTATGACCATGTAGTGATGATGCTGCGTATGTGCTGCTTGGTACG

TGTAAAGTGTGCTCCTTTTGTCGA

← PL
APPENDIX II. Calculation of $V_{\text{max}}$ and $K_m$ constants for nitrofurazone and NADPH in reactions catalyzed by NfsA

Double-reciprocal expression for ping-pong Bi-Bi mode of catalysis is:

$$\frac{1}{v} = \frac{1}{V_{\text{max}}} \left( 1 + \frac{K_{M2}}{[S_2]} \right) + \frac{K_{M1}}{V_{\text{max}}} \frac{1}{[S_1]} \quad (1)$$

where $V_{\text{max}}$ is the true maximal velocity for the reaction, whereas $K_{M1}$ and $K_{M2}$ represent Michaelis constants for substrates $S_1$ and $S_2$, respectively.

Taking $S_1$ and $S_2$ as nitrofurazone and NADPH, respectively, and constructing a double reciprocal Lineweaver-Burk plot of $1/v$ vs. $1/[S_1]$ (Figure 3.5 A) for three different $[S_2]$ yields the following:

<table>
<thead>
<tr>
<th>[NADPH] (μM)</th>
<th>100</th>
<th>75</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slope</td>
<td>1.642±0.043</td>
<td>1.654±0.047</td>
<td>1.653±0.048</td>
</tr>
<tr>
<td>Y-intercept</td>
<td>0.0279±0.0020</td>
<td>0.0303±0.0021</td>
<td>0.0331±0.0022</td>
</tr>
<tr>
<td>X-intercept</td>
<td>-0.0170</td>
<td>-0.0183</td>
<td>-0.0200</td>
</tr>
</tbody>
</table>

The Y-intercept in the above plot represents an apparent $V_{\text{max}}$ ($V'_{\text{max}}$) given by:

$$\frac{1}{V'_{\text{max}}} = \frac{1}{V_{\text{max}}} \left( 1 + \frac{K_{M2}}{[S_2]} \right) \quad (2)$$

which can be simplified to:

$$\frac{1}{V'_{\text{max}}} = \frac{1}{V_{\text{max}}} + \frac{K_{M2}}{V_{\text{max}} [S_2]} \quad (3)$$

Replotting $1/V'_{\text{max}}$ vs. $1/[S_2]$ in a secondary plot yields a line with a Y-intercept of $1/V_{\text{max}}$ and X-intercept of $-1/K_{M2}$ (Figure 3.5 B).

From Figure 2.5 B, Y- and X-intercepts are $0.0231±0.0013$ (μmol·min·mg enzyme) and $-0.0454$ (μM)$^{-1}$ thus giving $V_{\text{max}}$ and $K_{M2}$ (NADPH) of $43.3±2.4$ (μmol·min·mg enzyme$^{-1}$) and 22 μM, respectively.

$K_{M1}$ can be obtained from the slope in Figure 3.5 A, which is of the form $K_{M1}/V_{\text{max}}$ and averages to 1.650 this yielding:

$K_{M1}$ (nitrofurazone) = $V_{\text{max}}$·slope = $43.3 · 1.650 = 71.5$ μM

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References
References


Lei, B. and S-C. Tu. 1998. Mechanism of reduced flavin transfer from *Vibrio harveyi* NADPH-FMN oxidoreductase to luciferase. *Biochemistry* 37: 14623-14629


