Hypersensitivity of *Escherichia coli* Δ(*uvrB-bio*) Mutants to 6-Hydroxylaminopurine and Other Base Analogs Is Due to a Defect in Molybdenum Cofactor Biosynthesis

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We have shown previously that *Escherichia coli* and *Salmonella enterica* serovar Typhimurium strains carrying a deletion of the *uvrB-bio* region are hypersensitive to the mutagenic and toxic action of 6-hydroxylaminopurine (HAP) and related base analogs. This sensitivity is not due to the *uvrB* excision repair defect associated with this deletion because a *uvrB* point mutation or a *uvrA* deficiency does not cause hypersensitivity. In the present work, we have investigated which gene(s) within the deleted region may be responsible for this effect. Using independent approaches, we isolated both a point mutation and a transposon insertion in the *moeA* gene, which is located in the region covered by the deletion, that conferred HAP sensitivity equal to that conferred by the *uvrB-bio* deletion. The *moeA* operon provides one of a large number of genes responsible for biosynthesis of the molybdenum cofactor. Defects in other genes in the same pathway, such as *moa* or *mod*, also lead to the same HAP-hypersensitive phenotype. We propose that the molybdenum cofactor is required as a cofactor for an as yet unidentified enzyme (or enzymes) that acts to inactivate HAP and other related compounds.

The biological effects of many mutagenic agents are due to DNA base modifications, both in the DNA and the DNA precursor pool. A group of mutagens containing a preformed modified base, often referred to as base analogs (14), have received increasing attention recently. For example, 8-oxoguanine, in the form of 8-oxo-dGTP or 8-oxo-GTP, is a spontaneously arising guanine oxidation product that contributes substantially to the infidelity of DNA replication (13, 35, 37) or transcription (58). Specialized systems protecting the cell against 8-oxoguanine have been found in organisms from bacteria to humans (for reviews, see references 13 and 37), including the MutT enzyme, which is capable of hydrolyzing 8-oxo-dGTP, an activity referred to as pool sanitizing (2, 35). Other examples of mutagenic precursor pool contaminants are 5-hydroxy-dCTP (12) and 2-hydroxy-dATP (15), both oxidative stress products. The human MutT homolog hMTH1 has strong activity towards 2-hydroxy-dATP, suggesting that it, in addition to 8-oxo-GTP, could be an important threat if not actively removed (15). In addition, base analogs can be useful tools for probing the mechanisms of mutation avoidance during DNA replication, including base-base discrimination by DNA polymerases (51, 54).

An important group of base analogs are the N-hydroxy derivatives of adenine and cytidine (see reference 27 for a review). For example, 6-hydroxylaminopurine (6-HAP) and 2-aminooxy-6-hydroxylaminopurine (AHAP) are very powerful mutagens in phage, bacteria, yeast, and eukaryotic cells (42, 43), and they have been termed universal mutagens (42). These adenine derivatives are active when provided as bases or, in some organisms, nucleosides, as they are apparently converted efficiently into the corresponding deoxyribonucleoside triphosphates (dNTPs), which are then incorporated into DNA by DNA polymerase. Because of the ambiguous base pairing properties of these dNTPs, their incorporation is highly mutagenic.

We have previously performed studies on the genetic requirements of HAP mutagenesis in the bacterium *Escherichia coli* for the purpose of understanding at which levels cells may try to prevent mutagenesis by this and related agents (43). We found little or no protection by the exonucleolytic proofreading (*dnaQ* gene) or the postreplicative mismatch repair system (encoded by the *mutHLS* genes), two systems that play important roles in preventing mutations resulting from the mispairings of normal bases (50). This lack of discrimination is likely one of the reasons for the strong mutagenic potential of HAP. However, a strain carrying a deletion of the chromosomal *uvrB-bio* region had hypersensitivity to HAP for both mutagenesis and toxicity, implying the existence of a protective system. The deletion also conferred sensitivity to AHAP (43) and other analogs (31). Sensitivity of a *uvrB-bio* deletion strain for AHAP and related compounds has also been found in *Salmonella enterica* serovar Typhimurium (23–25). However, hypersensitivity is not conferred by the *uvrB*5 point mutation (43) or two different *uvrA* deficiencies (23, 24, 43). This argues against the *uvrABC* excision repair system being responsible for protection against HAP and related compounds. We concluded that certain genes in the *uvrB-bio* region, other than *uvrB*, were responsible for the observed sensitivity. In the present report, we investigate the nature of the gene or genes within this region of the *E. coli* chromosome that are responsible for this enhanced base analog sensitivity. The results point to an important role of the molybdenum cofactor, presumably through the action of a molybdenum cofactor-containing enzyme activity.

**MATERIALS AND METHODS**

**Bacterial strains, phage stocks, and plasmids.** Table 1 lists many of the *E. coli* strains used in this study along with their genotypes. Δ(*uvrB-bio*) of strain...
NR10107 is bioΔ261 of strain C261 (5). Strains NR10835 and NR10836 were created by introducing the F′pro-lac from strains CC105 and CC106 (9) into KA796 by conjugation. For convenience, these F′s were designated F′CC105 and F′CC106, respectively. NR11958 was obtained by localized mutagenesis of the bio region as described below. NR10771 is identical to NR11958 but contains, in addition, transposon insertion zbi-29:Tn6. It was constructed by inserting zbi-3058:Tn10 from CAG12034 by P1 transduction into NR11958, followed by retransduction of the moaA121::Tn10 insertion into KA796 (~5% linkage). Retention of the moaA (mut-1) allele was ascertained by checking individual transductants for HAP sensitivity using the spot test described below. NR12066 is a spontaneous streptomycin-resistant derivative of KA796. The derivation of NR12833 carrying a mini-Tn10cam insertion in moaA is described in detail below. Strains LC832 and NR1076 were transferred with a nearby Tn10 insertion and then transducing the moaA121::Tn10 combination into KA796, moaC4 was transferred with nadA57::Tn10 from CAG12034 by P1 transduction into NR11958, followed by retransduction of the moaA121::Tn10 insertion into KA796 (~5% linkage). Retention of the moaA (mut-1) allele was ascertained by checking individual transductants for HAP sensitivity using the spot test described below. NR12032 is a spontaneous streptomycin-resistant derivative of KA796. The derivation of NR12833 carrying a mini-Tn10cam insertion in moaA is described in detail below.

Isolation of a HAP-hypermutable strain by random mini-Tn10cam insertion mutagenesis. A random library of mini-Tn10cam insertions was obtained in strain NR10835 using phage λNK1324 as described by Kleckner et al. (29). A total of 6,000 chloramphenicol-resistant colonies were inoculated individually in wells of 96-well microtiter dishes (0.2 ml per well) containing LB with 0.50 μg of HAP/ml. After overnight growth, 10 μl from each well was spotted on an LB-riparian plate. Under these conditions, a HAP-hypermutable strain such as NR10107 produces about 30 rifampin-resistant mutants per spot, whereas a control strain produces none. Among the 6,000 isolates, one displayed high mutability in the presence of HAP but not in its absence. The responsible mini-Tn10cam insertion was transduced into NR12006 and Hfr mapping was performed using the tetracycline-resistant Hfr strain set as described by Singer et al. (57). This analysis placed the insertion site in the 10- to 20-min region of the E. coli chromosome. The allele was transferred with a nearby Tn10 insertion and then transducing the moaA121::Tn10 combination into KA796, moaC4 was transferred with nadA57::Tn10 from CAG12034 by P1 transduction into NR11958, followed by retransduction of the moaA121::Tn10 insertion into KA796 (~5% linkage). Retention of the moaA (mut-1) allele was ascertained by checking individual transductants for HAP sensitivity using the spot test described below. NR12032 is a spontaneous streptomycin-resistant derivative of KA796. The derivation of NR12833 carrying a mini-Tn10cam insertion in moaA is described in detail below.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source or reference</th>
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<tbody>
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<td>ara thi Δ(pro-lac)</td>
<td>52</td>
</tr>
<tr>
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<td>43</td>
</tr>
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<tr>
<td>NR10836</td>
<td>ara thi Δ(pro-lac) F′CC106</td>
<td>This work</td>
</tr>
<tr>
<td>NR11958</td>
<td>ara thi Δ(pro-lac) moaA120(Am)</td>
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<td>ara thi Δ(pro-lac) StrR</td>
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<td>NR12383</td>
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</tr>
<tr>
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RESULTS

A previous study on the genetic factors controlling mutagenicity and sensitivity to the base analog HAP revealed that strains carrying a deletion of the uvrB-bio region were hypersensitive to this agent (43). This hypersensitivity was not due to the uvrB excision repair defect (43), and we therefore undertook a search for the identity of the responsible gene(s) within the deleted area. Due to its large size (about 2 min) and undefined right endpoint (5) (Fig. 1A), the deletion was not readily suited for mapping and identification of the gene. We therefore embarked on the isolation of additional HAP-sensitive mutant alleles that would be more suitable for mapping procedures. Below, we describe two independent approaches, localized mutagenesis of the bio region, yielding a chemically induced point mutant, and a genome-wide search for a HAP hypermutability mutant, yielding a mini-Tn10cam insertion.

HAP hypersensitivity due to a moeA point mutation. A HAP-sensitive point mutant with a mutation in the uvrB-bio region was obtained by hydroxylamine-mediated localized mutagenesis of the bio region (see Materials and Methods). Testing of 50 Bio" transductants of strain NR10107 [Δ(uvrB-bio)] for hypersensitivity to the toxic effects of HAP yielded one mutant, initially called the mut-1 strain, whose sensitivity was indistinguishable from that of the deletion strain (see Fig. 2). This mutant strain, NR11958 (mut-1), also proved to be hypermutable by HAP (Table 2). P1 transductional mapping using known transposon insertions in this region (57) placed the responsible gene at around 18.8 min between insertions zbi-3108::Tn10kan (or zbi-29::Tn10) and zbi-3058::Tn10, located, respectively, at 17.67 and 19.34 min of the E. coli map (39) (Fig. 1A).

Further mapping of the mut-1 mutation was performed via recombination experiments (see Materials and Methods) with

<table>
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<th>Strain</th>
<th>Genotype</th>
<th>Avg mutant frequency</th>
<th>HAP concentration (µg/ml)</th>
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<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
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<td>Wild type</td>
<td>1.1</td>
<td>1.2</td>
</tr>
<tr>
<td>NR10107</td>
<td>Δ(uvrB-bio)</td>
<td>0.4</td>
<td>120</td>
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<tr>
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<td>moeAI20 (mut-1)</td>
<td>1.8</td>
<td>710</td>
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<td>NR12383</td>
<td>moeAI21::mini-Tn10cam</td>
<td>1.1</td>
<td>390</td>
</tr>
</tbody>
</table>

* See Table 1.
* Based on 12 independent cultures for each strain at each dose.
Kohara phages 206 through 211, which cover the region of the chromosome between 18.2 and 19.8 min (30, 49). This revealed that the HAP sensitivity of \textit{mut-1} could be overcome by recombination with phages 207 and 208, indicating that the putative HAP resistance gene was located on the overlap region of these two. This region has five open reading frames, the hypothetical genes \textit{ybiZ}, \textit{ybiK}, and \textit{yliA} and the two known genes constituting the \textit{moeAB} operon (18.6 min) (40, 49). The \textit{moe} operon is one of five operons (\textit{moa}, \textit{mob}, \textit{mod}, \textit{moe}, and \textit{mog}) responsible for molybdopterin (MPT) biosynthesis, an essential cofactor for several oxidoreductase enzymes (47).

These genes, collectively called \textit{mol} (53), were previously named \textit{chl} (for chlorate resistance), referring to the ability of strains lacking the molybdenum cofactor to grow under anaerobic conditions in the presence of chlorate, that cause the killing of wild-type \textit{E. coli} due to the production of lethal chloride (53). We found that both NR11958 (\textit{mut-1}) and NR10107 [\textit{uvrB-bio}] were chlorate resistant (see Materials and Methods), whereas the parental KA796 was chlorate sensitive. This suggested that the \textit{mut-1} defect resided in \textit{moeAB}.

The \textit{moeA} gene was cloned from Kohara phage 207 on plasmid pMoeA1 (see Materials and Methods) and introduced into strain NR11958 (\textit{mut-1}). The plasmid fully complemented the \textit{mut-1} defect because it made the strain HAP resistant and chlorate sensitive, consistent with the \textit{mut-1} mutation residing in the \textit{moeA} gene. pMoeA1 did not affect the HAP sensitivity and chlorate resistance of deletion strain NR10107. As seen in Fig. 1A, the \textit{uvrB-bio} deletion also comprises the \textit{moeAB} operon (as well as \textit{moeB}). This observation suggests strongly that HAP resistance as observed in a wild-type strain requires the active molybdenum cofactor and argues against an individual activity of \textit{moeA} towards HAP. DNA sequencing of the \textit{moeA} gene of NR11958 revealed a TGG → TAG mutation creating an (amber) nonsense codon at position Trp274, resulting in a truncation of the MoeA protein (Fig. 1B). The \textit{mut-1} allele was then renamed \textit{moeA120}(Am).

In addition, we tested the HAP sensitivity of established \textit{moeA} mutant \textit{moeA5} (formerly called \textit{chlE5}) (40). The mutation was transferred from strain JRG97 into KA796 (see Materials and Methods). This new strain was also HAP sensitive, in addition to its previously described chlorate resistance (40). Both the HAP sensitivity and chlorate resistance of this new strain were complemented by plasmid pMoeA1.

**HAP Hypersensitivity due to a mini-Tn10 Insertion in \textit{moeA}**

A HAP-sensitive mutant resulting from a mini-Tn10 insertion was sought as described in Materials and Methods. Six thousand random chromosomal mini-Tn10cam insertions were obtained, and the isolates were individually tested for hypermutability in the presence of HAP, scoring for high levels of rifampin-resistant mutants. One isolate (NR12383) whose spontaneous-mutant frequency was indistinguishable from that of the wild-type strain but that was hypermutable in the presence of HAP was obtained (Table 2). The mutant was also hypersensitive to killing by HAP and was chlorate resistant (Fig. 2). Mapping the insertion using \textit{Hfr} crosses and \textit{F1} transductions placed the responsible insertion between the \textit{zbi-29} (\textit{zbi-3108}) and \textit{zbi-3038} markers, as was the case for \textit{moeA120} (Fig. 1A). The HAP sensitivity was complemented by plasmid pMoeA1, corroborating that the insertion was in the \textit{moeA} gene. The location of the insertion site in \textit{moeA} was then determined by DNA sequencing (see Materials and Methods) and was found to be between nucleotides A280 and C281 (Fig. 1B). The allele was called \textit{moeA121::mini-Tn10cam}. Thus, using two independent approaches, we obtained two HAP-hypersensitive strains, each carrying a mutation in the \textit{moeA} gene.

**Mutations in other \textit{mol} genes also confer HAP sensitivity.**

The \textit{moeAB} operon is only one of several \textit{mol} genes and operons involved in the biosynthesis and activation of the molybdenum cofactor (47). We investigated whether HAP sensitivity is also conferred by other deficiencies in the biosynthesis of active molybdenum cofactor, including those due to mutations in \textit{moa} (17.6 min) and \textit{mod} (17.1 min) (Fig. 1A). These were initially tested in their original backgrounds (as obtained from the \textit{E. coli} Genetic Stock Center). This testing revealed significant variations in sensitivity even in the control strains for several of these strains, and we therefore transferred the alleles into our customary KA796 background (Table 1). The results in this background showed that the \textit{moaA1}, \textit{moaA18}, and \textit{modC4} alleles confer HAP hypersensitivity (Fig. 3) in addition to chlorate resistance (not shown). Thus, HAP sensitivity is due to the lack of an active molybdenum cofactor. Apparently, the molybdenum cofactor is required for some enzymatic activity protecting \textit{E. coli} against the toxic and mutagenic effects of HAP.

**DISCUSSION**

In this study, we have shown that mutations in \textit{moeA}, \textit{moaA}, and \textit{modC} lead to hypersensitivity to the base analog HAP. All these genes are involved in biosynthesis of the molybdopterin (MPT) guanine dinucleotide (MGD) cofactor, the essential molybdenum-containing cofactor for \textit{E. coli} molybdoenzymes (47). Synthesis of MGD proceeds in a number of steps. The genes of the \textit{moa} operon are responsible for the multistep synthesis of MPT, a dithiolene pterine derivative common to the molybdenum cofactor in all organisms. The \textit{mog} gene is responsible for inserting activated molybdenum into MPT (26), and the \textit{mobAB} operon is responsible for adding GMP to MPT to yield MGD (47). The \textit{mod} operon encodes a high-affinity molybdenum importer (11). Among the \textit{moeAB} products, MoeB is responsible for resulfurylation of MoaD, which provides the thiol groups on MPT. The function of \textit{moeA},...
highlighted here by two new HAP-sensitive, chlorate-resistant mutants, is not well known, but recent data have suggested a role in sulfurylation of molybdenum and generation of an activated form, possibly thiomolybdate (18). Thus, our results show that HAP hypersensitivity can result from defects in enzymes working at several stages of MGD biosynthesis. This argues that the molybdenum cofactor is required for base anabolic detoxification, most logically as a cofactor of a detoxifying enzyme.

At this time, the nature of the molybdenum-requiring enzyme responsible for HAP detoxification is unknown, as is the inactivating reaction. Molybdoenzymes comprise a broad, heterogeneous group, present in organisms from bacteria to humans, that assist in a variety of oxidation/reduction reactions (for a review, see reference 28). So far, nine *E. coli* molybdoenzymes have been described (see reference 16 for a review): DMSO reductase, TMAO (trimethylamine-N-oxide) reductase, biotin sulfoxide reductase, three nitrate reductases (NRA, NRZ, and NAP), and three formate dehydrogenases (FDH-N, FDH-H, and FDH-O). Most of these represent activities limited to anaerobic cells and controlled by the Frn oxygen sensor (17, 56). They are therefore unlikely candidates for the HAP-inactivating activity deduced by our aerobic experiments. Nevertheless, we have considered the possibility that a low basal level of either DMSO reductase (*dmsABC* product) (62) or TMAO reductase (*torCAD* product) (36), both anaerobic activities, could be responsible for our observed effect. Both enzymes have been shown capable of reducing a wide range of S- and N-oxides, including adenosine-5'-oxide and hydroxylamine (16, 62, 64). However, defective *dms* or *tor* mutants tested by us did not show increased HAP sensitivity (data not shown). The three known aerobic *E. coli* molybdoenzymes are biotin sulfoxide reductase (*BisC*) (10, 44, 45), the minor respiratory nitrate reductase NRZ (8, 22), and the minor formate dehydrogenase FDH-O (8, 16). A defective *bisC* mutant (10) tested by us was not HAP sensitive (data not shown). Thus, although not all known *E. coli* molybdoenzymes have been tested, the activity responsible for HAP inactivation most likely represents an as yet unidentified aerobic molybdoenzyme.

Clement and Kunze (7) described the reduction of HAP to adenine by xanthine oxidase, a well-studied mammalian molybdoenzyme which oxidizes hypoxanthine and xanthine to uric acid (19). Apparently, HAP is a compound capable of serving as an electron acceptor for this reaction. No defined xanthine oxidase in *E. coli* has been described (but see reference 63). Interestingly, the sequencing of the *E. coli* genome revealed three open reading frames whose proteins have some homology to known xanthine oxidases (33). These proteins may be candidates for a putative HAP reductase activity. The alternative possibility of a HAP-oxidizing activity must be left open as well.

The question arises why *E. coli* and *Salmonella* contain an activity to inactivate HAP. The activity is strong, at least based on a comparison of HAP-induced mutant frequencies in wild-type and molybdenum cofactor-defective strains. This difference is at least 100- to 1,000-fold (24, 43) (Table 2), suggesting that the activity is capable of destroying 99 to 99.9% of all HAP. The HAP-inactivating activity may be a side reaction of the as yet undescribed oxidation/reduction activity or, alternatively, targeted primarily at destroying HAP. HAP has been shown to be produced enzymatically from adenine by hepatic microsomal N-hydroxylation (6), and similar reactions could occur in *E. coli*. The intracellular enzymatic usage of hydroxylamine in purine biosynthesis could also lead to HAP production (3, 34). Interestingly, HAP may also be produced by DNA oxidation resulting from oxidative stress. Experiments exposing DNA and DNA bases to peroxyl radical (ROO•–), a major intracellular oxidant and oxidative stress product, showed HAP to be the major product (55).

Another suggestion that HAP may be physiologically relevant is the discovery in *Saccharomyces cerevisiae* of a putative dNTPase (*HAM1* gene product) involved in protection against HAP (41). The defective *ham1* mutant is hypersensitive to HAP, and a deoxy-HAP-triphosphatase activity for the Ham1 product is inferred based on certain homologies with known dNTPases (32). The homologous enzyme from *Methanococcus jannaschii* is capable of hydrolyzing nonstandard nucleotides such as xanthine triphosphate and inosine triphosphate to the corresponding monophosphates (21). Thus, protection against the mutagenic activity of HAP could take place on at least two levels by (i) oxidation/reduction of the base moiety as suggested by the present study and (ii) hydrolysis of the most dangerous form, the triphosphate.

The Δ(*uvrB-hio*) strain is sensitive not only to HAP but also to AHAP, hydroxylamine, and N4-hydroxyctydine (4-OH-C) in a manner that appears independent of the excision repair system (31, 42, 43). Thus, molybdoenzymes play a general role...
in the protection against N-hydroxylated base analogs and related compounds. It is of interest to note that a deletion of the uvrB-bio region is carried by several of the Ames tester strains of \textit{S. enterica} serovar Typhimurium, such as TA1530, TA98, and TA100 (1). These strains have been shown to be hyper-sensitive to the mutagenic action of HAP, AHAP, and 4-OH-C (23–25). The present data suggest that the simple interpretation of hypermutability of ΔuvrB strains in terms of susceptibility of the mutagenic agent to nucleotide excision repair is not always warranted. It is noted that N-hydroxylated compounds (hydroxylamines) are the active intermediates for the mutagenic action of both aromatic nitro compounds (by reduction) and aromatic amines (by oxidation) (48).

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