TusA (YhhP) and IscS are required for molybdenum cofactor-dependent base-analog detoxification

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Abstract
Lack of molybdenum cofactor (Moco) in Escherichia coli leads to hypersensitivity to the mutagenic and toxic effects of N-hydroxylated base analogs, such as 6-N-hydroxylaminopurine (HAP). This phenotype is due to the loss of two Moco-dependent activities, YcbX and YiiM, that are capable of reducing HAP to adenine. Here, we describe two novel HAP-sensitive mutants containing a defect in iscS or tusA (yhhP) gene. IscS is a major L-cysteine desulfurase involved in iron–sulfur cluster synthesis, thiamine synthesis, and tRNA thio-modification. TusA is a small sulfur-carrier protein that interacts with IscS. We show that both IscS and TusA operate within the Moco-dependent pathway. Like other Moco-deficient strains, tusA and iscS mutants are HAP sensitive and resistant to chlorate under anaerobic conditions. The base-analog sensitivity of iscS or tusA strains could be suppressed by supplying exogenous L-cysteine or sulfide or by an increase in endogenous sulfur donors (cysB constitutive mutant). The data suggest that iscS and tusA mutants have a defect in the mobilization of sulfur required for active YcbX/YiiM proteins as well as nitrate reductase, presumably due to lack of functional Moco. Overall, our data imply a novel and indispensable role of the IscS/TusA complex in the activity of several molybdoenzymes.

Introduction
Base analogs are modified nucleobases that can substitute for the natural bases in cellular metabolism, and their involvement can lead to toxic and/or mutagenic consequences. Base analogs may be generated in vivo during normal cellular metabolism or by the action of certain chemical and physical factors, such as alkylating agents or ionizing radiation. An important group of mutagenic base analogs are the N-hydroxylated derivatives of purines and pyrimidines, such as 6-N-hydroxylaminopurine (HAP) (Fig. 1A), 2-amino-HAP (AHAP), and N4-hydroxyctydine (HC). These agents have been shown to be strong mutagens in bacteria, yeast, and mammalian cells (Barrett 1981; Pavlov et al. 1991; Kozmin et al. 1998; Kozmin et al. 2000). HAP can be produced enzymatically from adenine by hepatic microsomal N-hydroxylation (Clement and Kunze 1990) and from the inadvertent use of hydroxylamine in purine biosynthesis (Lieberman 1956). It has also been shown to be a major product of exposure of DNA and nucleobases to peroxyl radicals (Simandan et al. 1998).

Our previous studies have shown that Escherichia coli strains lacking molybdenum cofactor (Moco) are hyper-sensitive to the toxic and mutagenic action of HAP (Fig. 1A) and related N-hydroxylated analogs, including AHAP, HC, and hydroxylamine (NH₂OH) (Kozmin et al. 2000; Kozmin and Schaaper 2007). Subsequently, we demonstrated that Moco-dependent base-analog detoxification is due to action of two Moco-dependent enzymes, YcbX and YiiM, which were shown capable of reducing HAP to nontoxic adenine (Kozmin et al. 2008). Further genetic studies revealed the participation of CysJ flavin
Figure 1. Base-analog sensitivity of Escherichia coli deficient in Moco biosynthesis, previously described Moco-dependent pathways for HAP detoxification, and structure of Moco. (A) The chemical structure of HAP and the results of a spot test when HAP (10 μg) was placed in the center of a plate seeded with either the wild-type (wt) strain (NR10836) or its Moco-deficient (ΔmoaE) derivative (NR16523) using a multiprong replicator device (see Experimental Procedures). (B) Genetic pathways for Moco-dependent base-analog detoxification. The diagram, adapted from Kozmin et al. (2008) and Kozmin et al. (2010), shows some of the synthesis steps of Moco, as well as its use in MPT pathways for the indicated substrates. The latter two perform in conjunction with flavin reductases CysJ (Kozmin et al. 2010) and Fre (S. G. Kozmin, E. I. Stepchenkova, J. Wang, and R. M. Schaaper, unpublished data). A third (minor) Moco-dependent HAP-detoxification activity was discovered in a ycbX, yiiIM-deficient background, which was identified as the bisC gene product (biotin sulfoxide reductase) (Kozmin et al. 2008). The three established pathways for HAP detoxification are outlined in Figure 1B. One important difference between the ycbX and yiiIM pathways and the bisC pathway relates to the precise structure of the Moco. Most E. coli molybdoenzymes, including BisC, belong to the dimethylsulfoxide (DMSO)-reductase family, which utilize a molybdopterin (MPT)-guanine-dinucleotide (MGD) form as cofactor (Iobbi-Nivol and Leimkühler 2012). While the structure of the cofactor in YcbX and YiiM is not yet established, it is clear that it does not require mobA-dependent MGD formation, and it may represent a simple MPT form (Fig. 1B and C) (Kozmin et al. 2008). Consequently, the YcbX and YiiM proteins were suggested to represent members of a distinct novel family of molybdoenzymes (Anantharaman and Aravind 2002; Kozmin et al. 2008). Two mammalian mitochondrial proteins, mARC1 and mARC2, are likely members of the same family (Anantharaman and Aravind 2002; Kozmin et al. 2008). As part of a continuing investigation of the cellular factors that determine resistance to toxic N-hydroxylated compounds, and to better understand the regulation of Moco and Moco-dependent enzymes, we have undertaken further searches for genetic mutants of E. coli that display altered sensitivity to the base-analog HAP. Here, we describe the properties of two such mutants, tusA and iscS, that express increased sensitivity to HAP and related compounds. While iscS encodes a L-cysteine desulfurase involved in various sulfur-dependent activities (Fontecave et al. 2008; Roche et al. 2013), such as iron–sulfur cluster biosynthesis, the only known function for TusA is as a sulfur carrier in the thiromodification of certain tRNAs, where it operates in complex with IscS (Ikekuchi et al. 2006). Our results reveal a novel correlation between the activity of Moco-dependent enzymes and cellular sulfur metabolism.

Experimental Procedures

Media and chemicals

Bacteria were cultivated in Luria-Bertani (LB) broth (Miller 1972) or minimal Vogel–Bonner medium (VB) (Vogel and Bonner 1956) containing 0.2% glucose as car-
bon source and supplemented with 12.5 \( \mu g/mL \) of nicotinamide and 1 \( \mu g/mL \) of thiamine. When indicated, minimal media was also supplemented with 0.33 mmol/L L-cysteine or 2 mmol/L sodium sulfide. Solid media contained 1.5% agar. For selection of antibiotic-resistant clones, media was supplemented with 35 \( \mu g/mL \) of kanamycin or 15 \( \mu g/mL \) of tetracycline, or 100 \( \mu g/mL \) of rifampicin. HAP, in form of free base, was purchased from Midwest Research Institute (Kansas City). All other chemicals were from Sigma-Aldrich.

**Bacterial strains**

The *Escherichia coli* strains used in this study are listed in Table 1, along with their source or derivation. All mutagenesis and base-analog sensitivity tests were performed using strain NR10836 and its mutant derivatives. The *tusA::kan* mutant was obtained from a genome-wide search for HAP-sensitive mutants using the EZ-Tn5\(^{TM}\) <R6Kytori/KAN-2>Tnp Transposome\(^{TM}\) Kit from Epicentre, Madison, WI. The ΔiscS, ΔiscU, ΔiscA, ΔiscUA, ΔsufS, ΔsufA, ΔcsdA(b2810), ΔnfuA, ΔtusBCD, and ΔtusE deletions were generated in strain BW25113/pKD46 by the polymerase chain reaction (PCR)-based gene replacement method of (Datsenko and Wanner 2000), using either the Kan' module of plasmid pKD13 (Datsenko and Wanner 2000) or the tetA tetR tetracycline-resistant (Tet') module of transposon Tn10 as a template. Primers for the PCR reactions were (upper case letters indicate the sequences of Tn10 or pKD13) iscS-p1 (5'-gta agc cat tat aaa ttc tcc tga ttc cga cG TGT AGG CTG GAG CTG-3') and iscS-p4 (5'-ggt agc ctc att ctc agg). The PCR products were ligated into plasmid pCP20 as described by Datsenko and Wanner (2000).

Table 1. *Escherichia coli* strains used in this study.

| Strain           | Genotype                        | Reference or derivation               |
|------------------|---------------------------------|---------------------------------------|
| BW25113 [pKD46]  | lacI\(^{+}\) rmb\(^{+}\)165 ΔlacZ::Tn16 hsdR514ΔaraBAD\(_{AH13}\)ΔhisB\(_{L78}\) [pKD46] | (Datsenko and Wanner 2000)            |
| NR10836          | ara-thi (Δpro-lac) F'/CC106      | (Kozmin et al. 2000)                  |
| NR15995          | ara-thi (Δpro-lac) F'/CC106      | (Kozmin et al. 2000)                  |
| NR15996          | ara-thi (Δpro-lac) F'/CC106      | (Kozmin and Schaaper 2007)            |
| NR16042          | ara-thi (Δpro-lac) F'/CC106      | This study                            |
| NR16045          | ara-thi (Δpro-lac) F'/CC106      | This study                            |
| NR16046          | ara-thi (Δpro-lac) F'/CC106      | This study                            |
| NR16072          | ara-thi (Δpro-lac) F'/CC106      | This study                            |
| NR16075          | ara-thi (Δpro-lac) F'/CC106      | This study                            |
| NR16195          | ara-thi (Δpro-lac) F'/CC106      | This study                            |
| NR16263          | ara-thi (Δpro-lac) F'/CC106      | This study                            |
| NR16523          | ara-thi (Δpro-lac) F'/CC106      | This study                            |
| NR16539          | ara-thi (Δpro-lac) F'/CC106      | This study                            |
| NR16758          | ara-thi (Δpro-lac) F'/CC106      | This study                            |
| NR17603          | ara-thi (Δpro-lac) F'/CC106      | This study                            |
| NR17604          | ara-thi (Δpro-lac) F'/CC106      | This study                            |
| NR17605          | ara-thi (Δpro-lac) F'/CC106      | This study                            |
| NR17606          | ara-thi (Δpro-lac) F'/CC106      | This study                            |
| NR17607          | ara-thi (Δpro-lac) F'/CC106      | This study                            |
| NR17613          | ara-thi (Δpro-lac) F'/CC106      | This study                            |
| NR17614          | ara-thi (Δpro-lac) F'/CC106      | This study                            |
| NR17615          | ara-thi (Δpro-lac) F'/CC106      | This study                            |
| NR17616          | ara-thi (Δpro-lac) F'/CC106      | This study                            |
| NR17617          | ara-thi (Δpro-lac) F'/CC106      | This study                            |
| NR17618          | ara-thi (Δpro-lac) F'/CC106      | This study                            |
| NR17619          | ara-thi (Δpro-lac) F'/CC106      | This study                            |
| NR17622          | ara-thi (Δpro-lac) F'/CC106      | This study                            |
| NR17633          | ara-thi (Δpro-lac) F'/CC106      | This study                            |
| NR17634          | ara-thi (Δpro-lac) F'/CC106      | This study                            |
| NR17635          | ara-thi (Δpro-lac) F'/CC106      | This study                            |

*In-frame deletions.

**Elimination of the Kan' module from moaE using plasmid pCP20 as described by Datsenko and Wanner (2000).
tga tgt acg gag ttt ata gag cca tga TTC GGA TCC GTC GAC C-3') for generating ΔiscS::kan; iscU-p1 (5′-caa ctc tta ttt tgg gaa ttt ccc ctc tta gta aag cac aag gaa aag tta tgg acc aac agc gac gct ctc gac atc ttg gtt acc g-3') and iscU-p4 (5′-cgg aat cag gag aat tta aac gaa aag tta tgg acc aac agc gat ggc c-3') and ΔiscU::kan; iscA-p1 (5′-aaa ccc cac gcc cac gcc gggt gat ttc cag ggg caa gat aag tca GAC C-3') for generating ΔiscA::kan; iscU-p1 and iscU-p4 for ΔiscUA::kan; sufS-p1 (5′-gac ctc tga cga ctt ttt ttc cgg tca aag tgc cgg tca agg gtt cgg tct tta cgt gtt ctg ggg caa gat aag taa TCA TTA TAT TTC G-3') and sufS-p4 (5′-gca gaa tga ttt tct ccc gac atg ctc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc gcc 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ond gene in an operon containing up to eight genes (Zheng et al. 1998) a precise in-frame iscS deletion was created (see Experimental Procedures). We found that the iscS mutant, like tusA, conferred HAP hypersensitivity (Fig. 2A) as well as sensitivity to AHAP, HC, and hydroxylamine (data not shown). Like for the tusA mutant, the iscS mutant displayed a reduced growth rate, which may likewise account for the increased apparent HAP sensitivity relative to the Moco-deficient moaE mutant (51 mm vs. 41 mm).

**iscS and tusA operate within the Moco pathway**

Previous studies on HAP sensitivity defined two parallel pathways for HAP detoxification, dependent on the YchX and YiiM proteins (Fig. 1B), as well as one minor pathway depending on the BisC protein (Kozmin et al. 2008), each of which was dependent on the availability of the Moco factor. Thus, it was of interest to determine whether the role of the TusA and IscS proteins is related to these pathways. In Figure 2, we show the HAP sensitivity of the tusA or iscS defects alone or in combination with the moaE defect. The tusA moaE double mutant showed essentially equal sensitivity to the single tusA mutant (47 mm vs. 46 mm). Likewise, the iscS moaE double mutant displayed similar activity as the single iscS mutant (53 mm vs. 51 mm). These results suggest that the tusA and iscS defects are likely epistatic to the moaE defect. This is further confirmed by the identical sensitivity of the triple tusA iscS moaE mutant relative to the double tusA iscS mutant (Fig. 2). Although the iscS and tusA strains appeared more HAP sensitive than the single moaE mutant, this may be due to the slower growth observed for iscS and tusA mutants, which may amplify their sensitivity in this test.

The epistasis of the various pathways was also investigated by looking at the mutagenic response of the various strains upon exposure to HAP. The results shown in Figure 3 indicate that the tusA moaE combination was
equally susceptible to HAP as the single tusA (or moaE) mutant, and that the iscS moaE double mutant, as well as the iscS tusA moaE triple mutant, was equally susceptible as the single iscS or the double iscS tusA mutant (Fig. 3). These data likewise support the operation of TusA and IscS within the Moco pathway. As in the toxicity test, iscS strains displayed slightly greater mutability than the moaE (and also tusA) strain, likely reflecting some indirect sensitization due to the pleiotropic characteristics of iscS (Fontecave et al. 2008; Roche et al. 2013).

**Chlorate resistance of tusA and iscS strains**

One major hallmark of lack of Moco is resistance to chlorate under anaerobic conditions (Shanmugam et al. 1992). Sensitivity to chlorate is generally attributed to the activity of nitrate reductase, Moco-dependent activities reducing chlorate to toxic chlorite (Stewart and MacGregor 1982). We tested the tusA and iscS mutants whether they are, like the previously established Moco-deficient strains, also resistant to chlorate. As shown in Figure 4 (top panel), the tusA and iscS strain are chlorate resistant, like the control ΔmoaE strain, indicating that they are also deficient in nitrate reductase activity.

**Inorganic sulfide or L-cysteine partially suppress the HAP sensitivity of iscS or tusA mutants**

A unique phenotype that we discovered associated with the iscS or tusA deficiencies is that their HAP sensitivity could be suppressed by addition of sulfide or L-cysteine to the medium (see Fig. 5). This phenotype is not observed for other HAP-hypersensitive mutants, like moa, moe, or ycbX (see moeB in Fig. 5). The HAP sensitivity of iscS or tusA strains is also partially suppressed in strains carrying the constitutive cysB<sup>C</sup> (T149P) allele (Kozmin et al. 2010). In this strain lack of repression of L-cysteine biosynthesis leads to overproduction of L-cysteine (and, likely, sulfide) (Kredich 1996). These observations suggest a defect in iscS and tusA strains in the mobilization of sulfur required for the activity of the YcbX/YiiM proteins. This function may parallel the described function of the TusA-IscS complex in the thiolation of certain unique tRNAs (Ikeuchi et al. 2006).

**No requirement for alternative L-cysteine desulfurases CsdA and SufS**

Other than IscS, *E. coli* possesses two additional L-cysteine desulfurases, CsdA and SufS (Fontecave et al. 2008; Roche et al. 2013). While IscS alone appears to be fully sufficient for HAP resistance, it seemed worthwhile to check the effect of the additional cysteine desulfurases. Our experiments showed that the single csdA and sufS mutants as well as the csdA sufS double mutant were fully HAP resistant (Fig. 6) as well as chlorate sensitive (Fig. 4), like a wild-type strain. Thus, the postulated need for sulfur transfer in the Moco-dependent activities is fully served by the IscS cysteine desulfurase.

**Role of iron–sulfur cluster biosynthesis in base-analog resistance**

IscS is a major L-cysteine desulfurase involved in iron–sulfur cluster biosynthesis (Fontecave et al. 2008; Roche et al. 2013). The first step of Moco biosynthesis requires MoaA protein (Fig. 1B), which contains a catalytically essential iron–sulfur cluster (Schwarz et al. 2009; Iobbi-Nivol and Leimkühler 2012). Important iron–sulfur clusters are also present in nitrate reductases (Schwarz et al. 2009; Iobbi-Nivol and Leimkühler 2012) as well as YcbX protein (Kozmin et al. 2008; J. Wang and R. M. Schaaper, unpublished data). Thus, the observed sensitivity of the ΔiscS strain to HAP, as well as its chlorate-resistant phenotype, may be due to a defect in the iron–sulfur cluster of one or more of these enzymes. During cluster biosynthesis, L-cysteine desulfurase provides a sulfur atom to a “scaffold” protein enabling synthesis of a nascent cluster (Fontecave et al. 2008; Roche et al.
2013). The cluster is then transferred to the target apoprotein, either directly or perhaps mediated through one of several carrier proteins. Several possible scaffold and carrier proteins have been described in *E. coli*, including IscU, IscA, SufA, and NfuA (Fontecave et al. 2008; Roche et al. 2013). To check their role, we inactivated the corresponding genes. No increase in HAP sensitivity was observed for any of these mutants, including several of their double mutants (Fig. 6). The strains were also chlorate sensitive (Fig. 4), with the exception of *iscU*. The *iscU* mutant displays a “split” phenotype: IscU is not required for HAP resistance, but is required, at least partially, for nitrate reductase activity (note the reduced size of the chlorate-resistant colonies in Fig. 4). Despite this split phenotype (see also Discussion), the overall results are strongly supportive of the idea that lack of iron–sulfur cluster biosynthesis per se is not the main reason for the HAP sensitivity of *iscS* and *tusA* mutants.

The effect of TusA is independent of the TusBCD-TusE sulfur transfer complex

The main role that is currently described for TusA is to act as sulfur-carrying protein in the 2-thiolation of 5-methylaminomethyl-2-thiouridylate (mnm$^5$s$^2$U) at the wobble position of certain tRNAs (Ikeuchi et al. 2006). In this pathway, TusA first accepts sulfur from IscS, which is then transferred via TusBCDE proteins to mnm$^5$s$^2$U methyltransferase MnmA, which ultimately performs the tRNA thiomodification (Ikeuchi et al. 2006). In our study, inactivation of *tusBCD* and *tusE* produced neither HAP sensitivity (Fig. 6) nor chlorate resistance (Fig. 4). This indicates that, in contrast to the tRNA thiomodification process, the activity of TusA in the Moco pathway does not require the TusBCDE proteins.

Discussion

In our efforts to understand the mechanisms by which cells can detoxify mutagenic and toxic chemicals such as N-hydroxylated compounds we have previously characterized novel activities that depended on at least two proteins, YcbX and YiiM, both requiring the Moco. Mammalian enzymes, termed mARC (Havemeyer et al. 2006), and a green algal protein, crARC (Chamizo-Ampudia et al. 2011) have been described that may be similarly involved in a variety of detoxification reactions, specifically via reduction
of \(N\)-hydroxylated substrates to the corresponding amines (Havemeyer et al. 2006, 2010; Grunewald et al. 2008; Wahl et al. 2010; Chamizo-Ampudia et al. 2011; Kotthaus et al. 2011; Krompholz et al. 2012; Neve et al. 2012). The genetic approaches that we have followed in the bacterium \textit{E. coli} have proven useful in identifying (a) the involvement...
of Moco as cofactor (Kozmin et al. 2000; Kozmin and Schaaper 2007), (b) the identity of the responsible Moco-dependent enzymes YcbX, YiiM, and BisC (Kozmin et al. 2008), and (c) additional factors involved in these reactions, such as CysJ protein, which functions as a specific electron donor for the YcbX-mediated reduction reaction (Kozmin et al. 2010). In the present study, we have identified two additional factors relevant to these processes, namely the TusA and IscS proteins, which are known to be involved in sulfur mobilization. We have shown that these two proteins act within the previously defined Moco-dependent pathways and, as will be argued below, within the biosynthesis pathway for Moco, most likely in the introduction of the dithiolene sulfur atoms of the MoCo molecule. This functioning of IscS and TusA has also been described recently by Dahl et al. (2013) using more biochemical approaches.

Biosynthesis of Moco is an area of active research (Schwarz et al. 2009; Iobbi-Nivol and Leimkühler 2012). Moco synthesis starts with guanosine-5'-triphosphate (GTP), which is converted to an intermediate called cPMP (cyclic pyranopterin monophosphate) by MoaA and MoaC proteins (Fig. 1B); in the next step the MoaDE/MoeB proteins convert cPMP to the MPT by addition of the dithiolene sulfurs (Fig. 1C), which are needed for coordinating the Molybdenum atom (Moco). Various forms of Moco exist depending on further ligand coordination at the Mo center, as well as possible formation of a dinucleotide with guanosine monophosphate (GMP) or cytidine monophosphate (CMP), followed by insertion in the corresponding apoenzyme (Schwarz et al. 2009; Iobbi-Nivol and Leimkühler 2012). The precise form of Moco in the YcbX and YiiM proteins has not yet been established, although it is clear that in contrast to most other E. coli molybdoenzymes it is not present as a MGD dinucleotide (Kozmin and Schaaper 2007).

The current observations on the HAP sensitivity and chlorate resistance of tusA and iscS mutants raise the question of the precise role of the TusA and IscS proteins in Moco-dependent pathways. In Figure 7, we have indicated several ways in which defects in sulfur metabolism could potentially interfere with Moco biosynthesis. Synthesis of Moco has a direct sulfur requirement as it contains two dithiolene sulfurs that serve to coordinate the Mo atom (Fig. 1C). In addition, its biosynthesis requires, among others, the MoaA protein, which is an iron–sulfur cluster protein (Schwarz et al. 2009; Iobbi-Nivol and Leimkühler 2012). The YcbX protein (for HAP resistance) as well as nitrate reductase enzymes (for chlorate sensitivity) are also iron–sulfur proteins, and their activity may be impaired in iscS/tusA strains for this reason. Finally, the hypothesis could be considered that Moco present in YcbX and YiiM contains a sulfur atom as an additional Mo-ligand, as demonstrated to be the case for enzymes of the xanthine oxidase family (Schwarz et al. 2009; Iobbi-Nivol and Leimkühler 2012).

With regard to the role in IscS, biochemical studies have shown this cysteine desulfurase to be the primary sulfur-donating factor in MPT synthesis (Zhang et al. 2010; Dahl et al. 2011), with no inferred role for the alternative CsdA and SufS cysteine desulfurases (Zhang et al. 2010; Dahl et al. 2011). Our results showing the HAP sensitivity and chlorate resistance of the iscS mutant are fully consistent with this possibility, including our observed lack of effect of the alternative cdsA or sufS cysteine desulfurase defects. In addition to this role, IscS is also the main (housekeeping) sulfur donor for the synthesis of iron–sulfur clusters in E. coli (Fontecave et al. 2008; Roche et al. 2013). In this function, IscS generally operates in conjunction with the IscU protein and a variety of additional factors like IscA, HscA, HscB, and Fdx, which are all expressed as part of the iscRSUA–iscSAB–fdxA operon (Zheng et al. 1998). Other Fe–S synthesizing systems, such as that encoded by the sufABCDSE operon or NfuA protein form alternative systems that may play a role in the repair of damaged clusters under stress conditions (Fontecave et al. 2008; Roche et al. 2013). Importantly, we showed that the iscU and iscA defects (or the alternative sufA or nfuA defects) did not produce any sensitivity to HAP (Fig. 6). As the IscU protein is required for Fe–S cluster biosynthesis by the IscS–IscU pathway, this leads us to two conclusions.

**Figure 7.** Possible roles of the IscS-TusA complex in Moco-dependent pathways. The possible stages where lack of IscS- and TusA-mediated sulfur mobilization could lead to sensitivity to HAP (loss of YcbX and YiiM activity) or to chlorate resistance (loss of nitrate reductase activity) are indicated in red font. The MoaD-SH box is outlined in red, as this stage is proposed to be the main critical step. See text for details.
First, the role of IscS in MPT biosynthesis, which does not require IscU, is clearly distinct from its role in Fe–S cluster synthesis, which does require IscU. This observation is consistent with emerging models in which IscS is able to participate in several different processes but using different partner proteins (Shi et al. 2010; Maynard et al. 2012; Dahl et al. 2013). It is plausible to assume that, for MPT synthesis, IscS partners with TusA, to provide the sulfur atoms needed by the MoaD protein for MPT synthesis. This would be similar to the described IscS-TusA partnership that performs the thiolation of the wobble position of certain tRNAs yielding the modified tRNA base 5-methylaminomethyl-2-thioridine (Ikeuchi et al. 2006). The physical IscS-TusA complex and its crystal structure have been described (Ikeuchi et al. 2006; Shi et al. 2010).

The second conclusion is that iron–sulfur cluster assembly per se does not appear to be a rate-limiting factor for the HAP-detoxification pathway, as the loss of the IscS-IscU pathway (as in the iscU mutant) does cause detectable HAP sensitivity. It appears that sufficient Fe–S centers must be present in the YcbX protein and MoaA protein (see Fig. 7). It is likely that some alternative IscU-independent pathway provides sufficient Fe–S clusters for these proteins to remain active. The one possible exception to this interpretation may be the observed chlorate resistance of the iscU mutant (Fig. 4). This aspect will be addressed further below.

The possibility (see Fig. 7) that an extra sulfur atom might be required as additional Mo–ligand in Moco in YcbX or YiiM, as found in the Xanthine Oxidase family (Schwarz et al. 2009; Iobbi-Nivol and Leimkühler 2012; Hille 2013), was also considered, as the precise nature of Moco in YcbX and YiiM is presently undetermined. Recent evidence indicating that the corresponding eukaryotic proteins, human mARC or green algal crARC, do not carry an additional Mo–S ligand on their Moco (Wahl et al. 2010; Chamizo-Ampudia et al. 2011; Rajapakshe et al. 2011). Also, our results show that iscs and tusA mutants are chlorate resistant (see Fig. 4), indicating a defect in nitrate reductase activity. All three E. coli nitrate reductase (NarGHI, NarZYZ, and NapABC-GDH) are members of the DMSO Reductase family of molybdoenzymes and carry the Mo-bisMGD form of Moco, which does not contain the extra sulfur ligand (Hille 2013; Iobbi-Nivol and Leimkühler 2012). Thus, a parsimonious interpretation of these data would rule against a requirement of Moco in YcbX and YiiM for an extra sulfur ligand. Thus, the combined data argue in favor of the hypothesis that the observed HAP sensitivity of iscs and tusA mutants results from lack of MPT synthesis (Fig. 7).

Overall, the results with the chlorate-resistant phenotype of iscs and tusA paralleled those obtained with the HAP-sensitivity phenotype (Fig. 4). However, an interesting split-phenotype result was obtained with the iscU mutant. This mutant proved fully HAP resistant (Fig. 6), indicating sufficient YcbX/YiiM activity, but it was chlorate resistant (Fig. 4), indicating some loss of nitrate reductase activity. However, the chlorate resistance of iscU appears, in fact, to be only partial, as judged from their significantly smaller colonies on the chlorate plates compared to, for instance, the fully resistant moae mutant (Fig. 4). We suggest that this partial nitrate reductase deficiency results primarily from a reduction of the iron–sulfur clusters on nitrate reductase, although an indirect effect on Moco via its effect on the MoaA iron–sulfur cluster could also play a role. A split phenotype of this kind (partial chlorate resistance along with full HAP resistance) has also been reported for certain polar mutants of the moa operon (Kozmin and Schaaper 2013). It appears that a modest reduction in nitrate reductase activity can already give rise to a significant level of chlorate resistance, while a much more robust reduction in YcbX/YiiM activity is required to observe HAP sensitivity (Kozmin and Schaaper 2013).

Another interesting observation of the current study is that the IscS/TusA-dependent step could be circumvented by the addition of sodium sulfide or L-cysteine to the growth medium (Fig. 5). It is possible that the MoaD protein can directly utilize inorganic sulfide, at least to a sufficient extent, to assure adequate Moco levels. Support for this idea comes from in vitro biochemical experiments in which activation of MPT synthase could be achieved by incubation with sodium sulfide (Leimkühler and Rajagopalan 2001). As our results show the same effect by addition of L-cysteine, L-cysteine may be catabolized in vivo to generate sulfide. Indeed, in E. coli excess of L-cysteine is readily removed by L-cysteine desulphydrase activities, of which tryptophanase (tnaA gene product) has been identified as a main activity (Snell 1975). We have investigated iscs tnaA and tusA tnaA double mutants for HAP sensitivity and observed that in those cases addition of cysteine had much reduced effects, in support of this hypothesis (data not shown). Likewise, an increase in the intracellular cysteine or sulfide concentration in the absence of any additions to the media was also achieved (data not shown) by constitutive expression of the CysB regulon, which normally controls the cysteine biosynthesis pathway (Kredich 1996).

The recent study by Dahl et al. (2013) has also described a role for TusA in the thiolation reaction that produces MPT. However, somewhat different conclusions were reached. Biochemical analyses of MPT content revealed a low MPT content in tusA cells growing aerobically, but only an about 50% reduction for MPT in anaerobically growing tusA cells. Hence, while it was
acknowledged that in aerobic cells TusA may be a preferred IscS partner for the cPMP thiolation reaction, TusA should not be regarded as an obligate IscS partner for this reaction in view of the anaerobic results. Instead, alternative sulfur carriers such as YnjE might play a role (Dahl et al. 2013). It was also observed that tusA cells suffer from altered gene expression patterns, possibly resulting from altered cellular Fe–S status (Maynard et al. 2012), and it was suggested that the effects of the tusA deficiency might result indirectly from these altered patterns (Dahl et al. 2013). Pleiotropic effects of a tusA deletion have also been reported in other studies (Yamashino et al. 1998; Ishii et al. 2000; Nakayashiki et al. 2013). It thus appears that the precise role of TusA in MPT synthesis requires further investigation. Our experiments measuring the effects of IscS and TusA on HAP resistance were performed under aerobic conditions and, hence, our results are consistent with the strong reduction in MPT observed under these conditions. On the other hand, chlorate-resistant experiments are performed under anaerobic conditions. Hence, our observation that tusA strains are chlorate resistant (Fig. 4) must indicate that even under these anaerobic conditions other hypothetical sulfur-carrier proteins cannot readily substitute for TusA. Experiments with a ΔynjE strain (results not shown) revealed normal HAP resistance (aerobic condition) as well as normal chlorate resistance (anaerobic condition), suggesting that the role of YnjE protein as alternative sulfur carrier may be limited.

Our genetic studies with the N-hydroxylated base analogs have provided a sensitive window into several important biological phenomena. Detoxification of hazardous agents, either produced endogenously or encountered in the environment, is an important cellular activity. The severe sensitivity of Moco-deficient strains has enabled the discovery of the YcbX and YiiM enzymes that represent a novel class of activities. The question of the physiological substrate of these enzymes is still an open question, and deserves further investigation, especially in light of the discoveries of corresponding mammalian enzymes (mARC) that have a mitochondrial membrane localization (Wahl et al. 2010; Klein et al. 2012). Furthermore, the precise metabolism of endogenous or foreign toxicants is an issue that can be further explored using this experimental system. Additional results from our laboratory have revealed that exposures to HAP have profound consequences for cellular DNA/RNA and nucleotide metabolism, including the involvement of cellular response systems, like the SOS system (Foster 2007). Most importantly, the results have allowed new insights into the role and metabolism of Moco in the cell and have defined an important, novel role of the TusA-IscS complex, as shown in the present study.

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Conflict of Interest

None declared.

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