# Identification of Guanosine as the Nucleoside Moiety of the Molybdopterin Cofactor of Arsenite Oxidase

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ABSTRACT

Arsenite oxidase converts arsenite ($As^{+3}$) to arsenate ($As^{+5}$), thereby enabling bacteria that produce the enzyme to tolerate high levels of arsenite in the environment. The electron transfer occurs via a molybdenum atom liganded to a molybdopterin cofactor associated with the enzyme. Molybdopterin cofactors from all bacteria that have been investigated contain an associated nucleotide. However, the nucleotide associated with the cofactor in arsenite oxidase has not been reported. To determine which nucleotide is attached to the cofactor in arsenite oxidase, the cofactor was released from the enzyme by heat denaturation in the presence of acid. The nucleotide moiety was hydrolyzed with pyrophosphatase and alkaline phosphatase, and separated on reverse phase high performance liquid chromatography. Four nucleotide standards were treated similarly, and their retention times compared to the one released from the enzyme. The results suggest that guanosine is the nucleotide associated with the molybdopterin cofactor of arsenite oxidase.

## INTRODUCTION

Arsenite, a toxic form of arsenic, is present as an environmental contaminant due in part to the large-scale use of arsenic-containing pesticides. Although these types of pesticides are now outlawed in the United States, many crops continue to exhibit stunted growth and other detrimental effects. In western states of the US, arsenite, among other toxic heavy metals, occurs as a by-product of smelting and other metal ore mining operations. Currently, no suitable methods exist to detoxify arsenite contaminated soils.

Because arsenic is an element, it cannot be broken down further and therefore must remain in the environment. Arsenical compounds differ in their toxicity, and arsenite can be converted to less toxic forms. Bacteria have been used to detoxify other environmental contaminants, and this process, known as bioremediation, has been quite successful, most notably in cleaning up marine oil spills. However, there are certain problems when using bacteria to detoxify arsenite in the environment. For example, other toxins or heavy metals present in the surroundings, along with the arsenite, may inhibit the growth of the bacteria. Although enzymes isolated from the bacterial cells can be used to carry out the same detoxification process in soils containing high levels of arsenite, such enzymes are sensitive to pH, temperature and other factors and therefore are not an efficient way to detoxify soil and water samples. By studying the detoxification mechanism of these enzymes, chemical models may be developed to achieve the same results chemically and more efficiently.

The toxicity of arsenite lies in its ability to bind to the sulfur groups of essential cysteines in proteins. These groups can be important for the three dimensional structure of proteins which affect the function of proteins and enzyme-substrate interactions. Because arsenite binds to two sulfur groups, it can cross link proteins, distorting their overall shape and impeding their function (Coddington 1986). If the proteins are critical to the survival of the cell, arsenite can cause death to the organism. BAL (British Anti-Lewisite) was developed as an antidote for gas warfare in World War I and is still used to combat arsenite poisoning. BAL (2,3-dithio-1-propanol) forms a five-member ring with arsenite (Fig. 1), which is more stable than the large structure formed from the crosslinked proteins via arsenite. In this manner, BAL competes for arsenite and sequesters it, thereby decreasing its toxic effects. This BAL-arsenite structure is then excreted from the body.

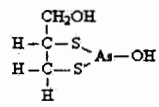


Figure 1. The BAL-arsenite complex. BAL (British Anti-Lewisite) is an antidote for arsenite poisoning. Arsenite cross-links cysteine residues in essential proteins distorting their overall shape and function. BAL competes for the arsenite, and the BAL- arsenite complex is excreted from the body.

Another form of arsenic, arsenate, is less toxic than arsenite. Because of the similarity in structure between arsenate and phosphate, arsenate acts as a phosphate analog and competes with the formation of ATP (Coddington 1986). ATP is used extensively in all living organisms as an energy storage molecule. The energy is released when ATP is hydrolyzed to form ADP and a phosphate group, thereby releasing a moderate amount of energy. The energy can then be used by the hydrolyzing enzyme to carry out chemical reactions in the cell. To form ATP, energy is used to add a phosphate group

to ADP. Arsenate can be enzymatically added to an ADP to form an ADP-arsenate molecule. Unlike ATP, this ADP-arsenate complex spontaneously hydrolyzes in the absence of enzymes, and the energy released will not be captured by a hydrolyzing enzyme (Fig. 2). Arsenate is, therefore, an uncoupler because it results in a futile cycle whereby the cell continues to exert energy in synthesizing this ADP-arsenate complex but cannot store the energy for future use.

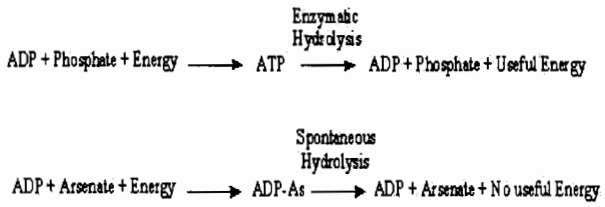


Figure 2. The toxicity of arsenate. Arsenate, as a phosphate analog, acts as an uncoupler. Energy is required to attach an arsenate to an ADP. This ADP-arsenate complex, unlike ATP, hydrolyses spontaneously producing no useful energy.

One strain of the bacterium, *Alcaligenes faecalis*, detoxifies arsenite by converting it to arsenate via an electron transfer reaction (Anderson 1992). The enzyme responsible for this reaction, arsenite oxidase, resides in the periplasmic space (the space between the two membranes of Gram-negative bacteria), so that arsenite is converted to arsenate before it can enter the cytoplasm. The two electrons from arsenite are transferred to a molybdenum atom which is bound to a cofactor in arsenite oxidase. The molybdenum in turn transfers the electrons to a set of intramolecular iron-sulfur clusters. The electrons are then transfered from arsenite oxidase to a copper-containing protein, azurin. The electrons from azurin are donated to a periplasmic cytochrome c (Anderson 1992). Presumably, they are then accepted by a heme-containing protein, cytochrome c oxidase, which then transfers the electrons to oxygen to form water. Concomitant with the electron transfer to oxygen, cytochrome c oxidase uses the energy from the electron transfer to force protons across the cell membrane where protons are already at a relatively high concentration. Another enzyme complex, ATP-synthase, then allows the protons to leak through the membrane to relieve the proton gradient, and the energy released is harvested and ATP is produced from the combination of an ADP and a phosphate. By this set of reactions, the bacteria can presumably generate ATP equivalents during the detoxification of arsenite.

The cofactor of arsenite oxidase, which performs the electron transfer reactions, belongs to a group of cofactors known as molybdopterin cofactors. Molybdopterin cofactors are found in several enzymes of both prokaryotes and eukaryotes (Hille 1996). For example, in humans, sulfite oxidase is involved in the metabolism of sulfur-containing compounds, and xanthine oxidase is important in the degradation of nucleotides. Certain bacteria use molybdopterin-containing enzymes, such as DMSO (dimethyl sulfoxide) reductase and aldehyde oxidase, to utilize various compounds as sources of fuel.

Molybdopterin cofactors have a similar core structure consisting of one or two pterin ring systems liganded to a central molybdenum atom (Fig. 3). Molybdopterin cofactors from

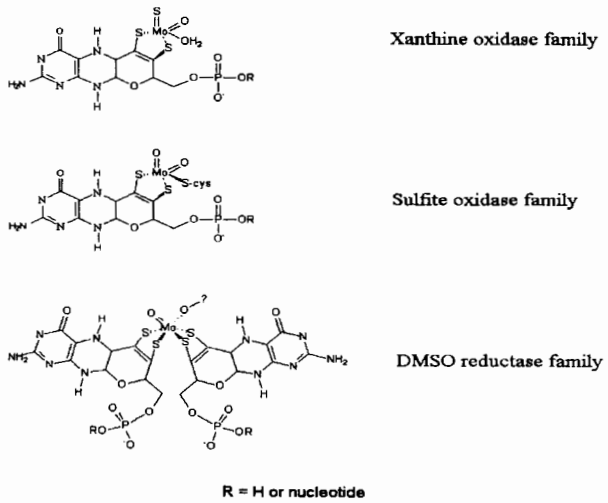


Figure 3. Major families of molybdopterin cofactor-containing enzymes. Molybdopterin cofactors consist mainly of a central molybdenum atom liganded to one or more pterin rings. In prokaryotes, the molybdopterin cofactors normally contain one or more nucleotides.

eukaryotes normally contain only one pterin ring, whereas prokaryotic molybdopterin cofactors may contain either one or two pterin rings and a nucleotide (Fig. 4) covalently linked to the pterin moiety. The nucleotide can contain any of four different bases, adenine, cytosine, guanine and uracil. The effect of the nucleotide on the chemical properties of the molybdopterin cofactor is not known. However, it is assumed that molybdopterin cofactors of similar structure have similar chemical properties. Since the structure of the molybdopterin cofactor of arsenite oxidase has not yet been reported, the determination of the number and the type of the nucleotide in arsenite oxidase would allow it to be classified and compared to other molybdopterin cofactor systems. This would provide insights into the chemical basis of the electron transfer at the cofactor of arsenite oxidase and may eventually lead to novel approaches in the detoxification of arsenite in the environment.

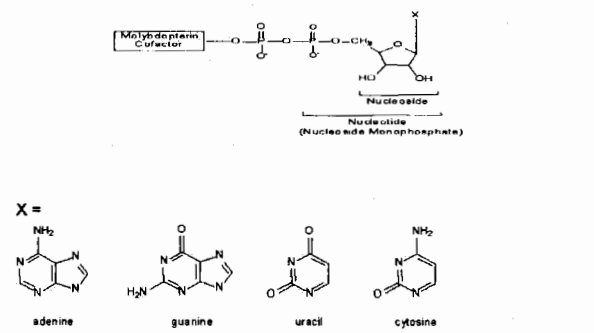


Figure 4. The nucleotide moiety associated with bacterial molybdoptein cofactors.Bacterial molybdopterin cofactors normally contain a nucleoside bonded via two phosophates. A nucleoside consists of a ribose and one of the four nitrogenous bases shown here. A nucleotide is a nucleoside which is bonded to one or more phosphates.

## Materials and Methods

*Alcaligenes faecalis* was grown aerobically as previously described (Anderson 1992) in a liquid broth medium (16 batches, 15 Liters each) containing 0.1% sodium arsenite, which induces the production of arsenite oxidase. Tangential flow ultrafiltration was used to separate the cells from the medium, and bacterial pellets were obtained by further centrifugation at 8000 RPM. The cells were washed three times with 20 mM TRIS pH 8.4, 0.9% NaCl. After a final centrifugation, the cells were suspended in the same buffer. Lysozyme (0.75 mg/mL) was added to break the glycoprotein cell coat, along with 0.05mg/mL of DNAse and 0.05mg/mL of RNAse to break down DNA and RNA polymers, respectively, in the presence of 2 mM $MgCl_2$. To isolate arsenite oxidase from the bacterial cellular contents, a DEAE (diethylaminoethane) ion-exchange column (5.0 x 200 cm) was used with 50 mM TRIS pH 8.4 as the buffer system. A sodium chloride gradient was applied from 0 to 0.5 M to compete with the enzyme for binding sites on the column and force arsenite oxidase to elute from the column. Fractions were collected, and those containing the enzyme were identified by a colorimetric assay containing arsenite and dichloroindolphenol. The enzyme was then concentrated by high pressure filtration, and the sample was purified further by an S200 column (2.5 x 250 cm), which separates molecules based on size, in 50 mM MES buffer at pH 6.0. Fractions were again collected and those containing arsenite oxidase activity were pooled and concentrated as before and applied to a C-8 agarose column (0.7 x 5 cm). Arsenite oxidase was eluted using a salt gradient from 0.5 M ammonium sulfate in 10 nM TRIS to 0 M ammonium sulfate in 10 mM TRIS buffer at pH 8.0, since this column takes advantage of the hydrophobic interactions between the enzyme and the column. The fractions collected from the C-8 column which contained the enzyme, were pooled, concentrated by centrifugal filtration and quantified spectroscopically. The purity of the enzyme was monitored by polyacrylamide gel electrophoresis.

To separate the cofactor from the enzyme, a sample of the enzyme purified by C-8 column chromatography was boiled in 0.1 N hydrochloric acid for 1 hour according to published procedures (Hilton 1996). After the pH was adjusted with sodium hydroxide to 8.5, the nucleoside moiety was separated from the cofactor using pyrophosphatase (0.5 U/mL), which breaks the phosphodieseter bond between the two phosphates that connect the nucleoside to the cofactor, and alkaline phosphatase (200 U/mL), which cleaves the terminal phosphate group. Four standards, each containing one of the possible nucleoside monophosphates, were incubated with alkaline phosphatase in the same manner in which the cofactor was treated. Reverse phase (C-18) high performance liquid chromatography (HPLC) was used to compare the digested arsenite oxidase cofactor to the four controls. The samples were eluted isocratically with a solvent system consisting of 93% 50 mM ammonium acetate and 7% methanol. The detector was at 290 nm, which corresponds to the maximum absorbance of the nucleosides.

## Results and Discussion

The bacteria *Alcaligenes faecalis* were grown aerobically in media containing sodium arsenite to induce the production of the arsenite oxidase. After the cells were lysed using lysozyme and sonication, the protein was isolated by a series of column chromatography techniques. An ion-exchange column was used to separate the enzyme based on its electrostatic interactions, and an S200 column was used to separate the enzyme from other proteins based on size. A C-8 hydrophobic-interaction column was used to further purify arsenite oxidase. Although only 20% of the total enzyme loaded on this column was recovered, the purity of the obtained arsenite oxidase was usually close to 95% as indicated by polyacrylamide gel electrophoresis (Fig. 5).

Figure 5. Polyacrylamide gel electrophoresis of arsenite oxidase. The heavy band on the top left part of the gel represents arsenite oxidase which was purified by a C-8 agarose column. The bands in the right lane are those of molecular weight standards ranging from 97.4 KD to 14.4 KD. The presence of only one band in the left lane indicates pure arsenite oxidase.

To analyze the molybdopterin cofactor, it must be released completely from the interfering enzyme matrix. The denaturation of the protein can be monitored visually by the disappearance of the brownish-red color due to the enzyme-bound iron-sulfur clusters. As the protein changes its conformational shape, the integrity of the iron-sulfur clusters can no longer be maintained. Although the molybdopterin cofactor is colorless in both the bound and unbound forms, the release of the cofactor is assumed to occur concommitantly with the release of the iron-sulfur clusters. Attempts to denature the protein using common denaturants, such as SDS, guanidine and urea, were not successful in releasing the cofactor. Upon boiling the enzyme in 0.1 N HCl for 1 hour, a change in color from reddish brown to clear was observed indicating that the cofactor was separated from the enzyme matrix in which it is held.

To separate the nucleotide moiety from the cofactor of arsenite oxidase, the enzyme pyrophosphatase was used to break the phosphodiester bond of the two phosphate groups which link the nucleoside to the cofactor (Fig 4). The release of the nucleoside was accomplished by digestion using the enzyme alkaline phosphatase, which cleaves the phosphate group from the generated nucleotide. Standards of the four possible nucleoside monophophates (adenosine, cytosine, guanosine and uridine monophosphate) were digested in the same manner as the molybdopterin cofactor, and the samples were analyzed using high pressure liquid chromatography (HPLC). Since the retention time of a compound (the time taken by a molecule to travel the length of the HPLC column) depends on the interactions between the molecule and the column, molecules with similar molecular structure will exhibit similar retention times. For this reason,

the retention time of the digested cofactor was compared to the treated nucleoside monophosphates (Fig. 6), as well as, nucleosides, nucleoside monophosphates and nitrogenous bases. According to the data obtained from HPLC analysis, the retention time of the digested molybdopterin cofactor of arsenite oxidase closely matched that of the guanosine and the digested guanosine monophosphate.

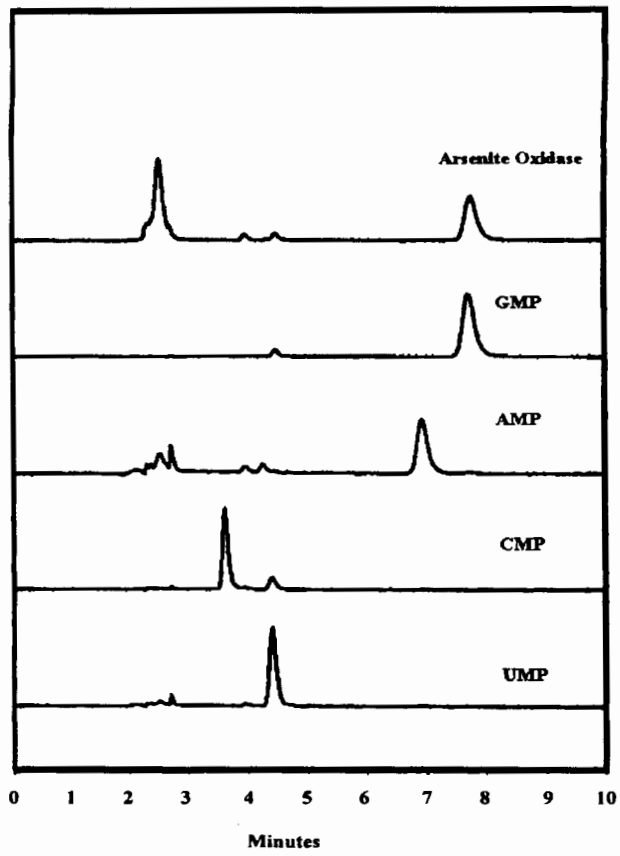


Figure 6. HPLC Chromatogram of digested arsenite oxidase cofactor and digested nucleoside monophosphates. A sample of arsenite oxidase was boiled in 0.1 N HCl to release the cofactor. After the pH was adjusted to 8.5, alkaline phosphatase and pyrophosphatase were added to release the nucleoside moiety. Standards of nucleotide monophosphates were treated with alkaline phosphatase to release their nucleosides. The retention times of the standards were compared to the digested sample of arsenite oxidase on HPLC. According to the chromatogram, the peaks for arsenite oxidase and the digested GMP have a similar retention time, suggesting that guanosine is the nucleotide associated with the cofactor of arsenite oxidase.

The retention time of the digested cofactor did not match that of guanosine monophosphate or guanine (Table 1). In contrast to previous reports (Hilton 1996), the treatment of the molybdopterin cofactor with 0.1 N HCl at $100^0$C for 1 hour, followed by the enzymatic digestion with pyrophosphatase and alkaline phosphatase, released the nucleoside and not the nitrogenous base. These data provide evidence that guanosine is the nucleoside associated with the molybdopterin cofactor of arsenite oxidase.

| Sample | Retention Time (Min) |
|---|---|
| Digested Cofactor[a] | 7.73 |
| Guanosine Monophosphate | 2.65 |
| Digested Guanosine Monophosphate[b] | 7.70 |
| Guanosine (Nucleoside) | 7.82 |
| Guanine (Nitrogenous Base) | 4.45 |

Table 1. The retention times of samples obtained from HPLC analysis.
[a]A sample of arsenite oxidase was boiled in 0.1 N HCl to release the cofactor and was treated with the enzymes pyrophophatase and alkaline phophatase to release the nucleoside moiety.
[b]A sample of guanosine monophosphate was treated with alkaline phophatase to release the nucleoside moiety.

## Conclusion

The toxic effects of arsenic are detrimental to both the environment and the economy. Large areas of land currently are nearly devoid of plant and animal life due to the presence of significant amounts of arsenicals in the soil. Although much of the chemistry of arsenic has been understood, still no significant strides have been made towards the effective detoxification of environmental arsenic. By investigating the structure of arsenite oxidase, which converts arsenite into the less toxic arsenate, a better idea of how the enzyme functions can be obtained. Using this enzyme as a model, chemical means of detoxifying environmental arsenic can be developed. The results of this study show that guanosine is the nucleoside associated with the molybdopterin cofactor of arsenite oxidase. We are currently investigating the number of guanosine moieties associated with each molecule of arsenite oxidase. This will be accomplished by the quantification of the number of phosphates per enzyme molecule using a modification of a colorimetric method described by Ames (Ames 1966). The quantification of the nucleoside associated with the molybdopterin cofactor of arsenite oxidase will provide a complete picture of the cofactor and will enable it to be classified among other molybdopterin containing enzymes.

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