HHS Public Access

Author manuscript

Chem Res Toxicol. Author manuscript; available in PMC 2017 November 15.

Published in final edited form as:

Chem Res Toxicol. 2017 July 17; 30(7): 1508–1514. doi:10.1021/acs.chemrestox.7b00158.

Arsenic Compromises Both p97 and Proteasome Functions

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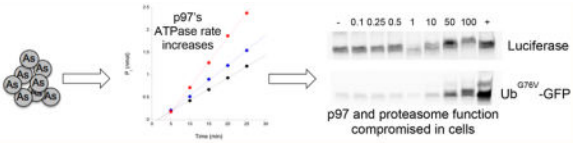
Abstract

Exposure to arsenic is a worldwide problem that affects more than 200 million people. The underlying mechanisms of arsenic toxicity have been difficult to ascertain due to arsenic's pleotropic effects. A number of recent investigations have shown that arsenic can compromise protein quality control through the ubiquitin proteasome system (UPS) or the endoplasmic reticulum associated protein degradation (ERAD) pathway. In this article, a link between arsenic and protein quality control is reported. Biochemical and cellular data demonstrate a misregulation of the ATPase cycle of the ATPase associated with various cellular activities (AAA+) chaperone, p97. Interestingly, the loss of p97 activity is due to the increased rate of ATP hydrolysis, which mimics a collection of pathogenic genetic p97 lesions. Cellular studies, using a well characterized reporter of both the proteasome and p97, show the proteasome to also be compromised. This loss of both p97 and proteasome functions can explain the catastrophic protein quality control issues observed in acute, high level arsenic exposures.

Graphical abstract



INTRODUCTION

It is estimated that over 200 million people throughout the world are chronically exposed to levels of arsenic that exceed the World Health Organization's (WHO) guidelines (currently

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.chemrestox.7b00158. The rate of ATP hydrolysis of various forms of p97 (PDF)

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Notes

The authors declare no competing financial interest.

set at 10 μg/L).[1] Exposure to arsenic occurs through food sources, water supplies, or inhalation.[2] The predominant species of arsenic in the environment is arsenic(V), also known as arsenate, but arsenate's mechanism of toxicity is complicated by its poor cellular permeability. Conversely, arsenic(III) or arsenite has shown much higher toxicity in cellular studies.[3] However, the ultimate toxic species is unclear, and the various arsenicals (a general term for arsenic derivatives that will be used when the precise form of arsenic is unknown) have been shown to be readily interconverted in cells.[4] The general effects of arsenicals on cells are pleotropic and include DNA damage, production of reactive oxygen (redox stress), compromised cellular respiration, and proteotoxicity.[5] Certainly, it remains that these conditions can be interrelated and are often related to the dose of an arsenical used in a given study.

Protein quality control, called proteostasis, is a critical element of cellular survival and includes the balance between protein folding and degradation.[6] The degradative arm of proteostasis is mediated through two pathways: the ubiquitin proteasome system (UPS) and autophagy. The UPS is a highly controlled protein degradative pathway that uses the small protein modifier, ubiquitin, to mark proteins for destruction by the 26S proteasome.[7] A critical subset of this pathway is the endoplasmic reticulum associated protein degradation (ERAD) pathway, which maintains the proteostatic health of the ER by removing troubled proteins from the ER membrane in a ubiquitin-dependent manner and feeding them to the proteasome.[8–11] This process is mediated by a collective of important machinery, but the force required to remove the ubiquitylated proteins from the ER is provided by the ATPase associated with various cellular activities (AAA+) chaperone, p97 (also called valosin containing protein or Cdc48 in yeast).[12] The second pathway, autophagy, is a bulk degradative pathway that also contains a critical p97-dependent subset: macroautophagy.[13–15] The exact relationship between p97 and autophagy remains uncertain, but it has been suggested that compromised p97 function can lead to compromised autophagosome maturation. This was discovered through biochemical investigations of p97 lesions that were found to lead to a rare autosomal dominant disorder called inclusion body myopathy associated with Paget's disease of bone and frontotemporal dementia (IBMPFD).[16] p97 is a ubiquitin targeted AAA+ protein that uses the energy of ATP binding and hydrolysis to extract proteins from other biomolecules such as biomembranes, protein complexes, or DNA.[17] In its functional state, p97 is a ring-shaped homohexamer that contains an N-domain and tandem AAA domains (D1 and D2) in each of its subunits.[18] Like all AAA proteins, p97 contains conserved Walker A and Walker B motifs in its AAA domains that are responsible for ATP-binding and hydrolysis, respectively.[19]

In the present work, we investigated the effects of arsenicals on p97 activity in biochemical and cellular assays. It was somewhat surprising to discover that p97's rate of ATP hydrolysis was enhanced in the presence of arsenate. Because, like many macromolecular machines, p97 relies on a well-timed ATPase cycle, any change in ATPase rate can have catastrophic consequences to the organism, such as those observed in the p97-altered IBMPFD mutants. The accelerated ATPase rate of p97 due to arsenate was noticed in both the D1 and D2 domains and was independent of the N-domains or the presence of IBMPFD mutations. Additionally, this rate of acceleration did not depend on p97's cysteines. Furthermore, cellular studies demonstrated compromised autophagosome maturation, which has been

implicated in the genetic knockdown of p97 and IBMPFD mutants and is a well-documented consequence of arsenic exposure.[16,20] Finally, a dual reporter system showed the loss of p97 and proteasome function in cells. These data provide, for the first time, a direct target to explain the loss of proteostasis in arsenic-exposed cells.

EXPERIMENTAL PROCEDURES

Materials

All chemicals and reagents were purchased from Thermo Fisher Scientific or Sigma-Aldrich. Primary antibodies against GFP and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology. NIH3T3 cells were purchased from the American Type Culture Collection (ATCC), where they were tested and authenticated by short tandem repeat (STR) analysis. HeLa cells stably expressing UbG76 V-GFP and ODD-Luc were generously provided by Dr. Tsui-Fen Chou (UCLA).

ATPase Assays

Assay buffer (50 mM Tris, 150 mM KCl, 10 mM MgCl2, and 1 mM DTT, pH 7.4; 100 μL) containing 250 nM p97 or p97 mutants was incubated with 0 μM, 50 μM, or 250 μM arsenate. Following incubation at 37 °C for 10 min, the ATPase assay was initiated by adding 1 mM ATP to each reaction followed by incubation at 37 °C. A 10 μL aliquot was taken from the reaction every 5 min up to 25 min, with the exception of the p97-A232E variant whose aliquots were taken every 2 min up to 10 min. This was then added into a cuvette (VWR) with 800 μL of malachite green solution (9.3 μM malachite green, 53 mM $(NH4)_2MoO_4$, 1 M HCl, and 10% Tween 20). After 5 min, 10 μL of 34% sodium citrate was added, and the OD_{660} was read on a GENESYS 10S Vis spectrophotometer. The results were represented as the slope determined by a linear curve fit using KaleidaGraph (Synergy Software).

Cellular Studies

Cell Culture—NIH3T3 cells were obtained from the ATCC and cultured in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS). In all cases, cells were grown at 37 °C with 5% CO_2.

Live Cell Fluorescent Microscopy—NIH3T3 cells were plated at 80% confluence in glass bottomed dishes and were left to adhere overnight. The mRFP-GFP-LC3 dual fluorescent reporter was transfected using the Lipofectamine 3000 reagent, according to the manufacturer's instructions. Twenty-four hours post-transfection, cells were treated with either DMSO or 1 μM arsenite in new media for 4 h. Multiple images were acquired for each treatment group using the Zeiss Observer.Z1 microscope with the Slidebook 4.2.0.11 imaging software (Intelligent Imaging Innovations, Inc.), but a single representative image was selected and presented.

Proteasome/p97 Reporter—HeLa cells stably expressing UbG76 V-GFP and ODD-Luc were generously provided by Dr. Tsui-Fen Chou (UCLA). Cells were cultured with arsenate

or MG132 (positive control) in DMEM supplemented with puromycin (2.5 μg/mL) and 10% FBS for 16 h. Cells were harvested in SDS-sample buffer (50 mM Tris-HCl at pH 6.8, 2% SDS, 10% glycerol, 100 mM DTT, and 0.1% bromophenol blue), lysed via sonication, and centrifuged to clear cellular debris. Samples were loaded on a 12% SDS–PAGE gel and transferred to nitrocellulose membranes. The blots were blocked in 5% milk for 1 h, then incubated with primary antibodies in 5% milk at 1:1000 anti-GFP (Gene Tex GTX113617), anti-Luciferase (Promega G745A), and anti-GAPDH (sc-32233, Santa Cruz Biotechnology). Blots were washed three times at 10 min intervals with wash buffer (1× PBS and 0.1% Tween 20) before the addition of 1:3000 goat anti-rabbit horse IgG horseradish peroxidase (HRP) conjugate (sc-2004, Santa Cruz Biotechnology) or goat anti-mouse IgG-HRP conjugate (sc-2005, Santa Cruz Biotechnology) for 1 h. The blots were washed three times at 10 min intervals with wash buffer, incubated in Supersignal West Pico, Dura, or Femto Substrate (Thermo Scientific), imaged using ChemiDocTM XRS (Bio-Rad), and analyzed using Quantity One 1-D Analysis software (Bio-Rad).

RESULTS

Arsenic Accelerates p97's ATPase Activity

In an effort to determine the relationship between arsenic and protein quality control, we examined the effects of arsenate on the p97-catalyzed hydrolysis of ATP. To study this, a malachite green based assay was employed. In this simple assay, the release of inorganic phosphate forms a complex with molybdate and reacts with malachite green to convert the solution from amber to green. In the present studies, it was noted that the presence of sodium arsenate led to an increase in the malachite signal, so a baseline value had to be determined (negative control) for each concentration of arsenate studied. In addition, we used a coupled ATPase assay following the procedure of Nørby and observed an equivalent enhancement of rates (data not shown).[21] To establish the negative control, 250 nM p97 hexamer and 1 mM ATP were used. These levels provide a reliable readout in this assay. Because arsenate is structurally similar to phosphate, it was originally thought that arsenate may act as an inhibitor of p97. Surprisingly, it was discovered that in the presence of arsenate, the rate of ATP hydrolysis was increased in a dose dependent manner (Figure 1A and B and Table S1).

Because arsenicals are known to interact with cysteines in many proteins and lead to oxidative modulation of thiols, it was reasoned that the increased rate of ATP hydrolysis may be due to an interaction with one of the 12 cysteines in the p97 monomers. To test this, a p97 construct devoid of all cysteine residues was produced: p97-Cys0. Each of the cysteines was converted to valine, except for the cysteines in the ATP-binding pockets, which were converted to alanine or serine. Although the alanine and serine mutants in the ATP-binding pockets were both tested and gave similar results, the data reported here are for the alanine mutants. Although the overall rate of ATP hydrolysis was lower in the p97-Cys0 mutants, the effect of arsenate was maintained, as it caused an increase in the rate of hydrolysis in a dose-dependent manner (Figure 1A and B and Table S1). Next, because the D2 domain of p97 is known as the major ATP-hydrolyzing domain, we sought to determine if the effects of arsenate were propagated through D2. To do so, we removed the D2 domain but left the N

and D1 domains, as well as the D1-D2 linker, intact (ND1L). Recently, this construct was shown to maintain activity independent of D2.[22] Again, in this case, the effects of arsenate were maintained (Figure 1A and B and Table S1). Finally, because IBMPFD mutants have shown increased ATPase activity that is likely due to enhanced N-domain dynamics, we examined the importance of the N-domains. Two constructs were made: one had the N-domains removed and left the linker between the N-domains and D1 intact (p97–188), and the other had the N-domains and the linker removed (p97–208). As shown in Figure 1B, the N-domain truncated states, especially p97–188, have increased rate of hydrolysis, which is in agreement with the literature.[23] As shown, the presence of arsenate approximately doubled the rate of ATP hydrolysis in these two constructs (Figure 1B and Table S1), suggesting a mechanism of acceleration that does not involve enhanced N-domain dynamics.

Arsenate Seems to Affect ATP Hydrolysis in Both D1 and D2

As indicated above, the use of the ND1L to report on ATPase activity in the D1 domain indicated that arsenate affected ATPase activity in D1. To further probe the interplay between D1 and D2 domain ATPase activity, we introduced a series of mutations to the Walker A or Walker B motifs. The Walker A motif is involved in ATP-binding, and the Walker B motif is involved in ATP hydrolysis.[19] Extensive studies on p97 and other AAA+ family members have shown that mutation of a conserved lysine to alanine in the Walker A motif or glutamate to glutamine in the Walker B motif (Figure 2A) led to compromised ATP-binding or hydrolysis in the respective D1 or D2 domain.[24] As a result, the effects of each mutation can be studied. Although some of the mutants had dramatically reduced ATPase rates, generally, an increase in the rate of ATP hydrolysis in response to arsenic was still observed (Figure 2B and Table S1).

Arsenic Further Increases IBMPFD Mutants' ATPase Activity

It was observed that the IBMPFD variants of p97 have an enhanced ATPase rate and that this can lead to certain cellular phenotypes being observed with arsenic exposures.[25–28] We decided to examine a panel of IBMPFD variants to see if the effects of arsenate would be mitigated or enhanced. First, a panel of IBMPFD mutants was generated and tested for basal levels of ATPase activity. As shown and reported previously in the literature, not all IBMPFD mutants share the same effects on the rate of ATP hydrolysis (Figure 3A).[29] Nonetheless, the ATPase activity of these mutants was enhanced upon the addition of arsenate (Figure 3B). Studies of IBMPFD mutants have shown that the N-domain dynamics of these mutants are different from those of wt-p97, and this might explain why the ATPase activity is increased.[27] However, as discussed above the observed effects of arsenate are independent of the N-domains, which is consistent with the IBMPFD data.

Arsenic Compromises Autophagy

Arsenic exposure leads to enhanced ATPase activity in wild type p97. Additionally, in the IBMPFD mutants, it has been shown that IBMPFD lesions lead to compromised autophagosome maturation.[16] So, we looked at this phenomenon in the presence of arsenite, which is a commonly used arsenical due to the poor cellular penetration of arsenate. In order to assess the difference between enhanced autophagy and blocked autophagosome-lysosome fusion, we used a dual LC3-GFP-RFP reporter in NIH3T3 cells.[30] In untreated NIH3T3

cells, both GFP and RFP were diffusely localized indicating no enhancement of autophagy (Figure 4, top row). However, when cells were treated with arsenite, both GFP and RFP puncta are observed to colocalize (Figure 4, bottom row). These data indicate an increase in the number of autophagosomes, but these are failing to mature into autolysosomes, as has been reported previously.[16]

Arsenic Compromises the Cellular Activity of Both p97 and the Proteasome

Finally, to differentiate p97 activity from proteasome activity in cells, we employed a dual reporter assay developed in the Deshaies' laboratory.[31] In this assay, the hypoxia-inducible factor 1 alpha (HIF1α) oxygen-dependent degradation domain (ODD) is fused to luciferase (ODD-Luc). As a separate complex, ubiquitin, with a G76V mutation to prevent deubiquitinase activity, is fused to GFP (Ub^{G76V}-GFP) (Figure 5). Both complexes are expressed in stable DTC25 cells. ODD-Luc is degraded independent of p97, so an increase in luciferase levels indicates loss of proteasome activity. Conversely, Ub^{G76V}-GFP requires p97 to be degraded. As shown, after treatment of cells for 16 h, a dose dependent increase in ODD-Luc and Ub^{G76V}-GFP was observed relative to a DMSO control, indicating that both p97 and proteasome function are compromised. Next, using the two highest doses of arsenate, 50 and 100 μM, we looked at time dependence. We found maximal inhibition of proteasome and p97 activity at 8 h postarsenate treatment.

DISCUSSION

Despite the enormous societal significance, the root cause of arsenic toxicity remains without a mechanistic explanation. Studies have shown a variety of effects, many of these on central quality control and stress response systems.[32] This likely indicates a lack of specificity and makes it hard to differentiate what begets what. A number of recent investigations have shown deleterious effects on protein quality control in cells treated with arsenic, but how this comes to pass is not clear.[32–34] Because arsenic can lead to oxidative stress and increased ROS, this certainly could lead to protein damage that could in turn lead to proteostatic stress, or this could proceed in the opposite direction, which is to say that proteostatic stress could lead to ROS.[35] Indeed, we have recently shown a relationship between p97 and the major oxidative stress response pathway, the KEAP1-NRF2-ARE axis.[36] Moreover, it has been shown that treatment with arsenic can lead to compromised autophagic function, one of the major stress response pathways.[20,32]

The effects on autophagy we observed, coupled with recent reports on protein quality control, led us to consider if arsenicals may interact with p97, which is likely, we hypothesized, by mimicking phosphate and leading to inhibition. To our surprise, we actually observed an increase in the rate of ATP hydrolysis. This was intriguing because a series of genetic p97 lesions have been described in patients, and biochemical and cellular investigations of the mutants have shown increased ATPase activity and compromised autophagosome maturation, respectively. Upon extensive dissection of this interaction, we found that arsenate affects both the D1 and D2 ATPase activity and that it seems to be mechanistically distinct from the IBMPFD mutants. The latter observation based on the fact that acceleration was independent of the N-domains and was not lost in IBMPFD mutants. It

is known, in the case of IBMPFD mutations that the increased rate of ATP hydrolysis is related to the movement of the N-domains.[27] The N-domain truncated states likely mimic the IBMPFD state and likely lead to the increased rate of hydrolysis, which is in agreement with the literature.[23]

In cellular studies, we were able to observe compromised autophagosome maturation in the presence of arsenic, similar to the IBMPFD mutants. This is certainly sufficient to lead to proteotoxic stress. However, we also observed a compromise of the UPS system. By using a dual reporter system, we could show that not only was p97's activity but also the function of the proteasome seemed to be compromised. Although the dual nature of these effects makes it difficult to conclude the meaning, it is clear that the effects on the DTC25 cells treated with arsenic are quite different from the effects on the same cells treated with the known proteasome inhibitor MG132. The effects of arsenic were greater than the effects of MG132 on the proteasome reporter, whereas the effects of arsenic were less than the effects of MG132 on the p97 reporter.

In addition to the pleotropic effects of arsenic, pinning down the exact arsenical responsible for compromised cellular function or cellular transformation has been a challenge. Certainly, the predominant environmental species is arsenate, but this species has been shown by some to be nontoxic in cellular and *in vivo* models.[37] However, in conflict with this view, more recent investigations have shown this to be an issue of cellular penetration, which can be overcome by using a low phosphate buffer.[36] Once arsenate penetrates the cells, toxicity occurs.[38] In metabolic studies, a series of metabolic routes have been proposed, and a number of arsenicals have been discovered in the urine of test cases.[39] Arsenicals discovered include a variety of organic arsenic compounds of both +3 and +5 oxidation states. In our studies, we see an effect of inorganic arsenate on p97's ATPase activity. The true physiologic significance of this is hard to determine because of the complex metabolic processing of arsenicals, but it certainly adds to the effects arsenicals can have on cells. In addition, we have used inductively coupled plasma mass spectrometry to study cells treated with either arsenite or arsenate and found both species to be present in the cell lysates regardless of starting species (data not shown).

In conclusion, we report here for the first time the direct effects of an arsenical on a central component of several protein quality control pathways including the UPS, the ERAD pathway, and the autophagic pathway. Although we do not report the precise arsenical responsible for cellular function, we do show how arsenate, the predominant environmental species can lead to p97 compromise and that this compromise leads to cellular effects predicted by genetic and chemical biological data.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Raymond Deshaies for CB-5083 and Tsui-fen Chou for the DTC25 reporter cells.

Funding

This research was funded by grant ES023758 (to E.C. and D.D.Z.) and grant GM008804 (to A.A.).

ABBREVIATIONS

UPS	ubiquitin proteasome system
ERAD	endoplasmic reticulum associated protein degradation
ER	endoplasmic reticulum
AAA+	ATPase associated with various cellular activities
WHO	World Health Organization
IBMPFD	inclusion body myopathy associated with Paget's disease of bone and frontotemporal dementia

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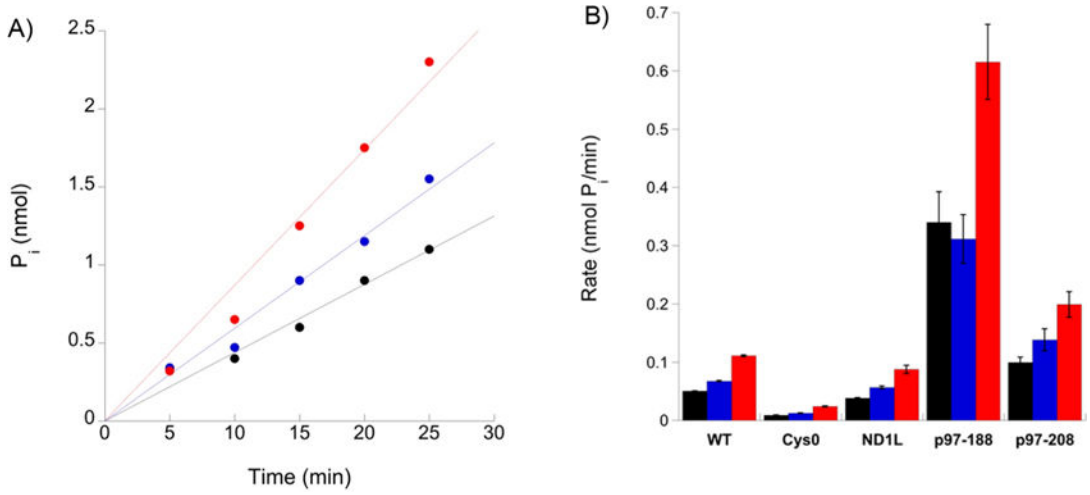

Figure 1.

Arsenate increases p97's ATPase activity. (A) The rate of p97 catalyzed ATP hydrolysis with no added arsenate (black; 0.0437 nanomol/min), in the presence of 50 μM arsenate (blue; 0.0594 nanomol/min), or in the presence of 250 μM arsenate (red; 0.0868 nanomol/min). In each case, 250 nM p97 (hexamer) and 1 mM ATP were used, and the inorganic phosphate released was measured with a malachite green assay. (B) Rates of inorganic phosphate production in the absence of arsenate (black), 50 μM arsenate (blue), or 250 μM arsenate (red). Five constructs were employed: wild-type p97, p97 with no cysteines (Cys0), p97 with no D2 domains but with the linker connecting D1 and D2 (ND1L), p97 with no N-domains but with the linker (p97–188), and p97 without the N-domains or linker (p97–208).

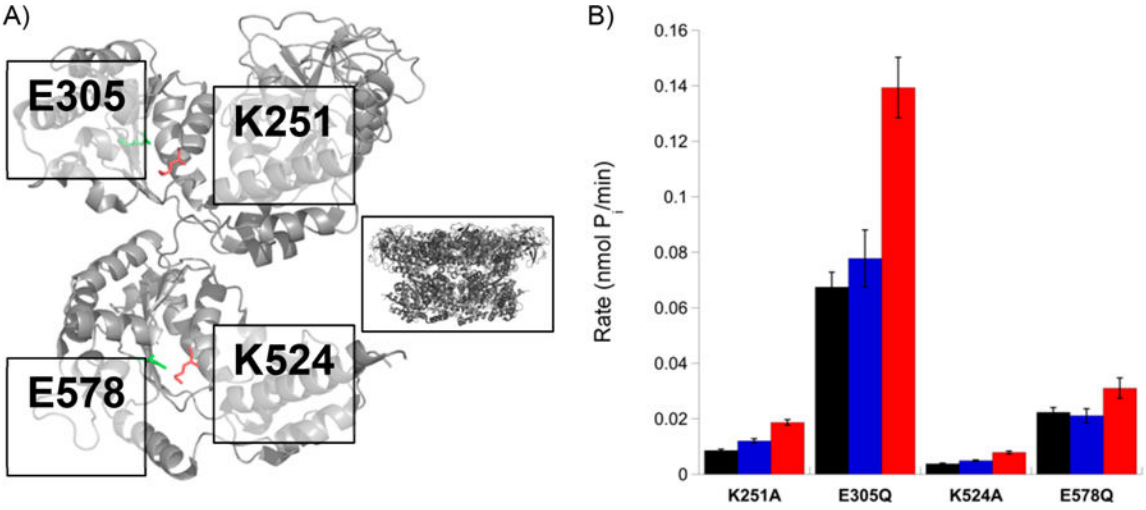

Figure 2.

Arsenate seems to affect ATP hydrolysis in both D1 and D2. (A) A monomer of p97 showing residues to be mutated in both D1 (K251 and E305) and D2 (K524 and E578). The Walker A residues are shown in red, and the Walker B residues are shown in green. (b) Bar graph showing the rate of inorganic phosphorus formation per minute for each of the Walker A and Walker B mutations in D1 and D2. The black bars are without arsenate, the blue bars are with 50 μM arsenate, and the red bars are with 250 μM arsenate. In each case, 250 nM p97 hexames was used and 1 mM ATP.

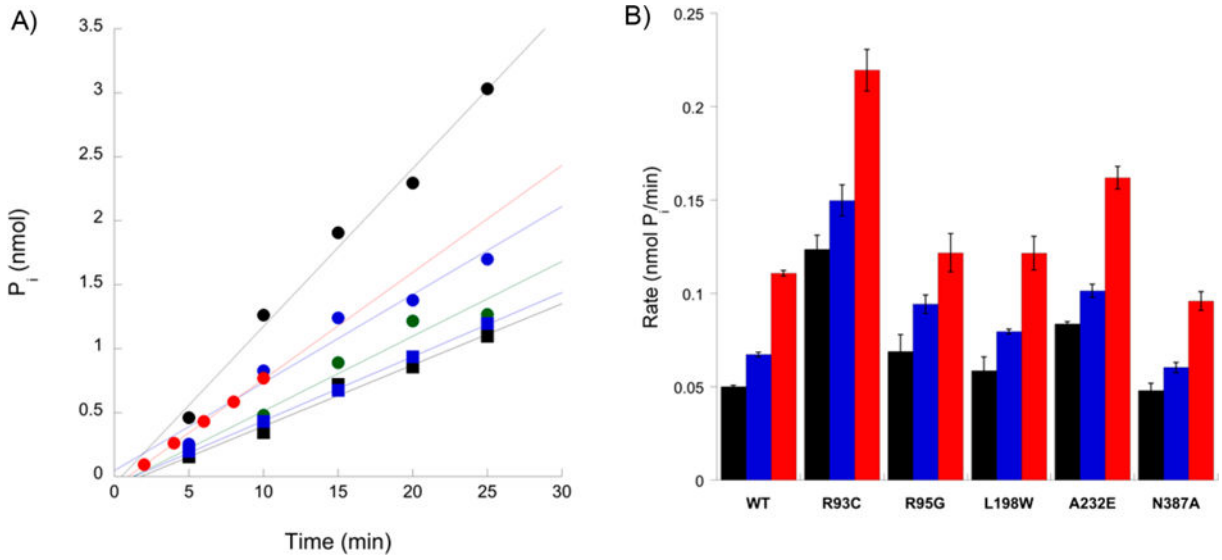

Figure 3.

Arsenate further accelerates ATPase activity in IBMPFD mutants. (A) The ATPase rates of the IBMPFD mutants used in the present studies showing the increase in rate over wt-p97. The p97 variants are given as follows: wt-p97, blue squares; R93C, black circles; R95G, blue circles; L198W, green circles; A232E, red circles; and N387A, black squares. (B) Rates of ATP hydrolysis for p97 and its variants in the absence of arsenate (black), in the presence of 50 μM arsenate (blue), or in the presence of 250 μM arsenate. The mutants being used are indicated below the bar graph.

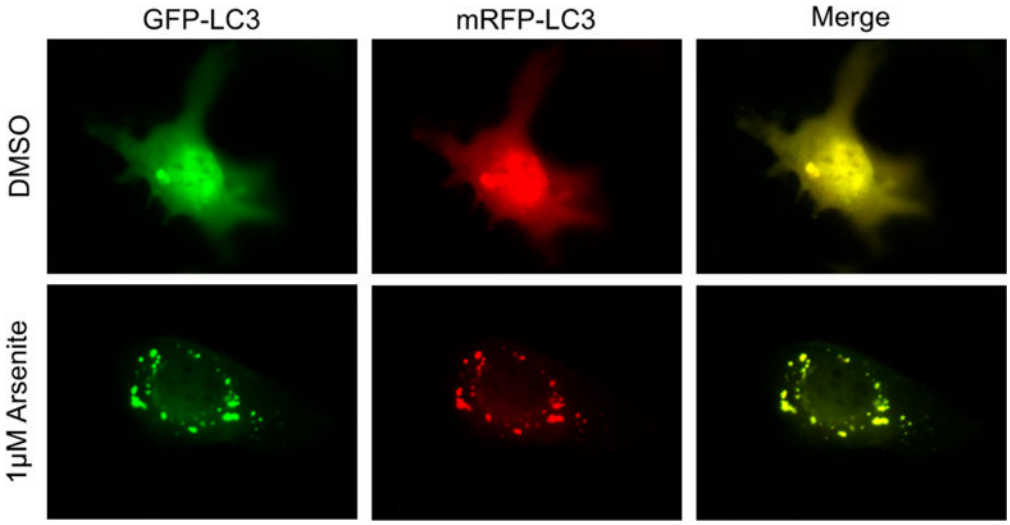

Figure 4.

Treatment of cells with arsenite leads to the loss of autophagosome formation. To measure autophagosome maturation, NIH3T3 cells were transfected with an LC3-GFP-RFP fusion. Co-localization of the two signals indicates compromised lysosome autophagosome fusion, as indicated by yellow puncta in the arsenic treated cells in the right-hand panel.

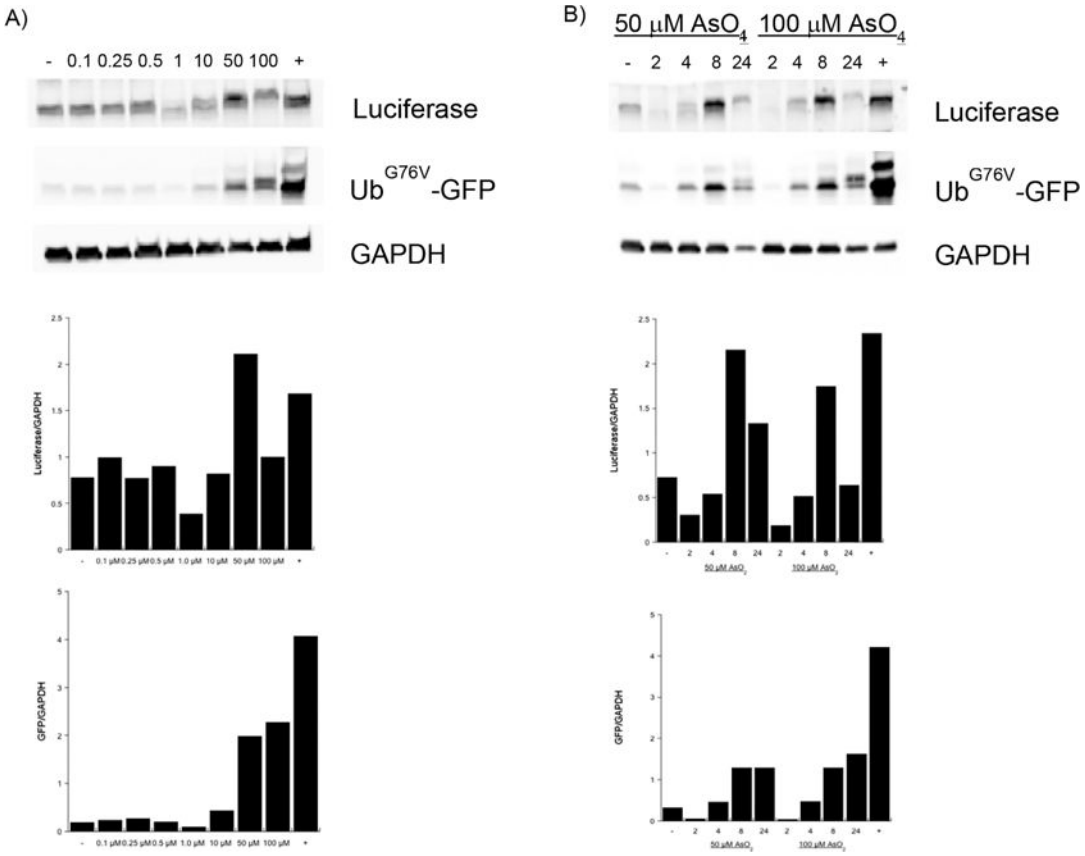

Figure 5.

Arsenate compromises p97 and proteasome function in a dose and time dependent manner. (A) DTC25 cells treated with increasing levels of arsenate for 16 h were analyzed by Western blot. DMSO was used as a negative control, and 1 μM MG132, a proteasome inhibitor, was used as a positive control. (B) DTC25 cells were treated with 50 μM arsenate and harvested for Western blot analysis at the times indicated. DMSO was used as a negative control, and 1 μM MG132 was used as a positive control.