

ABSTRACT

SAVINO, RONALD JOSEPH.—The Impact of Methionine on Avian Myoblast Proliferation and Differentiation (Under the direction of Drs. Paul E. Mozdziak, James N. Petite, Christopher Ashwell, and Bartosz Kempisty).

Methionine's role as an amino acid is essential in a number of cellular functions, particularly as the initiator of protein synthesis, and has long been understood to be indispensable. More recently, its function within cells and proteins has been further expanded to protein stabilization, antioxidant, and cell and protein activity modulating abilities. However, it has been found that cysteine, a similarly structured sulfur-containing amino acid, has the potential to substitute for methionine as a protein stabilizer, cell and protein activity modulator, and endogenous antioxidant. As it seems chemically justifiable that cysteine may be able to take the place of methionine in the role of protein stabilization, redox signaling, and antioxidant function, the essential function of methionine may lie within the initiation of protein synthesis. New discoveries in the field of protein translation initiation have identified a number of alternative pathways that may allow translation machinery to bypass traditional methionine-dependent initiation. These alternatives include the use of non-methionine amino acids for initiation, internal ribosome entry sites, and ribosome recycling factor and the use of these may allow the synthesis of proteins to occur in the absence of methionine, challenging the essential nature of methionine further. In this experiment, myoblasts derived from avian embryonic muscle tissue were grown in a variety of methionine sources at varying concentrations. L-methionine is the commonly supplemented form of methionine in cell culture and was used as the positive control group (15 mg/L), Methionine deficient RPMI was used as a negative control, and varying concentrations of DL-methionine (DLM; 1.125 mg/mL or 0.56 mg/mL) and methionine hydroxy analog (MHA; 1.28 mg/mL or 0.64 mg/mL) were used as treatment groups in a proliferation

assay Unexpectedly, cell division in the absence of methionine was not significantly different than that observed for the positive control. Significantly, myoblast fusion showed greater myotube diameter between methionine supplemented groups compared to the methionine deficient negative control. It appears that in the absence of methionine, avian myoblasts may be able to employ alternative amino acid initiators, internal ribosome entry sites, ribosome recycling factor, a novel mechanism, or a combination of these in order to continue protein synthesis and survive in nutrient-stressed environments. The reduced myotube diameter in the methionine deficient group compared to all methionine supplemented groups implies that methionine is, however, necessary for the level of protein synthesis required for normal myotube hypertrophy. Further research into the avenues of alternative protein synthesis initiation and cell survivability in the absence of methionine within avian myoblasts is warranted.

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The Impact of Methionine on Avian Myoblast Proliferation and Differentiation

by
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BIOGRAPHY

Since coming to North Carolina State University in the Fall of 2020, I have developed a passion and appreciation for cell culture that has extended into my career aspirations. Coming here with no experience in the field of cell culture, my work with Dr. Mozdziak and Dr. Petite has developed my skills to the point where I have become proficient in working with both avian and mammalian derived myoblasts. With this, I have become enamored with the field of cellular agriculture and after graduation I will be starting a position for a company in Raleigh, NC working to create a lab cultivated meat product for eventual commercialization.

ACKNOWLEDGMENTS

I would like to acknowledge the guidance and contributions of Dr. Paul Mozdziak, Dr. James Petite, and Dr. Bartosz Kempisty during this project and over the last two years that I have spent at NC State. They are a large reason for the career I am currently pursuing; this is certainly not where I thought I would've ended up coming out of my undergraduate degree, but it is more than I could have hoped for.

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The Role of Methionine in Cells and Proteins and Survivability in the Absence of Methionine

1. Introduction

The understanding of the scope of methionine's function has been a subject of much debate for a number of decades. It is often considered an essential amino acid due to its inability to be synthesized *in vivo* as well as its necessity for the initiation of protein synthesis. Beyond that, its stabilizing role in the hydrophobic core of proteins was believed to be replaceable by any other hydrophobic amino acid. Unlike methionine, cysteine has been explored as a multifunctional amino acid that has a number of properties regularly used by cells. Besides cysteine being the rate-limiting component of the well-known antioxidant glutathione [1], the antioxidant function of cysteine appears to be an endogenous protection mechanism used by proteins. Cysteine was also identified for its stabilizing ability in endogenous and exogenous proteins as well as its participation in certain signaling pathways of cells [2-10]

Within the past two decades, research has revealed a number of newly discovered properties of methionine that expand its role in proteins and within cells. An in-depth review by Lim, Kim, and Levine characterized many of the unique and novel aspects of methionine, giving way to further exploration into its importance outside of protein synthesis [32]. Unique *s*-aromatic interactions formed by methionine granted to the amino acid by its inclusion of a sulfur atom have been characterized and found to contribute to the stability of intracellular proteins [11,12]. Additional work has shown antioxidant properties similar to that of cysteine that serve important protection within proteins which prevent damage by reactive oxidative species [13], with the reduction of methionine antioxidant activity being related to cell aging and certain diseases [14,15]. Methionine has also shown an ability to serve as a modulator of cellular activity during

times of oxidative stress such as calmodulin activity to prevent further cell damage via the production of additional ROS [16]. With a clearer picture of the array of roles that methionine has for protein and cell function; the focus shifts into understanding whether these functions are essential to proteins and cells. New research looking into the properties of cysteine reveals similarities in hydrophobicity [17] while the ability to form similar stabilizing motifs as methionine has been previously identified. If it is indeed possible that cysteine may be able to take the place of methionine in many of these roles related to structure, antioxidant, and modulation then once again the focus of the essential function of methionine within protein synthesis. Recently, novel research in the field of eukaryotic and prokaryotic protein translation has provided evidence for the initiation of protein synthesis with alternative amino acids and initiator tRNAs as well as the complete bypassing of the initiation stage by use of unique mRNA structures. In this review, the aforementioned functions of methionine are inspected in the context of their discovery and commonality. The potential for cells and proteins to remain functional in the absence of methionine is discussed based on the shared attributes between methionine and cysteine as well as recent evidence contesting methionine's essential nature in initiating protein synthesis.

2. Methionine's role in protein structure

Methionine resides among a unique class of amino acids due to its incorporation of a sulfur atom. There are four sulfur-containing amino acids: methionine, cysteine, taurine, and homocysteine (fig. 1) of which only methionine and cysteine play a role in the synthesis and structure of proteins. Early studies have shown that supplementing the diets of animals with methionine alone had rates of growth similar to animals fed diets supplemented with both

cysteine and methionine, while animals fed diets supplemented with exclusively cysteine saw significantly lower rates of growth [18]. In follow-up studies it was shown that, at suboptimal levels of methionine, cysteine was able to compensate for this lack of methionine and normal rates of growth were observed [19]. These early findings suggest a cysteine compensatory action in a methionine-deficient environment which allowed for normal rates of growth to occur. If only a minimal amount of methionine is needed for growth, these findings suggest that methionine may only be necessary to perform its most essential function of translational initiation while cysteine compensates for the non-essential functions of methionine.

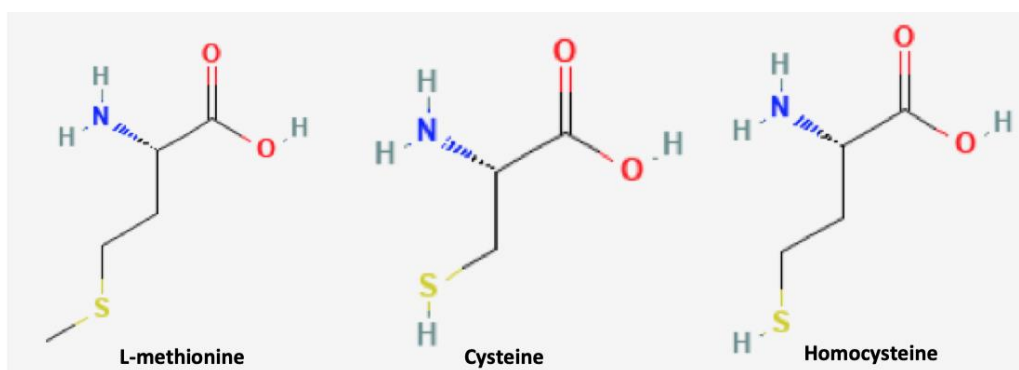


Figure 1. Structure of the sulfur-containing amino acids.

(Left) L-methionine (PubChem CID 6137), (Center) Cysteine (PubChem CID 5862), (Right) Homocysteine (PubChem CID 91552).

It has been many years since these initial studies, and in that time knowledge of the function of methionine in proteins has grown. It is commonly known that methionine is the first amino acid added during protein synthesis as well as regularly incorporated to the growing peptide chain. The two roles of methionine in protein structure, the initiating methionine and internal methionine will be described separately.

In both eukaryotes and prokaryotes, the initiating tRNA encodes for L-methionine or N-formylmethionine respectively (Fig. 2). Though crucial for the initiation of protein synthesis,

shortly after the onset of elongation this same methionine is removed from the polypeptide chain. As discussed by Wingfield [20], N-terminal methionine in eukaryotes is co-translationally cleaved by the enzyme methionine aminopeptidase (MAP) soon after it initiates protein synthesis (fig. 3). A similar mechanism can be seen in prokaryotes which first requires the removal of the formyl group by formylmethionine deformylase which leaves an N-terminal methionine ready to be removed via MAP. In any given proteome, about two-thirds of the proteins represent potential targets for MAP cleavage and in bacteria and yeast, the inhibition of MAP activity is lethal. Other experiments have further shown that the removal of the N-terminal methionine or N-formyl methionine is essential for the function and stability of proteins [21-23]. So not only is N-terminal methionine not essential for the structural integrity of proteins, but its removal is also a necessity in many cases.

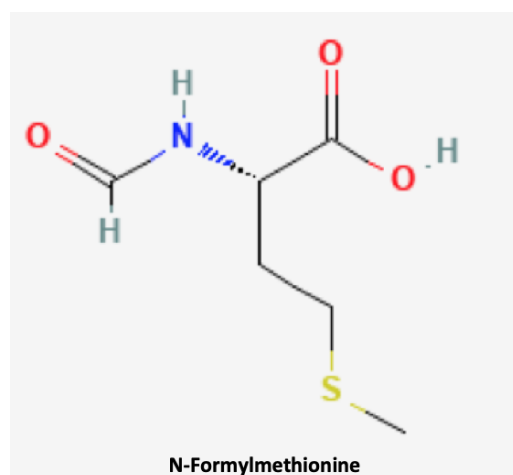


Figure 2. Structure of N-Formylmethionine.

The image above displays the structure the N-terminal methionine that is encoded for during the initiation of prokaryotic protein synthesis (PubChem CID 439750).

Since methionine is a hydrophobic amino acid, it has often been assumed that its main role within proteins is maintaining the structure of the hydrophobic core. It was believed for many

years that other hydrophobic amino acids could replace methionine within a protein with little consequence. More recently, the role of the sulfur atom of methionine has been explored for its ability to form stabilizing bonds within a protein. Motifs known as S-aromatic motifs are formed by the sulfur atom found in the side chain of methionine and aromatic residues within the protein have been characterized as far back as 1985 [24]. Almost 20 years later the protein stabilizing contribution of S-aromatic motifs was shown by Zauhar, Morgan, and Welsh [11] leading to additional research into the motifs by Valley and colleagues [12]. This research served to characterize the features of methionine-aromatic interactions and found that they occur at a greater distance than salt bridges (5-7 Å and 4 Å respectively) while the energies of these interactions are comparable. Work by Orabi and English [25] modeled S-aromatic motifs in proteins which showed that the redox capability of these interactions provides dynamic flexibility to proteins which is an important feature for their activity. As S-aromatic motifs are quite common within proteins, it is likely that they represent a significant stabilizing feature. The oxidation of methionine to methionine sulfoxide as is discussed later is another important function of methionine, however, how this oxidation affects S-aromatic motifs within proteins is very interesting. It was later discovered [26] that oxidation of methionine actually strengthens the methionine-aromatic interaction rather than weakening it. It is also worth noting that with increased rates of methionine oxidation, changes in the protein structure including increased protein surface hydrophobicity occur which can be an age-related effect occurring over time or during specific periods of oxidative stress [27]. Clearly, methionine's capacity within proteins extends beyond merely contributing to the hydrophobic core of proteins; its stabilizing function should not be discounted.

3. Antioxidant function of methionine

3.1. Discovering Methionine's Antioxidant Function

Within certain proteins, methionine residues may be exposed to the outer surface, making them susceptible to interaction with ROS. In 1982, Brot and Weissbach described the oxidation of methionine which produces methionine sulfoxide and, when further oxidized, produces methionine sulfone [28]. It was also observed that free and protein-bound methionine sulfoxide could be reduced back to methionine by methionine sulfoxide reductase while methionine sulfone was unable to be reduced (Fig. 4). The oxidative capacity of methionine gave birth to the hypothesis that cells could utilize this property as an endogenous antioxidant for protection against ROS. Testing of methionine antioxidant hypothesis began by observing the effect of exposing glutamine synthetase derived from *E. coli* to hydrogen peroxide [13]. The results showed that methionine residues on the surface of the protein that were susceptible to oxidization were arranged in a way that prevented the oxidation of the active site on the protein.

Additionally, it was postulated that due to the ability to reduce oxidized methionine residues using methionine sulfoxide reductase, these methionine residues could protect against oxidization repeatedly [29]. Confirmation of the methionine antioxidant hypothesis was later done by Luo and Levine in an experiment where methionine was substituted with norleucine (Fig. 5) in *E. coli* [30]. While enzymatic activity or growth rates were not significantly different in colonies supplemented with norleucine vs methionine, when exposed to oxidizing agents, cells with norleucine substitution died at rates significantly faster than those containing methionine. Later testing of the oxidative function of methionine found that eukaryotic cells increased methionine incorporation within proteins in times of oxidative stress [31]. A review by Lim, Kim, and Levine [32] looked even further to the adapted antioxidant function in the context of α -

2-Macroglobulin; a protein that was found with methionine residues lining its active site as a purposeful protection mechanism. This all implies that use of methionine for protection against oxidation is an adapted function utilized by cells rather than a secondary function.

3.2 Reversible Oxidative Ability of Methionine

The enzymes that give methionine its repeated oxidative capacity are as important to the endogenous antioxidant ability of proteins as methionine itself. The oxidation of methionine to methionine sulfoxide yields both an R-MetO and S-MetO form which requires a different set of enzymes for their reduction [33]. Methionine sulfoxide reductase A (MsrA) is responsible for reducing S-MetO [34-36] while MsrB reduces the R-MetO [37]. Though MsrA is able to reduce both free and protein-bound methionine, there are two separate enzymes that are needed to reduce R-MetO. MsrB reduces the protein-bound R-MetO but has low activity on free R-MetO; it is the enzyme free methionine-R-sulfoxide reductase (fRMsr) that has high specificity and activity on free R-MetO [38-40]. Interestingly, the activity of fRMsr has only been observed in prokaryotic organisms whereas plants and mammals lack this enzyme. The importance of these enzymes has been noted in experiments where knocking out MsrA in mice [41] and bacteria [42,43] led to states of oxidative stress. In cases where the expression of MsrA was over-expressed, human T-cells showed increased resistance to oxidative stress [44] and was even found to double the lifespan in *Drosophila* [45]. The activity of these enzymes since their discovery has also been known to decrease with age and in certain diseased states [14,15,46,47]. Many new therapies have emerged that have focused on the antioxidant function of methionine and the reducing enzymes to counter increased oxidative states present during aging and disease [48-51]. These findings reveal that the role of methionine oxidation and subsequent reduction by

these enzymes is not only important for the survivability of both prokaryotic and eukaryotic cells but that their antioxidant capacity can be used as a preventative measure against age-related and disease-related damage.

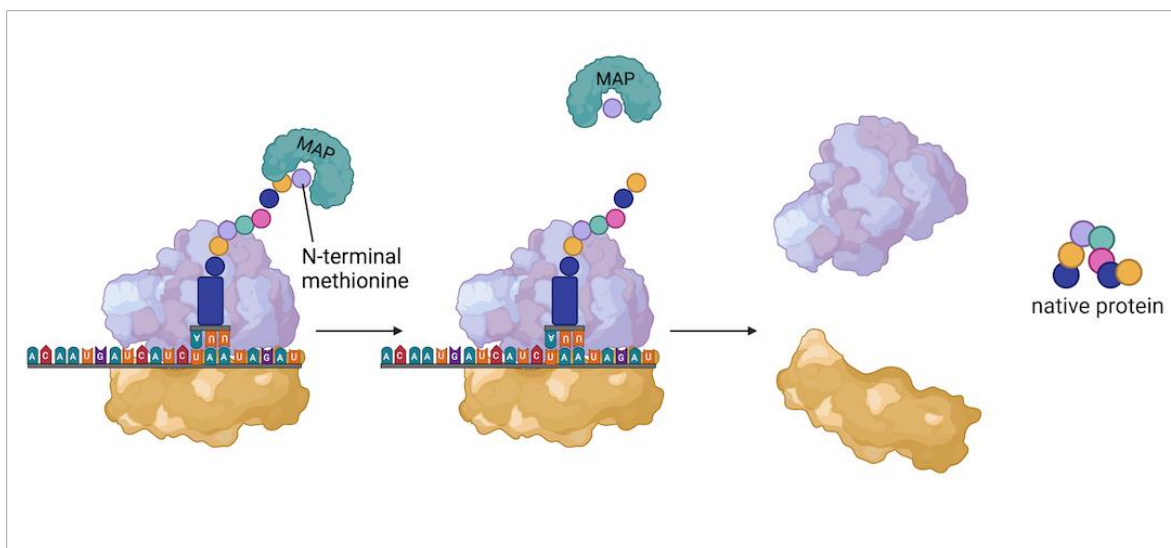


Figure 3. Methionine aminopeptidase acting on N-terminal methionine.

Above is the general function of methionine aminopeptidase (MAP) in eukaryotic protein synthesis. From left to right shows the binding of MAP to the N-terminal methionine, the removal of the methionine, and the disassociation of the ribosomal subunits leaving the native protein.

4. Methionine as a modulator of cellular activity

In addition to protecting cells from oxidative stress, the reversible nature of methionine oxidation led to questions regarding whether it could serve as a mechanism of cell signaling and regulation. Early assumptions were that, in most cases, the oxidation of protein-bound methionine was detrimental to protein enzymatic activity [28]. It was not until almost a decade later that the oxidation of methionine was found to be able to activate and deactivate some proteins as a sort of

on-off switch to maintain homeostasis [16]. Since then, a plethora of examples of the oxidative state of methionine being involved in the activity state of cells have been identified.

4.1 Methionine and Calmodulin

Calcium regulatory proteins such as calmodulin (CaM) and plasma membrane Ca-ATPase (PM-Ca-ATPase) during states of oxidative stress have been observed to have reduced activity associated with the oxidation state of their methionine residues [52] (Fig. 6). The reduced activity in the presence of high levels of reactive oxidative species (ROS) is believed to be a survival mechanism as it prevents the production of additional ROS created as by-products of cellular metabolism. Calmodulin has additional multifunctional activity within a cell as a part of important calcium-regulated signal transduction pathways so the discovery of oxidative activity on methionine within CaM led to further inquiries. It was found that the Met77 of CaM was susceptible to oxidation mediated by MsrA which was found to regulate its interaction with one or more of its targets [53]. Using a mutant M77Q which mimicked a perpetually oxidized Met77 revealed that this mutant CaM was less effective in activating CaMKII α [54]. The identification of a direct correlation between CaM oxidation and decreased activity is evidence and confirmation of the modulatory ability of methionine oxidation with CaM which changes cellular function.

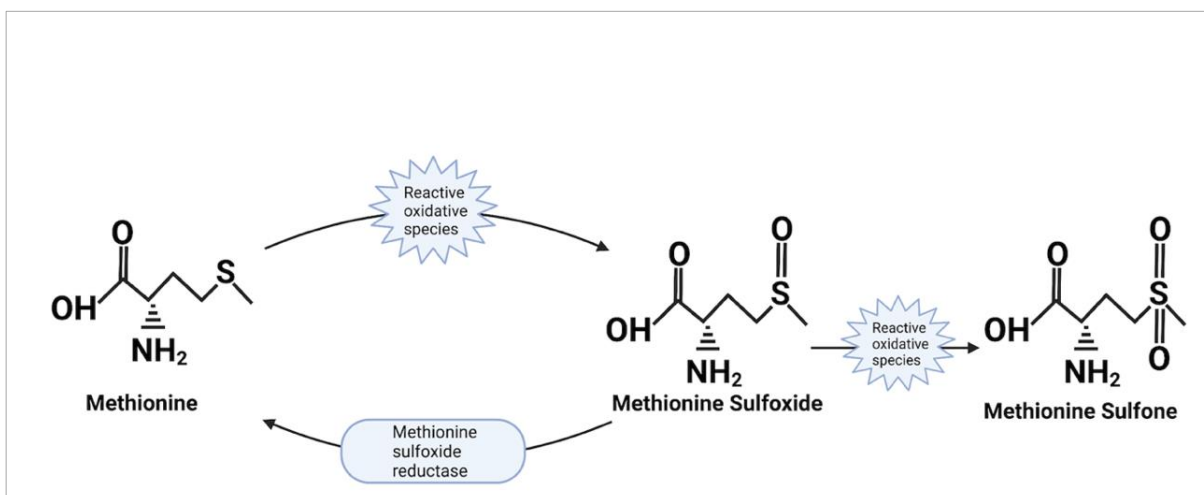


Figure 4. The oxidation and reduction of methionine.

This figure displays the action of methionine as an antioxidant. Reactive oxidative species oxidize the sulfur side chain of methionine to produce methionine sulfoxide and may further oxidize methionine sulfoxide to produce methionine sulfone. Also shown is the action of methionine sulfoxide reductase to reduce methionine sulfoxide back into methionine though it is unable to reduce methionine sulfone.

4.2 Methionine and Actin

The actin that is a major component of the cytoskeleton within almost all eukaryotic cells has also been found to be a target for methionine oxidation [55]. The polymerization and depolymerization of actin within cells is a highly regulated process. Of the 16 methionine residues found in actin, 6 have been found to convert to methionine sulfoxide in the presence of oxidizing agents: Met44, Met47, Met176, Met190, Met269, and Met355. Further, Met44 and Met47 exhibit the greatest vulnerability to oxidation [56]. Hung, Pak, and Terman used oxidizing proteins known as MICALs [58] to oxidize the Met44 of actin which led to the filament severing and a decrease in polymerization ability [57]. More recently it has been discovered that this oxidation leading to reduced polymerization is a reversible process. The oxidation of Met44 is stereospecific, generating the R-MetO form of methionine sulfoxide which can be reduced back

to methionine by MsrB methionine sulfoxide reductase [59,60]. The implications of the oxidative and reductive states of F-actin are a capability to modify the polymerization to G-actin thus changing cytoskeletal activity based on the needs of the cell.

4.3 Methionine and Ion Channels

The redox potential of the sulfur-containing amino acids also has an effect on the activation states of certain ion channels which control the excitability state of the cell. In certain cases, the oxidation of cysteine and or methionine within Ca^{2+} activated K^{+} channels (KCa) have shown to decrease channel activity with normal ion flow restored upon reduction [61,62]. Interestingly, there have also been examples found of the opposite effect occurring where KCa channels were activated upon oxidation [63]. Though in these cases the redox effect was not confirmed to be caused by either cysteine or methionine specifically, there have been more recent studies where channels that activate and deactivate depending on whether cysteine or methionine is oxidized [64]. In Na^{+} channels, the oxidation of Na^{+} channels in skeletal muscle and cardiomyocytes of mammals inhibited their inactivation [65]. The inhibition of inactivation in Na^{+} channels of muscle cells can result in changes in the electrical activity, which in the case of cardiomyocytes can lead to the fatal prolonging of action potentials [66,67].

Though these are only a few of the many examples of modification of cellular activity based on methionine's oxidative state, they exemplify that cells have evolved to use the oxidative capacity of this sulfur-containing amino acid for a number of functions. Since in all of these cases the reduction; and therefore, resumption of activity following methionine oxidation is dependent on the activity of methionine sulfoxide reductase, it has been postulated that this mechanism of oxidation and reduction could serve as a longer-lasting post-translation modulator of cellular

activity [68]. Many of the scenarios that have been described are of a negative feedback mechanism that downregulates the activity of cells during times of oxidative stress to prevent further damage. It is evident now that methionine not only serves as an antioxidant but also plays an important part in the balance of cellular activity.

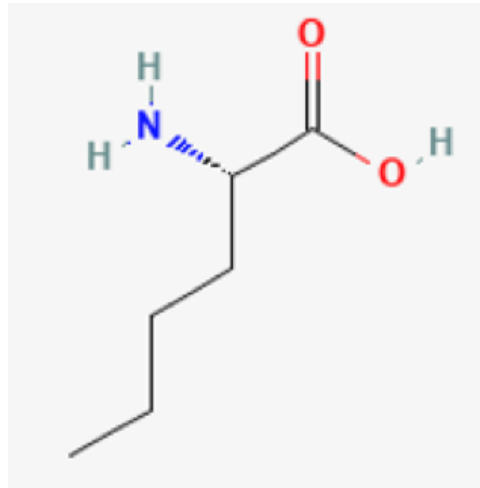


Figure 5. The chemical structure of norleucine (PubChem CID 21236).

5. Cysteine playing the part of methionine

5.1 Transmethylation and Transsulfuration

The expansive role of methionine within proteins has been the subject of recent work to exemplify that it is indeed important beyond the initiation of protein synthesis. However, whether these functions are essential or merely useful adaptations is remains to be demonstrated. The relationship between methionine and cysteine is a balance, with methionine being used for the synthesis of cysteine. An early study by Vigneaud, Kilmer, Rachele, and Cohn demonstrated the metabolic conversion of methionine to cysteine using radiolabeled sulfur and found that 80% of sulfur found in cysteine had been derived from the labeled methionine [69]. What was later discovered by Cantoni was the intermediate S-Adosylmethionine (SAM) which aided in the

understanding of the pathway of methionine metabolism [70]. The first series of reactions begins with the conversion of methionine to SAM using the enzyme methionine adenosyl transferase. The newly formed SAM then utilizes a methyl transferase to donate its methyl group to an acceptor which results in the formation of an additional intermediate: S-Adenosylhomocysteine (SAH). SAH is then hydrolyzed by the enzyme S-Adenosylhomocysteine hydrolase which produces homocysteine marking the end of the transmethylation pathway. Homocysteine lies at a key junction in the synthesis pathway between methionine and cysteine. Depending on cysteine and methionine levels in the cell, homocysteine may be methylated back into methionine by methionine synthase, or it may undergo transsulfuration to produce cysteine (Fig. 7). In studies where excess cysteine was supplemented, parenteral methionine supplemental requirements dropped to 70% of the enteral requirements which was coupled with an increase in the re-methylation pathway of homocysteine [71,72]. This highlights the interconnection and internal balance that is maintained between these two amino acids and that cysteine production by methionine is not an essential function of methionine.

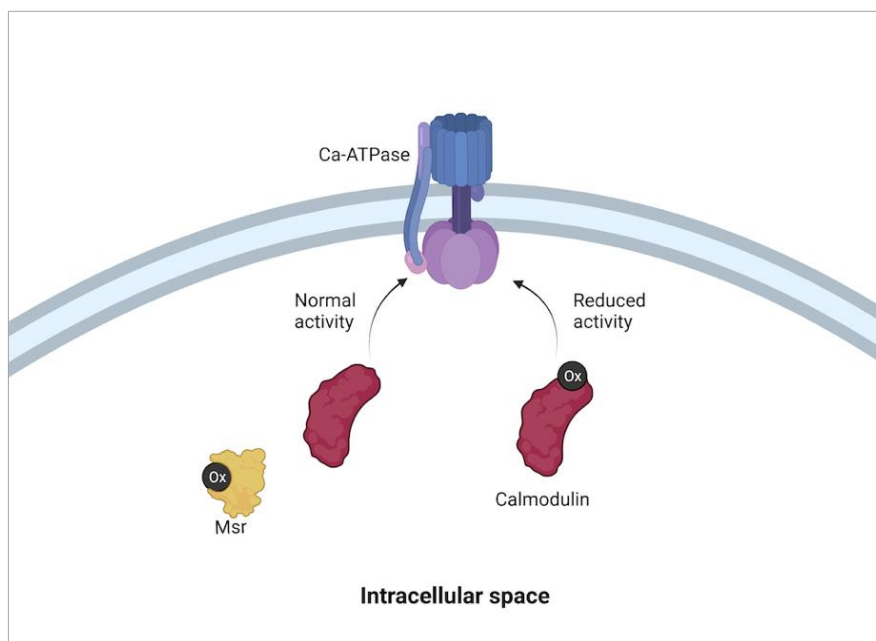


Figure 6. Modulation of calmodulin activity with oxidative state.

When reactive oxidative species (Ox in black) concentration is high in a cell, the oxidation of methionine residues within calmodulin modifies its activity. This in turn reduces the activity of Ca-ATPase pumps embedded within the plasma membrane. The reduction of Ca-ATPase pump activity alters intracellular Ca balance which then causes a reduction on cell metabolism. The reduction of methionine residues on calmodulin by methionine sulfoxide reductases (Msr) then returns normal activity to calmodulin and therefore cell Ca-ATPase pumps and cell metabolism [52].

An important characteristic of this pathway is that, while homocysteine can be converted to either methionine or cysteine, once homocysteine undergoes transsulfuration it cannot be converted back to methionine. This principle relates to the study by Womack, Kemmerer, and Rose because cysteine cannot be converted to methionine so, with only cysteine supplementation, protein synthesis was unable to be initiated at optimal rates, and growth was significantly reduced [18]. Again, referring to the work of Womack and Rose, the observation of normal growth in animals supplemented with suboptimal methionine and excess cysteine provide

the basis of the theory that all of the previously discussed roles of methionine are non-essential beyond the initiation of protein synthesis [19].

5.2 Cysteine and Methionine in Protein and Cell Structure

When looking at the structural contributions that both of these amino acids provide to proteins, there are many similarities. Like methionine, the sulfur atom of cysteine is subject to oxidation. Once oxidized, cysteine has the ability to form disulfide linkages within a protein. These disulfide linkages help to stabilize the tertiary and quaternary structures of proteins and exogenous proteins with more disulfide linkages are able to maintain biological function in more extreme environments (Fig. 8) [73]. Though this ability does showcase an example of cysteine's protein stabilizing properties, it is only relevant for extracellular proteins as the formation of disulfide bonds is restricted within intracellular proteins [74]. Both the hydrophobic property of cysteine as well as its potential to form S-aromatic bonds similar to methionine is important. The hydrophobic nature of cysteine was first described by Nagano, Ota, and Nishikawa who found that free cysteine residues were located in the hydrophobic core of 3D-modeled proteins with the likes of methionine [36]. Cysteine is unique as well in that its side chain provides both hydrophobic and polar properties. The polar nature of cysteine would seem to directly contrast its contribution to the hydrophobic core of proteins, but recent evidence has shown that the hydrophobic properties of cysteine are energetically favorable to its polar nature [17]. Additional evidence for the replaceable nature of methionine with cysteine emerges when considering the S-aromatic motifs. While S-aromatic motifs are valuable protein stabilizing features of methionine inclusion, they are not unique to methionine. S-aromatic interactions are simply non-covalent complexes formed between aromatic residues and sulfur-containing groups. Cysteine has been well documented to be able to form these motifs within proteins [24,75,76].

Given the hydrophobic nature of cysteine as well as the ability to form S-aromatic motifs, it certainly suggests that on the basis of the protein stabilizing action of methionine; cysteine can serve this same role in methionine's absence.

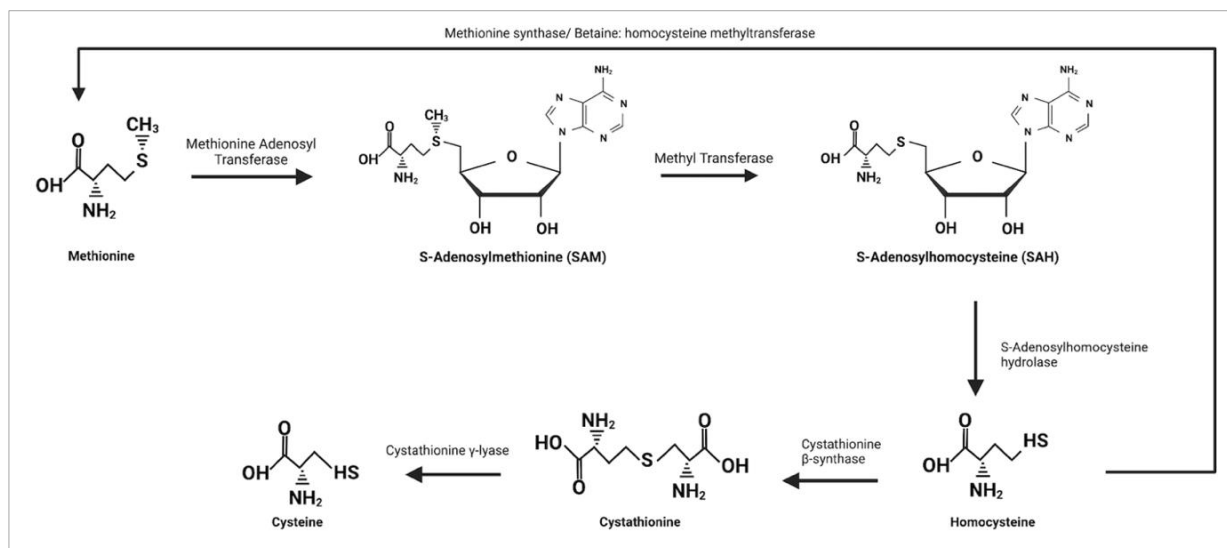


Figure 7. The conversion of methionine to homocysteine and cysteine.

Conversion of methionine to S-Adenosylmethionine (SAM) begins with methionine adenosyl transferase which is an ATP-dependent reaction requiring the removal of all 3 phosphate groups from ATP. SAM then donates its methyl group to a methyl acceptor using a methyl transferase to form S-Adenosylhomocysteine (SAH). SAH is hydrolyzed into homocysteine and adenosine by S-Adenosylhomocysteine hydrolase which marks the end point of the transmethylation reactions. At this point homocysteine may be methylated by either methionine synthase or Betaine: homocysteine methyltransferase to be converted back into methionine or undergo transsulfuration to form cysteine. Transsulfuration begins with the conversion of homocysteine to cystathionine by cystathionine β- synthase and then ends with the formation of cysteine from cystathionine by cystathionine γ-lyase.

5.3 Cysteine's Antioxidant Function

The endogenous antioxidant feature of methionine, much like the S-aromatic motifs, is centered around methionine's sulfuric side chain. As such, cysteine shares many of these antioxidant abilities and some that go even beyond sulfur oxidation. Cysteine differs from methionine in that, the sulfur of cysteine is in a thiol form compared to methionine's thioether form. The functional significance of the thiol form on cysteine is that it is ionizable and contains a negatively charged thiolate group that is generated after deprotonation thus boosting its reactivity. Additionally, this thiol group is subject to alkylation and is regularly targeted for oxidation by reactive oxygen and nitrogen species [77]. These thiol groups come into play when cysteine is incorporated on the surface of a protein thus exposing these thiol groups. A study conducted by Requejo, Hurd, Costa, and Murphy found that the majority of free thiols in rat liver and heart cells were from surface-exposed cysteines and not glutathione-incorporated cysteines [78]. These findings suggest that the greater antioxidant function as it relates to cysteine is within protein-incorporated cysteine and not glutathione.

As was alluded to, cysteine's antioxidant function goes beyond its endogenous role as it is a key component of the antioxidant glutathione. Glutathione is a tripeptide that consists of cysteine, glutamic acid, and glycine and is present in most mammalian tissue. It serves as a free radical scavenger and detoxifying agent that protects proteins against oxidation via ROS and the reduction of glutathione increases oxidative stress within an organism [79]. In glutathione production, cysteine often serves as the rate-limiting component which suggests that a reduction of cysteine supplementation would greatly increase oxidative stress within a cell. Cysteine's antioxidant activity is potentially more important outside of its incorporation within glutathione. As an antioxidant, cysteine is both capable of serving a similar purpose of endogenous

antioxidation as methionine as well as being critical to glutathione production which would suggest that cysteine's antioxidant capability is much greater than methionine's.

5.4 Cysteine Modulating Cell and Protein Activity

Finally, when considering the ability of methionine redox potential to serve as an instrument for the inactivation of certain cellular mechanisms, it seems cysteine may likely be able to fill in here as well. In rat brains, the oxidation of cysteine has been observed to be an inhibitor of fast inactivation by preventing the closure of the pore by the gate mechanism of K⁺ channels [80]. The formation of disulfide bridges between cysteine residues on the channel and cysteine residues within the inactivation gate maintain an open conformation (Fig. 9). Later work by Heinemann, Rettig, Wunder, and Pongs found additional evidence for cysteine oxidation and reduction controlling inactivation within the β subunits as well [81]. This very closely mirrors the disruption of inactivation for K⁺ channels detected by the oxidation of methionine residues [102], which implies that these mechanisms may work in concert but also be able to replace one another in the event of their removal. Sullivan identified cysteines within the NR1 subunits of NMDA receptors in the brain that control their sensitivity to the neurotransmitter glutamate as well as H⁺ based on their redox status [82]. Within the endoplasmic reticulum (ER), additional control mechanisms using cysteine have been identified that regulate the formation of ROS during disulfide bond formation. Exogenous proteins released by the ER form disulfide bonds before they are released to improve protein stability in the exogenous environment. The formation of these bonds is within the ER is catalyzed by protein disulfide isomerase (PDI) and endoplasmic reticulum oxidoreductin 1 (Ero1). PDI oxidatively folds extracellular bound proteins and oxidase transfers electrons to Ero1 which then transfers those electrons to a terminal

acceptor oxygen. The transferring of electrons to oxygen by Ero1 produces a single molecule of H₂O₂ per de-novo disulfide bond formed [104]. In order to prevent the overproduction of oxidative H₂O₂, cysteine residues within the Ero1 will form intramolecular disulfide bonds during times of high H₂O₂ production which constrict Ero1 activity and therefore reduce the oxidative stress within the cell [83-85] (Fig. 10). These findings all show that it is the redox capacity of cysteine that is controlling the activity in these different structures, much like what was observed in methionine modulated activity. Because methionine and cysteine have characteristics of hydrophobicity and aromatic residue interactions, it is possible that in the absence of sufficient methionine, cysteine could take its place with little change in protein structure, antioxidant capability, or protein activity modulation.

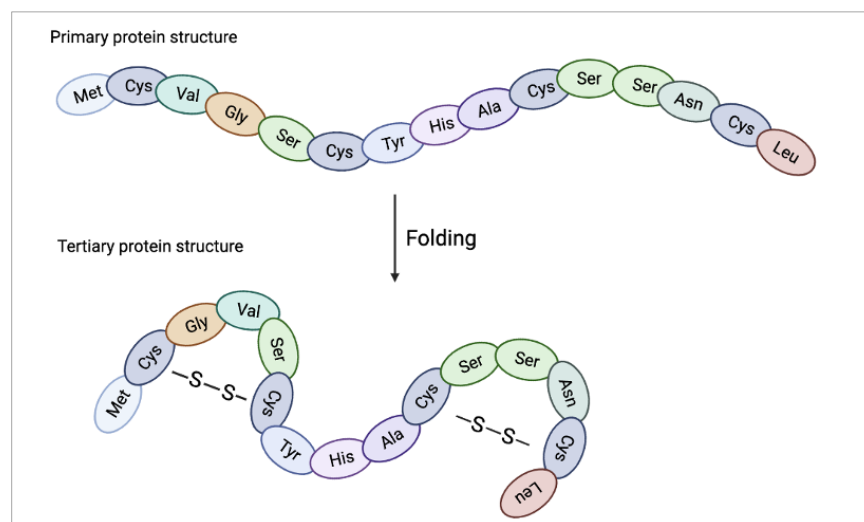


Figure 8. Disulfide bonds between cysteine in exogenous proteins.

This image demonstrates the function of cysteine in the folding and structural integrity of proteins. The top of the image shows the primary structure of the peptide chain while the bottom of the image shows the formation of the tertiary structure partially through the formation of disulfide bonds between cysteines within the protein.

6. Alternative mechanisms for protein synthesis initiation

6.1 Initiating Protein Synthesis with Alternative Amino Acids

With proper cysteine supplementation, it appears likely that the auxiliary functions of methionine can be replaced in a methionine deficient environment. Once again, the true essential nature of methionine seems to be within the context of protein synthesis initiation. However, in recent years, evidence has emerged challenging even this notion and suggesting alternative initiators of protein synthesis. Methionine has been known as the initiator of protein synthesis for a number of decades, and during this time, researchers have defined the features of methionine and formyl methionine tRNA that give it this unique ability as is well described in work by Mayer, Stortchevoi, Köhrer, Varshney, and RajBhandary [86]. Modern studies have used this information in an attempt to identify amino acids with similar properties and manipulate these structures that could serve as a replacement for methionine tRNA *in vivo*. In *E. coli*, substitutions of the methionine initiating anti-codon CAU with alternative anti-codons have shown an ability to initiate protein synthesis using valine, phenylalanine, isoleucine, tryptophan, and glutamine [87-90]. In a recent review [91] and subsequent experiments [92,93], the initiation of protein synthesis using non-canonical amino acids with mis-acetylated formyl methionine tRNAs both *in vivo* and *in vitro* is shown to be possible. These findings are very new; however, they display incredible plasticity and adaptability of translation machinery.

As methionine lies at a critical junction of the central dogma of eukaryotic translation, it wasn't until the functional adaptability in prokaryotic translation was identified that this mechanism in eukaryotes was tested. Among the first of these studies in eukaryotes was done by Drabkin and Rajbhandary using mammalian COS1 cells, they identified mutant initiator tRNAs, AGG and GUC which were able to initiate protein synthesis using methionine and valine respectively [94].

Though these alternative initiator tRNAs were synthesized artificially by experimenters rather than occurring naturally, the results are significant because they showed that there were no downstream detrimental effects on the structure or function of proteins produced or cell viability in the case of valine substitution. In a similar manner that the prokaryotic *E. coli* translational machinery showed incredible adaptability during initiation, the findings of these studies suggest that eukaryotic cells share a similar plasticity.

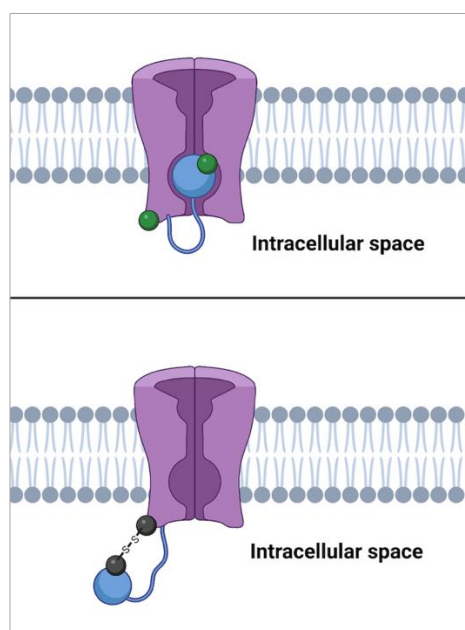


Figure 9. Inhibition of voltage-gated potassium channel inactivation.

The top half of this figure shows a voltage gated K^+ channel (in purple) in a state of inactivation. This state occurs when an auto-inhibitory peptide (blue ball) that is tethered to the N-terminus of the K^+ channel binds to the open channel pore by competing with intracellularly applied channel blockers [103]. When cysteine residues located on both the blocking peptide and the channel itself are in their reduced state (green ball), inactivation activity is rapid. The lower half of this image shows the inhibition of inactivation by the auto-inhibitory peptide. When the cysteine residues on the inhibitory peptide and channel are oxidized (black ball), they form disulfide bridges. These disulfide bridges effectively tether the auto-inhibitory channel blocking peptide to the N-terminus of the K^+ channel which prevents it from blocking K^+ ion flow leaving the channel in a state of activity [80].

6.2 Ribosome Recycling Factor

Though the study by Drabkin and Rajbhandary does showcase a surprising ability to utilize mutant tRNAs for initiation of protein synthesis, they still require a tRNA initiator nonetheless. What may be even more surprising is a study by Sasaki and Nakashima detailed the *Plautia stali* intestine insect virus which bypasses completely the need for an initiator tRNA to produce a capsid protein [95]. What was found was a specialized mRNA pseudoknot structure upstream of the glutamine codon which was necessary for this ability. A study by Malarkannan, Horng, Shih, Schwab, and Shastri showed a similar ability in MHC I molecules of a cryptic peptide where protein synthesis was initiated using a leucine CUG without the use of an initiating tRNA [96]. A factor that may be relevant to this ability is ribosome recycling factor (RRF) which has been identified in *E. coli* and is bound to the 70S monosomes and aids in ribosomal dissociation at the termination codon [97]. The deactivation of RRF led to the random translation of sequences downstream of the termination codon [97] which displays an ability of the ribosome to re-initiate translation independent of an initiation sequence of initiator tRNA. Deactivation of RRFs potentially allow for these 70S monosomes to remain attached to mRNA rather than dissociate. Because of this, it has been found that these bound 70S monosomes may re-initiate translation at leaderless mRNAs (mRNAs with only a few or no nucleotides preceding the AUG start sites) rather than requiring a re-initiation phase by the 30S ribosomal subunit [105]. A number of leaderless mRNAs have been identified, some of which confer to antibiotic resistance [106]. This adaption of bacteria using leaderless mRNAs as a survival mechanism suggests that the deactivation of RRFs could be employed by other cell types in stressed environments as well. Though these RRFs are not found ubiquitously in eukaryotic cells, they have been identified in

eukaryotic mitochondria which are presumed to have originated from the symbiotic development of eukaryotic cells [97].

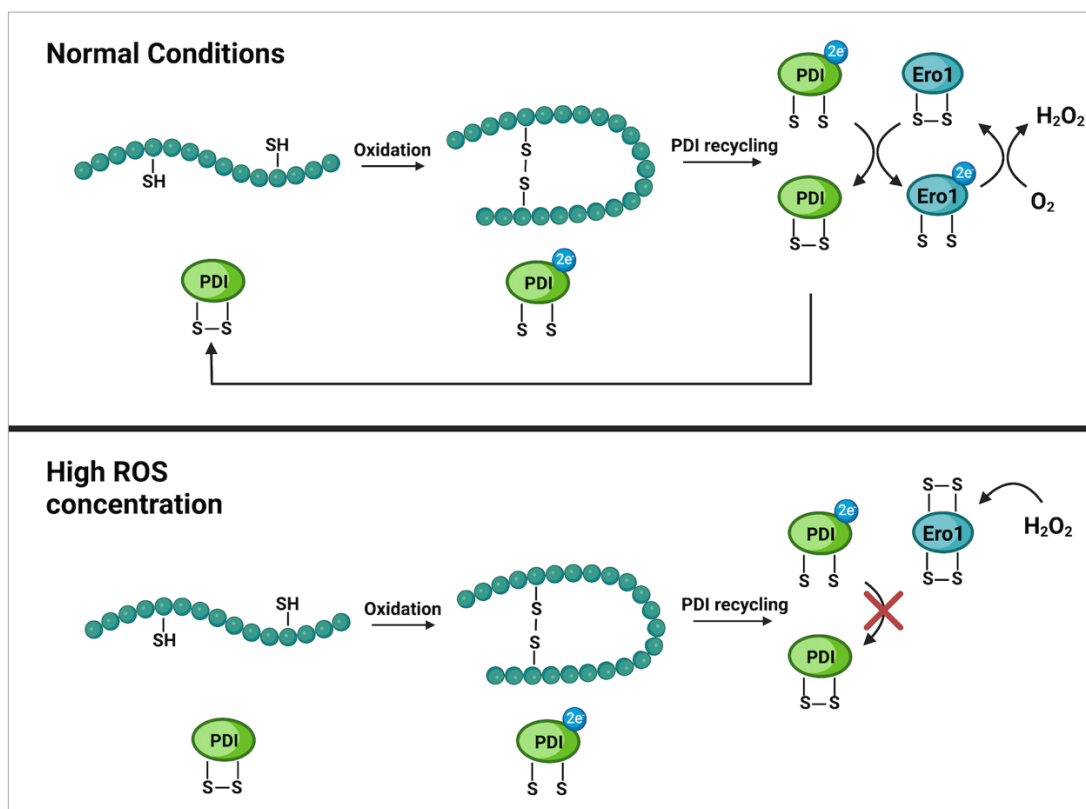


Figure 10. Cysteine oxidation of Ero1 as a protection mechanism.

The top portion of this figure displays the formation of disulfide bridges within proteins bound for the extracellular environment. This takes place in the endoplasmic reticulum of the cell and is mediated by the protein disulfide isomerase (PDI). Upon disulfide bridge formation, PDI is reduced and must transfer its electrons to the disulfide oxidase enzyme endoplasmic reticulum oxidoreductin 1 (Ero1) in order to return to its active state. The now reduced Ero1 then transfers electrons to a terminal acceptor oxygen which returns Ero1 to its active state and forms H₂O₂ as a byproduct [104]. The bottom portion of this figure shows the formation of intramolecular disulfide bridges between cysteines within Ero1 due to high concentrations of H₂O₂. This inhibits regular activity of Ero1 which then in turn prevents the recycling of PDI and therefore the formation of disulfide bridges within proteins. This serves as a protection mechanism to prevent the over-production of reactive oxidative species (ROS) during times of oxidative stress in order to protect the cell from damage [83-85].

6.3 Internal Ribosome Entry Sites

An additional mRNA motif that is of interest is the internal ribosomal entry sites (IRES) (Fig. 11). These are RNA structures located on the 5' end of untranslated regions within polycistronic mRNAs. They have a diversity of forms which can range from 9 to 1000 nucleotides in length as well as being unstructured or forming secondary and tertiary structures [98]. IRES are able to bypass the typical scanning phase of the ribosomal initiation complex so that initiation can occur independently of the 5' cap of mRNA. These complexes also remodel the small ribosomal subunit which then allows it to include the internal template into the RNA-binding channel [99]. This mechanism is commonly utilized by viruses which will suppress the normal translational activity of the host cell and then bind to the IRES to monopolize translation to only viral particles. While viruses are where IRES were discovered, it is now understood that mammalian cells take advantage of them as well. Under stressed conditions that downregulate cap-dependent translation such as hypoxia and nutrient deficiencies, cancer cells have been seen to use IRES to continue translation [100]. Later, the identification of thousands of viral and human sequences that initiate translation using IRES which is essential both in stressed conditions as well as during normal development [101]. Though most of this research is very new, it uncovers that there are an increasing number of motifs that may be utilized to continue translation in both nutrient-deficient environments as well as in non-stressed conditions.

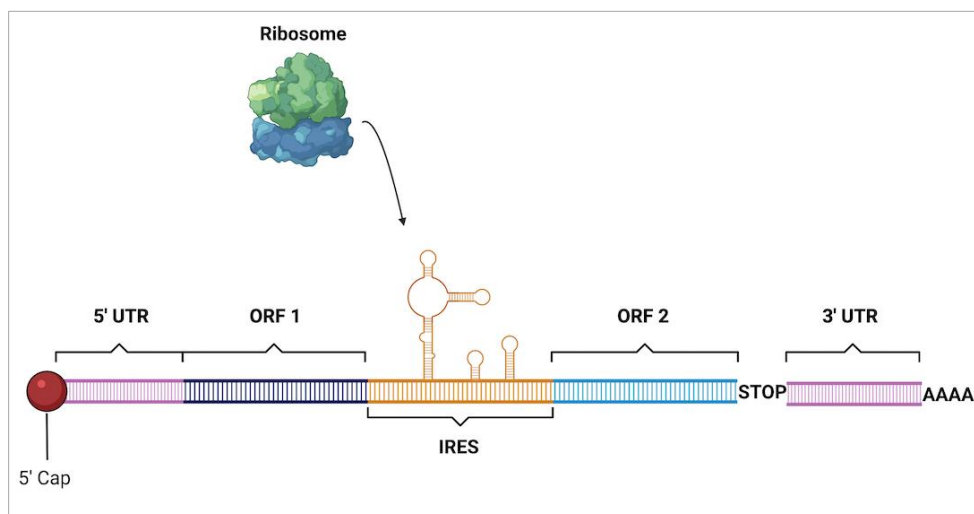


Figure 11. Internal ribosomal entry site.

Internal ribosomal entry sites (IRES) are complex secondary structures that form within mRNA that allow mammalian ribosomes to bind and begin translation. These motifs allow for initiation of translation to occur in a cap-independent manner.

7. Conclusions

As the properties of methionine continue to be explored it is clear that the uses for methionine go beyond simply initiating protein synthesis. The ability of the sulfur atom within methionine to form S-aromatic motifs within proteins has been shown to be a significant stabilizing factor helping to prevent protein denaturation. Evidence of methionine as a compelling antioxidant has not only showcased that the replacement of methionine with norleucine increased oxidative stress within cells but also that cells have adapted to take advantage of methionine's antioxidant properties by adding methionine residues around vulnerable receptors. Methionine has also been found to be a potent regulator of enzymatic activity once again highlighting that methionine serves a number of vital protective roles within cells. However, though cells utilize methionine for a growing variety of functions, they also maintain the ability to adapt to environments where methionine may not be present. Many of the functions of methionine center around its inclusion

of a sulfur atom: a feature that is shared with cysteine. Cysteine is also able to form stabilizing S-aromatic motifs in intracellular proteins as well as disulfide bonds in extracellular proteins, and with the hydrophobic nature of cysteine having recently been identified, it may likely be able to serve a similar stabilizing function as methionine within the protein core. The antioxidant features of cysteine seem to be even further reaching than that of methionine as cysteine has the same intrinsic antioxidant ability granted to it by a sulfur atom but with the added factor that it is essential for the synthesis of the intracellular antioxidant glutathione. Though the examples of methionine and cysteine modulating cell activity were not identical, it is possible that the potential for cysteine to replace methionine structurally as well as showing an ability to modulate cell activity based on its oxidative or reduced state could allow for cells to replace methionine with cysteine within proteins and maintain the same signaling features in a methionine deficient environment. Due to recent exploration into the mechanisms initiating protein synthesis, it now seems possible that in the absence of methionine that cells could use an alternative initiating amino acid or even circumvent the initiation process by using unique structural motifs within mRNAs. Though maximal cell and protein function is under conditions where all amino acid requirements are met, both prokaryotic and eukaryotic cells have shown impressive plasticity under environmental stress. The capability of cells and proteins to function in the absence of methionine is a topic worthy of additional research and many in the field have already uncovered exciting new adaptations which challenge long-held beliefs of methionine's role in proteins, cells, and protein synthesis.

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The influence of L-methionine, DL-methionine, and a methionine hydroxy analog on proliferation and differentiation potential of avian myoblasts

1. Introduction

Methionine is an essential amino acid that serves an array of roles in both eukaryotes and prokaryotes. It is commonly known as the initiator of protein synthesis and plays an essential role as a methyl donor during the synthesis of fatty acids, polyamines, and biotin [1]. Its importance in cellular function makes its supplementation essential in animal feed as well as cell culture media. Three forms of methionine are typically used for supplementation *in vivo*: L-methionine (LM), DL-methionine (DLM), or liquid DL-methionine hydroxy analog-free acid (MHA). L-methionine is biologically active and commonly found in most tissues and is, therefore, the common form used for supplementation [2]. DL-methionine is a racemic mixture of D-methionine and L-methionine. Although D-methionine is the biologically inactive form, birds have the ability to utilize both D- and L-methionine [3]. Therefore, birds may be supplemented with either powdered DL-methionine, which is typically 99% pure DLM, or liquid DL-methionine hydroxy analog-free acid, which typically contains 88% DL-hydroxy-4-(methylthio) butanoic acid (DL-HMB). Chemically, LM and DM are identical with differences only in their chemical structure based around their central carbons as seen in figure 1. An MHA on the other hand differs chemically from L-methionine in that it contains a hydroxyl group in place of the amine group (Fig. 1) and contains one asymmetrical carbon which means contains a mixture of 50% L-isomer and 50%-D-isomer. Converting D-methionine to L-methionine requires the enzyme D-amino acid oxidase to remove the amino group forming an alpha-keto-methionine intermediate which is finally converted to L-methionine by the addition of an amino-

group via a transaminase (Fig. 2). The conversion of an MHA consists of converting both the L-isomer and D-isomer and therefore involves additional enzymes. The conversion of the L-isomer utilizes L-hydroxy acid oxidase while the D-isomer utilizes D-hydroxy acid dehydrogenase, both of which converts the hydroxyl group of their respective isomers into a keto group (Fig. 2). This conversion is interesting in that it forms the same intermediate as the conversion of D-methionine: alpha-keto methionine. Finally, much like in D-methionine, a transaminase attaches an amino group which forms L-methionine from this intermediate [4]. A commonly used methionine supplement, Alimet (Novus International, St. Charles, MO), is one of these liquid MHAs that serves as a feed additive in livestock. Previous studies have shown that the use of MHA, such as Alimet, shows no difference in the biological response in methionine bioactivity and growth efficiency compared to supplementation with DL-methionine or L-methionine in birds [4,5,6,7,8]. Though the difference of these supplements on growth efficiency and methionine activity is negligible *in vivo*, supplementation with DL-methionine and MHAs *in vitro* has not been previously investigated. The aim of this study was to analyze the effect of supplementation with DL-methionine hydroxy analog-free acid and crystalline DL-methionine on the proliferation and differentiation of avian myoblasts *in vitro*.

2. Materials and Methods

2.1 Standard plating and proliferation media

Plating media was made containing 94% Dulbecco's Modified Eagle's Medium (Gibco Burlington, ON, Canada), 5% horse serum (Gibco, Auckland, New Zealand), and 1% antibiotic/antimycotic (A5955 Sigma-Aldrich). Complete proliferation media was made containing 84% RPMI medium 1640 from powder (Gibco), 15% chicken serum (Gibco,

Auckland, New Zealand), and 1% antibiotic/antimycotic (A5955, Sigma-Aldrich, St. Louis, MO). Media was made based on previous formulation by MacFarland et al [9].

2.2 Media for proliferation assay

Media used as the positive control was RPMI complete proliferation media containing L-methionine. Media which served as the negative control for the proliferation assay was made with 84% methionine deficient RPMI (Gibco Burlington, ON, Canada), 15% chicken serum (Gibco, Auckland, NZ), and 1% antibiotic/antimycotic (A5955 Sigma-Aldrich). DL-methionine media was made using 84% methionine deficient RPMI (Gibco), 15% chicken serum (Gibco, NZ), 1% antibiotic/antimycotic (Sigma-Aldrich), and 1.125 mg/mL DL-methionine (Novus International, St. Charles, MO). DL-methionine media was diluted 1:1 with methionine deficient RPMI negative control media. This media served as 0.56 mg/mL DL-methionine treatment.

Media with Alimet for the proliferation assay was made using 84% methionine deficient RPMI (Gibco), 15% chicken serum (Gibco, NZ), 1% antibiotic/antimycotic (A5955, Sigma-Aldrich), and 1.28 mg/mL liquid Alimet (Novus International). Alimet media was diluted 1:1 with methionine deficient RPMI negative control media. This media served as the 0.64 mg/mL Alimet treatment. Alimet and DL-methionine treatment media were formulated to have equivalent molar concentrations of methionine.

2.3 Differentiation media used for fusion and myotube diameter assay

Negative control media was made with 96% methionine deficient RPMI (Gibco Burlington, ON, Canada), 3% horse serum (Gibco, Auckland, New Zealand), and 1% antibiotic/antimycotic (A5955 Sigma-Aldrich St. Louis, MO). Treatment media with DL-methionine was made using

96% methionine deficient RPMI (Gibco), 3% horse serum (Gibco, NZ), 1% antibiotic/antimycotic (A5955, Sigma-Aldrich), and 1.125 mg/mL DL-methionine (Novus International, St. Charles, MO). DL-methionine media was diluted 1:1 with methionine deficient RPMI negative control media. This media served as 0.56 mg/mL DL-methionine treatment. Treatment media with Alimet was made using 96% methionine deficient RPMI (Gibco), 3% horse serum (Gibco, NZ), 1% antibiotic/antimycotic (A5955, Sigma-Aldrich), and 1.28 mg/mL liquid Alimet (Novus International). Alimet media was diluted 1:1 with methionine deficient RPMI negative control media. This media served as the 0.64 mg/mL Alimet treatment. To avoid mature myotubes contracting and detaching from the culture dish, .01 mg/mL gelatin and 1 mg/mL bovine serum albumin (BSA fraction V, Fisher Scientific, Pittsburgh, PA) were added to all differentiation media based on a previously described protocol [10].

2.4 Avian myoblast isolation

Fertilized turkey embryos were incubated at 37.5°C and 55% relative humidity (RH) until embryonic day (ED) 21. The egg was sprayed with 70% EtOH after which the embryo was placed in a dish and euthanized by cervical dislocation. A small sample of muscle was dissected from the *pectoralis major* and placed into 2 mL of Hanks Balanced Salt Solution (HBSS, Sigma-Aldrich, pH 7.4). The muscle sample was mechanically disassociated while still suspended in HBSS using sterile scissors. Once minced, 8 mL of a 0.17% trypsin (Sigma-Aldrich, St. Louis, MO) and 0.085% collagenase (Sigma-Aldrich) solution were added to the muscle in HBSS. The sample of muscle was then incubated at 37°C and 5% CO₂ for 20 minutes to enzymatically dissociate muscle tissue and myoblast cells. Following incubation, the muscle homogenate was transferred to a snap cap tube and centrifuged for 5 minutes at 1,500 RPM. Following

centrifugation, the supernatant was aspirated, and the pellet was resuspended in proliferation media. The cell homogenate was passed through a 100 μm Swinnex filter (Millipore Corporation, Darmstadt, Germany) where large tissue debris was removed, and myoblast cell isolate was collected. A complete cell count was conducted using a hemacytometer to calculate the appropriate seeding density for each assay.

2.5 Avian myoblast proliferation assay

Primary turkey myoblasts were plated into 12 .1% gelatin-coated 24-well cell culture plates (Primaria, Corning, Durham, NC) at a density of 11,400 cells per well in 2 mL of media. One additional plate was seeded with 114,000 cells/well in 2 mL of media. This high-density plate served to verify the myogenicity of the isolate. All cultures were placed in an incubator at 37°C and 5% CO₂. After 48 h, media was completely aspirated from two plates and the cells were fixed with 70% EtOH to serve as the day 0 control. Of the remaining plates, 1 mL of spent media was replaced in 4 wells per plate with 1 mL of positive control, negative control, or the associated methionine treatment media. This process was repeated every 48 h with two plates fixed each time until the final two plates were fixed. This provided a total of n=8 replicates per treatment for each day. The high-density plate was fixed with a paraformaldehyde and phosphate-buffered saline solution once confluency was reached for later visualization of myotubes. After plate fixation, wells were rinsed with phosphate-buffered saline (PBS) to rehydrate cells before staining. PBS was aspirated and replaced with propidium iodide (PI, Invitrogen, Washington, DC) at a concentration of 50 $\mu\text{g}/\text{mL}$ as described previously [10]. The high-density plate fixed was stained with a phalloidin solution following a protocol provided by the manufacturer (Sigma-Aldrich, St. Louis, MO). Stained wells were placed under a fluorescent

inverted microscope under a 20x objective lens with a rotamine filter set. Using SPOT imaging software (SPOT imaging, Sterling Heights, MI), 10 images were taken at random from each well. Using Image-Pro Plus image analysis software (Media Cybernetics, Rockville, MD), illuminated nuclei were segmented and counted from each image and the average of all 10 images was calculated to determine average cell count per well. Images of the differentiated cells stained with phalloidin confirmed myogenicity of cells and showed fluorescent actin (Fig. 3).

2.6 Mammalian myoblast proliferation assay

Mammalian myoblast proliferation assay C2C12 cells (ATCC; CRL-1772) derived from frozen stock were plated onto 6 .1% gelatin coated 6-well culture plates (Primaria, Corning, Durham, NC) at a density of 30,000 cells/well in 3 mL of media. All cultures were placed in an incubator at 37°C and 5% CO₂. After 24 h, media was completely aspirated from one plate and cells were fixed with 70% EtOH to serve as the day 0 control. Of the remaining plates, 2 mL of spent media was replaced with either methionine supplemented or methionine deficient media. This process was repeated every 24 h until the final plate was fixed. This provided a total of n=3 replicates per treatment for each day. After fixation of the final plate, cells were rinsed with PBS for rehydration before staining. PBS was aspirated and replaced with PI at a concentration of .3 µg/mL according to a protocol provided by the manufacturer (ThermoFisher Scientific, Waltham, MA). Stained wells were placed under a fluorescent inverted microscope under a 20x objective lens with a rotamine filter set. Using SPOT imaging software (SPOT imaging, Sterling Heights, MI), 10 images were taken at random from each well. Using ImageJ analysis software (<https://imagej.nih.gov/ij/index.html>), illuminated nuclei were segmented and counted from each image and the average of all 10 images was calculated to determine average cell count per well.

2.7 Myotube fusion assay

Primary myoblasts isolated from turkey embryos were plated into 6 .1% gelatin coated 24-well cell culture plates (Primaria, Corning, Durham, NC). Each well was seeded with 19,000 cells and 2 mL of plating media and placed into a 37°C, 5% CO₂ incubator. Spent media was replaced with 1 mL of complete proliferation media every 48 h until cells were nearing 100% confluency. Once confluent, media was aspirated completely from one plate and cells were fixed with 70% EtOH to serve as the day 0 control. For each remaining plate 1 mL of spent media was replaced with 1 mL of positive control, negative control, and treatment differentiation media with four replicate wells per plate. Media was replaced every 24 h with one plate fixed with 70% EtOH each time for a total of n=4 replicates per treatment for each day fixed. This process was repeated until the final plate was fixed. Following fixation, cells from the day 4 plate were permeabilized with phosphate buffered saline (PBS) containing .25% Tween 20 (Fisher Bioreagents, Pittsburgh, PA). Cells were then blocked using PBS with Tween 20 and 3% goat serum (Gibco, Auckland, NZ) before being incubated with MF 20-c antibodies (DSHB, Iowa City, Iowa) at 4°C overnight. Cells were then incubated with IgG Goat anti-mouse secondary antibodies conjugated with FITC (Invitrogen, Washington, DC) after which a counter stain using propidium iodide (PI, Invitrogen, Washington, DC) was performed. Stained wells were placed under a fluorescent inverted microscope and viewed using a 20x objective lens and a rotamine filter set. Using SPOT imaging software (SPOT imaging, Sterling Heights, MI), 10 images were taken at random from each well. To determine the percent of myoblasts fused into myotubes a fusion index was completed on each image. For every 1000 cells counted, fused myoblasts were determined as three or more myoblasts aligned and encompassed within the same membrane. The number of fused myoblasts per 1000 cells counted was used as the percent fusion index. To determine myotube diameter, 5

myotubes were chosen at random from each well of treatment of images taken from the day 4 plate. Chosen myotubes were measured from each end and center using ImageJ (<https://imagej.nih.gov/ij/index.html>) and the average diameter was determined from the three measurements of each myotube following a method previously described by Thapaliya et al. [11].

2.8 Statistical analysis

Results of the avian and mammalian proliferation assays were determined by calculating the mean cell count and standard error by day per treatment from the mean cell count determined from each well. Results from the avian proliferation assay are expressed as mean fold-changes in cell count by day relative to both the LM supplemented positive and methionine deficient negative controls, \pm SEM (n=8 replicates per treatment). Overall differences in mean cell count of 80 replicates comparing cell count by treatment against both LM supplemented positive control and methionine deficient negative control groups was determined using a Proc GLM 1-way ANOVA test ($\alpha= 0.05$). Additional Proc GLM 1-way ANOVA tests were completed which compared the mean cell count of the high concentration treatments against one another and the low concentrations against one another ($\alpha= 0.05$). For the mammalian myoblast proliferation assay, mean cell count for RPMI and MdrRPMI treatments were compared by day using a Proc GLM 1-way ANOVA test ($\alpha= 0.05$). For both the avian and mammalian proliferation assays, statistical significance of data is given as Prob > F.

Fusion index data was quantified using a Proc GLM 1-way ANOVA to determine differences in percent fusion among all treatments and controls ($\alpha= 0.05$). An additional Proc GLM 1-way

ANOVA test was completed using the mean fusion percentage of the four wells that received treatment media ($\alpha = 0.05$) with significance given as Prob > F.

Myotube diameter data was compared using a least significant differences (LSD) test to determine differences between the mean myotube diameter from each treatment and control group. Means that are not significantly different ($\alpha = 0.05$) between groups were covered by the same bar while means that are significantly different were covered by separate bars.

All statistical analysis tests were completed using either SAS Studio 3.8 (SAS Institute Inc, Cary, NC), Microsoft Excel 16.50 (Microsoft, King County, WA), or ImageJ 1.53a (National Institute of Health, USA).

3. Results

3.1 Supplemental methionine and myoblast proliferation

The avian myoblast proliferation curves are shown in Fig. 4. Over the 10 days of the avian myoblast proliferation assay, the average cell counts of both RPMI positive control and MdrRPMI negative control wells were significantly higher than the cell count of wells treated with supplemental methionine ($P < .0001$). Further comparison of cell counts by changes every 48 h showed wells containing LM supplemented positive and methionine deficient negative controls had increasing cell counts over the course of the experiment while wells receiving supplemental methionine treatments all showed decreasing cell counts (Fig. 5 A-E). In comparing only wells treated with high concentrations of DL-methionine versus treatment with high concentrations of Alimet (Fig. 6A) wells treated with DL-methionine showed significantly higher cell count overall ($P < .0001$). Comparison of low concentration wells for DL-methionine

and Alimet (Fig. 6B) yielded the same result with significantly higher cell counts in wells treated with DL-methionine than wells treated with Alimet ($P < .0019$).

3.2 Mammalian myoblast proliferation in the presence and absence of supplemental methionine

Results of the mammalian myoblast proliferation curve is shown in Fig. 7. Over the course of the 5 days of the mammalian myoblast proliferation assay, cell counts in wells supplemented with methionine and those in methionine deficient media showed growth rates that were not significantly different until day 3. Beginning at day 3, wells treated with methionine supplemented RPMI showed significantly higher rates of growth compared to wells treated with MdRPMI ($P < .0225$) as shown in Fig. 8. The difference in cell counts between the two treatment groups continually increased throughout the remainder of the assay.

3.3 Fusion index shows no significant difference across all wells

The percent of myoblasts fused into myotubes was calculated for wells of each treatment from which the average fusion index was determined for each plate by treatment (Fig. 11A-F). As shown by Fig. 9A, no significant difference in fusion was found across all treatments and controls. Additional comparisons of fusion indices of only treatment wells (Fig. 9B) again revealed no differences in the rate of fusion at any concentration with either DL-methionine or Alimet supplements.

3.4 Supplemental methionine sources yielded larger myotube diameter than methionine deficient control

Myotube diameter was measured from the day 4 control and treatment wells and the average diameter was determined from the four wells of each treatment and control columns. Grouping of mean myotube diameter by treatment (Fig. 10) showed that wells receiving the positive control containing LM and wells receiving treatment methionine supplements were significantly larger than the methionine deficient negative control myotubes ($P < .0001$). Wells receiving DL-methionine had the greatest myotube diameter and were significantly larger than wells receiving positive control LM and LA treatment. The HA treatment group also contained myotube diameters significantly larger than positive control LM wells but not significantly larger than any other treatment.

4. Discussion

Previous studies have concluded that the efficacy of DLM and MHA as sources of L-methionine in birds is very similar [4,5,6,7,8]. However, these experiments focus on the *in vivo* ability of birds to metabolize these forms of methionine rather than *in vitro*. The efficacy of these sources of methionine to be converted and utilized by cells *in vitro* is still unknown. Alimet, being an MHA, contains a precursor to L-methionine called DL-hydroxy-4- (methylthio) butanoic acid (DL-HMB) which differs from LM in that it contains a hydroxy group on the alpha carbon instead of an amino group. The conversion of DL-HMB into LM is a previously studied process [12] which requires the enzymes L-hydroxy acid oxidase (L-HOAX), D-Hydroxy acid dehydrogenase (D-HADH), and D-amino acid oxidase (D-AAOX) to initiate the conversion of L-HMB isomer, D-HMB isomer, and D-methionine respectively. The enzymes L-HOAX and D-

AAOX are both found in the peroxisomes of cells, and in birds, are found in high concentrations in the liver and kidneys and in low concentrations elsewhere in the organism [13]. L-HOAX functions to convert L-HMB into 2-keto-4-(methylthio) butanoic acid; an intermediate in the conversion to LM [14]. Interestingly, D-AAOX converts D-methionine into the same intermediate: 2-keto-4-(methylthio) butanoic acid [15] with both steps being the first conversion of L-HMB and D-methionine. D-HADH differs from L-HOAX and D-AAOX since it is a dehydrogenase rather than an oxidase and it is a mitochondrial enzyme that has been found in all tissues including skeletal muscle [16]. The known disparity between the cell types in which these enzymes are found may have an impact on the ability of an *in vitro* culture of myoblasts to process these various sources of methionine.

Data collected from the proliferation of myoblasts assay has shown that in both treatments containing varying concentrations of Alimet and DL-methionine, cell count continually decreased throughout the course of the experiment. Meanwhile, myoblasts cultured with methionine provided in the LM form in positive control wells saw cell count steadily increasing. A likely explanation for this is that methionine supplemented to treatment groups reached a concentration inhibitory to myoblast proliferation. A previous study using fibroblasts derived from mice found that the optimal concentration of methionine for cell proliferation was .05 $\mu\text{moles/mL}$ with proliferation decreasing with increased methionine concentration past this point [17]. Additionally, a study using CHO cells concluded that the maximum methionine concentration for animal cell culture media is 153 mg/L [18]. In this experiment, Alimet was supplemented at a concentration of 1.28 mg/mL (7.5 mM) and .64 mg/mL (3.8 mM) for high and low concentration treatments respectively. Additionally, DLM was supplemented at a concentration of 1.125 mg/mL (7.5 mM) and .56 mg/mL (3.8 mM) for high and low

concentrations respectively. It has been suggested that the transaminative pathway utilized by cells at high intracellular methionine concentrations produces methanethiol as a breakdown product [19]. Methanethiol is highly toxic to cells and may be partly responsible for the significant reduction in cell proliferation witnessed in DLM and Alimet treatment groups. While it is clear that methionine concentration was inhibitory for normal myoblast proliferation, rates of proliferation between DLM and Alimet treatment groups of surviving cells revealed a significantly higher rate of proliferation in DLM treated myoblasts than Alimet treated myoblasts. A possible explanation for this difference is that supplemented DLM was in a 99% racemic mixture with 50% of the mixture being LM. Comparing this to Alimet which contains 88% DL-HMB where myoblasts only can convert D-HMB which comprises only half of the available DL-HMB. Conversion efficiency of D-HMB into LM may be worth exploring which may have further contributed to the difference in proliferation.

Data collected from the methionine deficient negative control wells did yield unexpected results. It was found that myoblast proliferation in these wells was significantly higher than all treatment wells and was not significantly different from the LM supplemented positive control. A study previously conducted by Reidy et al. [20] using older humans supplemented with essential amino acids (EAA) or non-supplemented found that supplemented groups saw increased satellite cell proliferation in type I muscle fibers but no difference in type II fibers. Though this study partially suggests that supplementation with essential amino acids does not impact proliferative ability, the effects of specific essential amino acids were not explored and therefore methionine's impact could not be determined. There have been reported cases of normal rates of proliferation of cells in the absence of methionine, though the supplementation of homocysteine was required [21,22]. To determine whether this methionine-free proliferation was specific to avian myoblasts,

a similar assay was completed using mammalian C2C12 cells. Data collected from this experiment revealed a significantly greater rate of proliferation in wells supplemented with methionine revealing a methionine dependence. As previously mentioned, the *in vitro* ability for birds to utilize varying sources of methionine as well as the ability to utilize homocysteine as a methionine precursor highlights the phenotypic adaptability of myoblasts during proliferation, though the exact mechanism utilized in the absence of methionine is unknown. A number of mechanisms have been identified in more recent studies that show protein synthesis initiation using alternative start codons. In prokaryotes, successful protein synthesis has been initiated after the replacement of the methionine anti-codon CAU with alternative anticodons allowing for initiation by valine, phenylalanine, isoleucine, tryptophan, and glutamine [23-26]. Further evidence shows mis-acetylated formyl methionine tRNAs were also able to initiate protein synthesis with non-canonical amino acids [27,28]. In eukaryotes, mutant initiator tRNAs for methionine and valine have also been identified where no detrimental downstream effects on protein synthesis were observed [29]. Though mutagenesis of RNAs in this experiment was conducted by the researchers, all of the studies listed showcase impressive plasticity of translation machinery that may be used by cells in methionine-stressed environments for survivability. Other reports have found the complete circumvention of traditional protein synthesis initiation. In a study by Malarkannan et al., MHC class I molecules were found to present cryptic peptides that had been synthesized both using leucine as the initiating amino acid and independent of upstream translation initiation machinery [30]. Whether avian myoblasts have the ability to utilize alternative start codons or bypass the initiation phase is unknown, but it does seem that they are able to proliferate in a methionine deficient environment.

The impact of methionine availability on myoblast differentiation is known for several species. Previous studies have shown that deprivation of methionine in mammalian myoblasts [31] maintained a state of satellite cell quiescence with MyoD being downregulated and Myf-5 expressed at a high level. When reintroducing methionine to quiescent satellite cells, the levels of MyoD and Myf-5 were inverted, and cells began to differentiate showing methionine's direct role in the process of differentiation. Additional studies looking at methionine's role in satellite cell differentiation in fish [32] concluded similar results that a lack of methionine resulted in lower levels of myogenin and myod1, which are known as the important markers of myogenesis. Introduction of methionine in fish satellite cells also showed the restoration of differentiation activity, again presenting methionine's direct role in the process. This direct relationship between methionine and induction of differentiation in avian systems however has not been as well documented. It has been observed that chicks supplemented in-ovo with methionine have increased body weights and muscle production of post-hatch [33-35]. These findings suggest that methionine plays a role during the early stages of myofiber development though none of the studies determined the impact to be on differentiation specifically. Differentiation data collected in the current experiment however did not concur with these studies. A fusion index (FI) of three or more nuclei contained within one membrane was used to determine rates of differentiation among the different treatment groups. The data collected showed no significant differences in the rates of fusion across all treatments and both LM supplemented positive control and methionine deficient negative control. Since differentiation was able to occur at rates equal to cells supplemented with methionine it is possible that methionine may not be necessary to trigger the onset of differentiation in avian myoblasts. Differentiation is known to begin when myoblasts exit the cell proliferation cycle and begin to fuse into multinucleated myotubes with the

expression of myogenic regulatory factor (MRF) proteins. MRF proteins include Myf5 and MyoD which are expressed during active proliferation of myoblasts while myogenin and MRF4 are expressed during and post-differentiation respectively [36,37]. As mentioned previously, the introduction and removal of methionine have had a direct impact on the concentration of these MRFs and therefore have been seen to have a direct correlation with rates of differentiation. With the results of the methionine deficient control group in this experiment still showing high rates of differentiation, it is possible additional factors play a role in regulating differentiation. The results from the myotube diameter data are interesting in that the methionine deficient negative control had a significantly smaller average size, clearly demonstrating that while methionine may not be essential for satellite cell proliferation, it has an essential role in protein synthesis. Hypertrophy of myotubes is a result of increased RNA synthesis and protein synthesis by existing nuclei or by adding additional nuclei. As this relates to methionine, a lack of sufficient methionine source would limit the rate of protein within growing myotubes as methionine is essential as the initiating amino acid of all protein synthesis. As evident by the data, the rates of myotube hypertrophy differed between the different methionine supplements and concentrations at which they were supplemented. Wells receiving treatments were generally larger than myotubes from the LM positive control wells with both LD and HA treatments having significantly larger diameters. It is possible that the reason for this is because treatment methionine supplementation was at a much higher concentration than the positive control. As methionine is often a limiting amino acid of protein synthesis; greater methionine supplementation would likely allow for greater protein accretion and therefore larger myotubes. It was also found that LD treatments had significantly larger myotube diameters than LA treatments. The reason for this may have to do with the bioavailability of DL-methionine

compared to Alimet as previously mentioned, but the exact reasoning is unknown. What this data does show is that Alimet supplementation may serve as a functional replacement for traditional methionine sources during the terminal differentiation stages of myotube development *in vitro*. Further research into the functionality of Alimet as a methionine supplement to improve protein synthesis in myotube cultures is warranted.

5. Conclusions

In summary, data collected during this study presents evidence that DL-methionine (DLM) and liquid DL-methionine hydroxy analog-free acid (MHA) may serve as viable alternatives to L-methionine during differentiation of *in vitro* culturing of myoblasts. Furthermore, the results obtained from the myotube fusion assay suggest the involvement of an alternative factor other than methionine influencing rates of differentiation. Finally, the proliferation of avian myoblasts in the absence of methionine suggests the utilization of an alternative source of methionine or the ability to proliferate through the use of alternative translational start codons or complete circumvention of translation initiation events. These findings merit further investigation into the mechanism behind methionine-free proliferation of avian myoblasts and methionine's direct role in the proliferative function of satellite cell and myoblast proliferation and differentiation.

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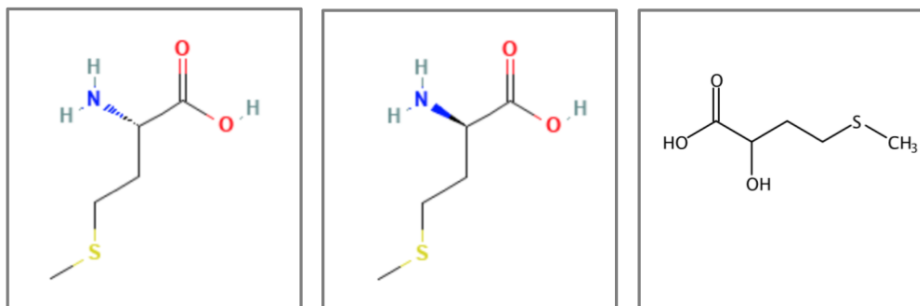


Figure 1. Three forms of methionine supplements

The images above represent the chemical structures of the three forms of methionine used in this experiment. From left to right: L-methionine (PubChem CID 6137), D-methionine (PubChem CID 84815), and methionine hydroxy analog (Alimet, Novus International, St. Charles, MO).

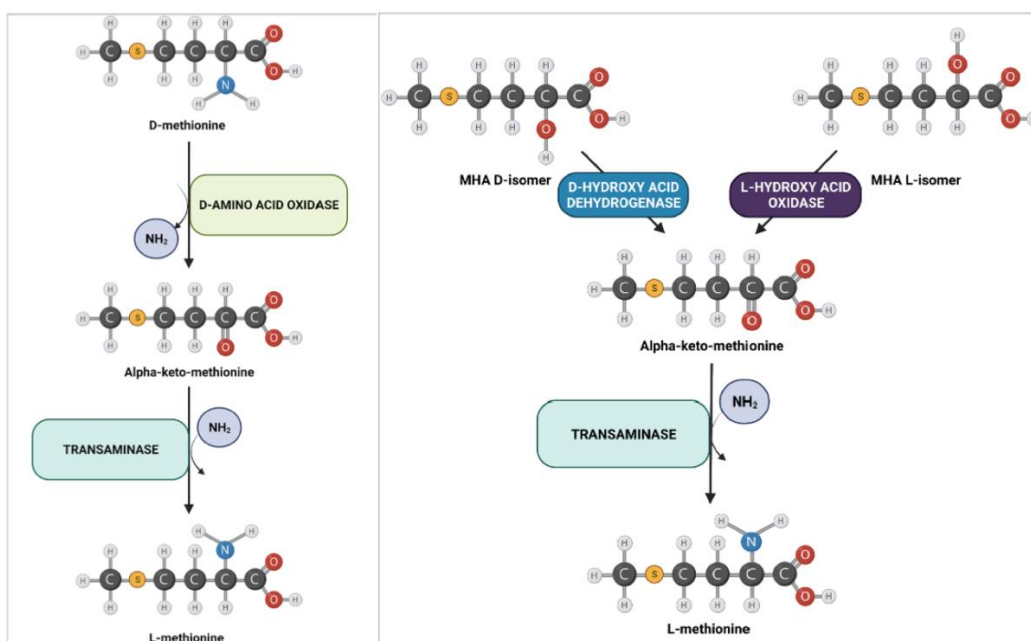


Figure 2. Conversion of DL-methionine and MHA to L-methionine

The image on the left hand side of the figure describes the pathway of the conversion of D-methionine to L-methionine. The image on the right hand side of the figure describes the conversion of both the D-isomer and L-isomer of methionine hydroxy analog (MHA) to L-methionine.

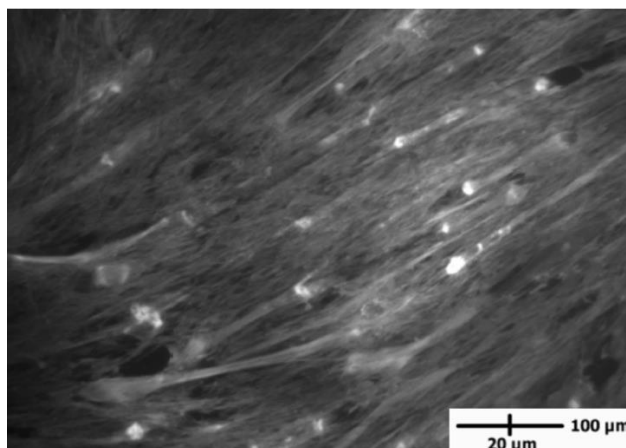


Figure 3. Myotube staining with phalloidin

Photomicrograph of phalloidin-stained myotubes. Myoblasts were seeded at high-density and incubated for 10 days until 100% confluency and differentiation was observed. Phalloidin-stained myotubes show fluorescent actin protein confirming myogenicity of cells.

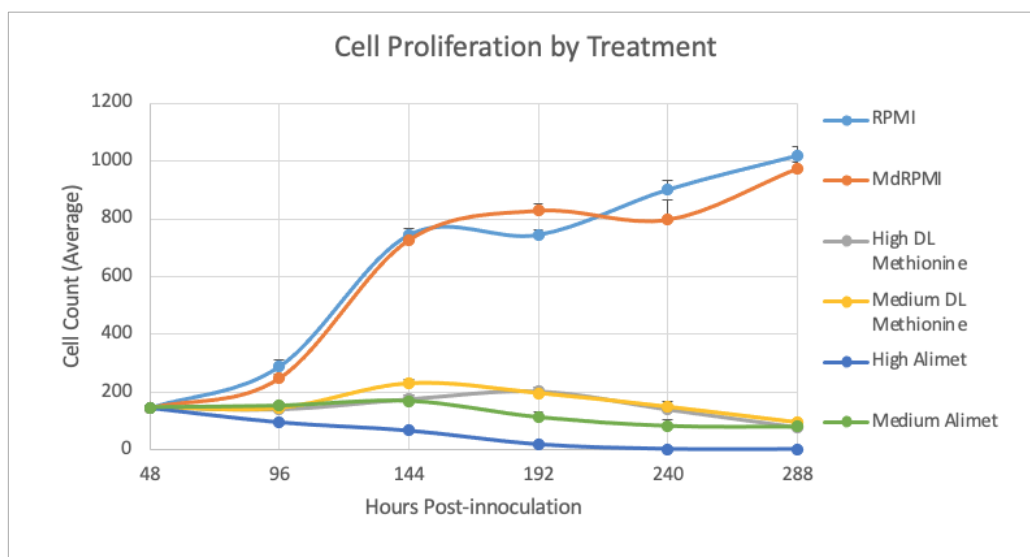


Figure 4. Rates of avian myoblast proliferation (line graph)

Proliferation of myoblasts isolated from the *pectoralis major* from embryonic day 21 turkey embryos. Average cell (\pm SEM) count by treatment 48 h post-inoculation. High Alimet (1.125 mg/mL Alimet), High DL-Methionine (1.28 mg/mL DLM), Medium Alimet (0.64 mg/mL Alimet), Medium DL-Methionine (0.56 mg/mL DLM), MdRPMI (negative control Methionine-deficient RPMI), and RPMI (15 mg/L positive control LM RPMI).

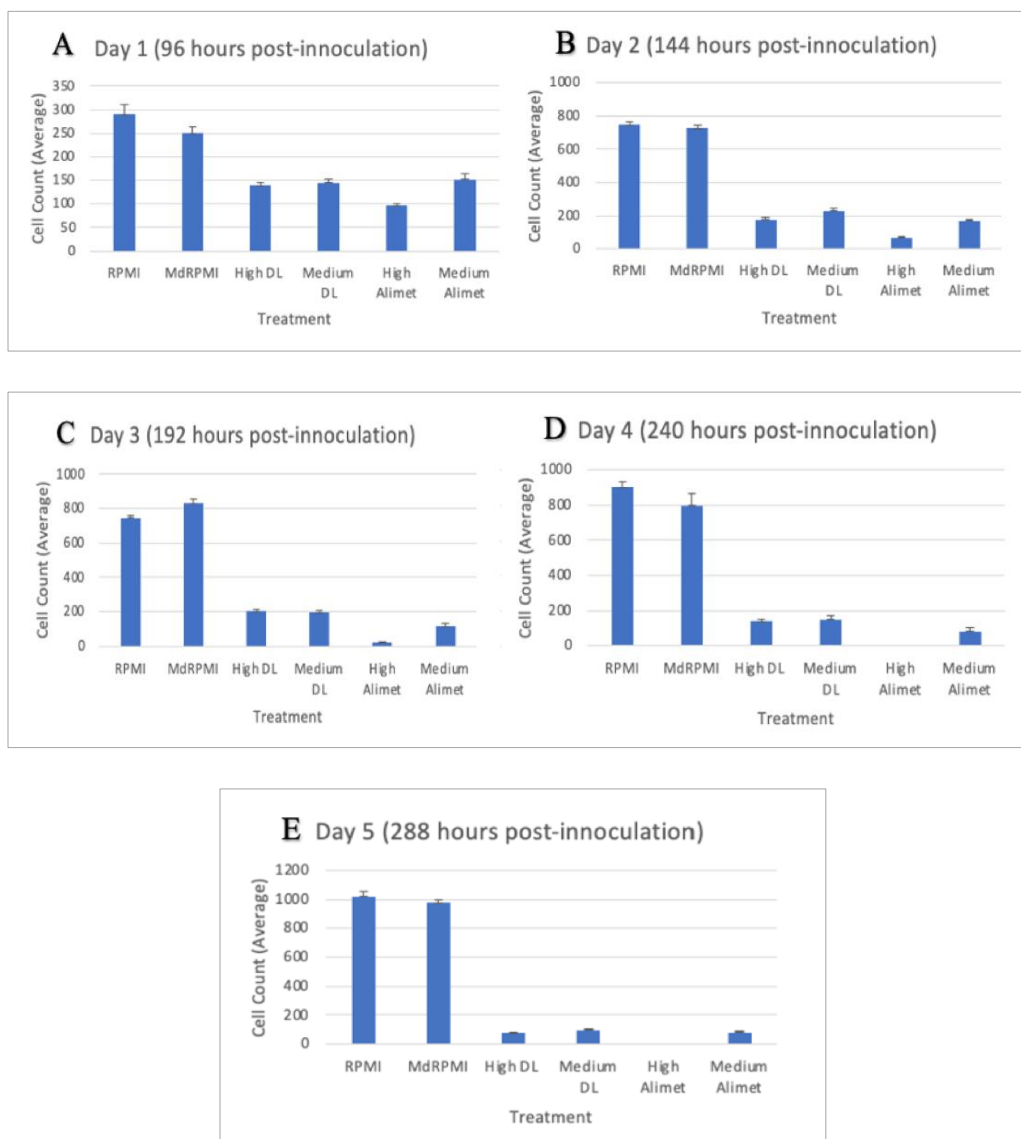


Figure 5. Rates of avian myoblast proliferation (bar graph)

Proliferation of myoblasts isolated from the *pectoralis major* from embryonic day 21 turkey embryos. High Alimet (1.125 mg/mL Alimet), High DL-Methionine (1.28 mg/mL DLM), Medium Alimet (0.64 mg/mL Alimet), Medium DL-Methionine (0.56 mg/mL DLM), MdRPMI (negative control Methionine-deficient RPMI), and RPMI (15 mg/L positive control LM RPMI). Each image represents cell counts at different time points: (A) 96 h post-inoculation, (B) 144 h post-inoculation, (C) 192 h post-inoculation, (D) 240 hours post-inoculation, (E) 288 hours post-inoculation.

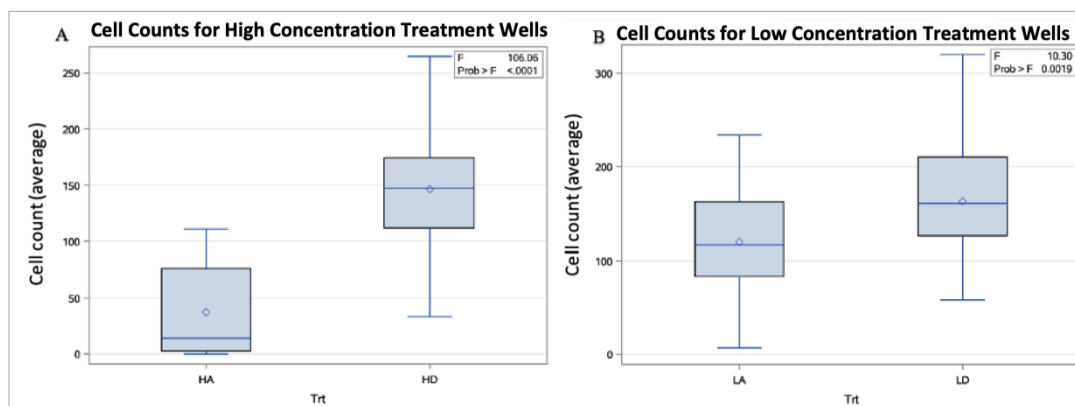


Figure 6. Avian myoblast cell count by treatment

Proliferation of myoblasts isolated from the *pectoralis major* from embryonic day 21 turkey embryos. Average cell count combined data from 96 h post-inoculation to 288 h post-inoculation. (A) Comparing only treatments that received either Alimet at 1.125 mg/mL (HA) or DLM at 1.28 mg/mL (HD). (B) Comparing only treatments that received either Alimet at 0.56 mg/mL (LA) or DLM at 0.64 mg/mL (LD). Statistical significance is indicated by $\text{Prob} > F$ ($\alpha < .05$). Mean, upper quartile, and lower quartile of cell counts were calculated for each treatment. Standard errors and outliers included. Proc GLM 1-way ANOVA was conducted using SAS Studio 3.8 (SAS Institute Inc, Cary, NC).

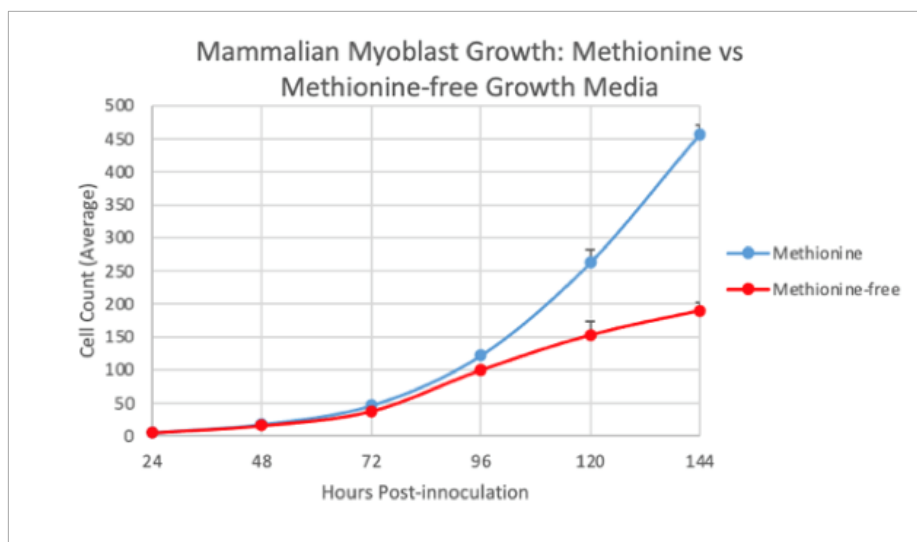


Figure 7. Rates of mammalian myoblast proliferation

Proliferation of mammalian C2C12 cell line sourced from frozen stock. Average cell (\pm SEM) count by treatment 24 h post-inoculation. Treatments include Methionine-free (Methionine-deficient RPMI), and Methionine (15 mg/L LM RPMI).

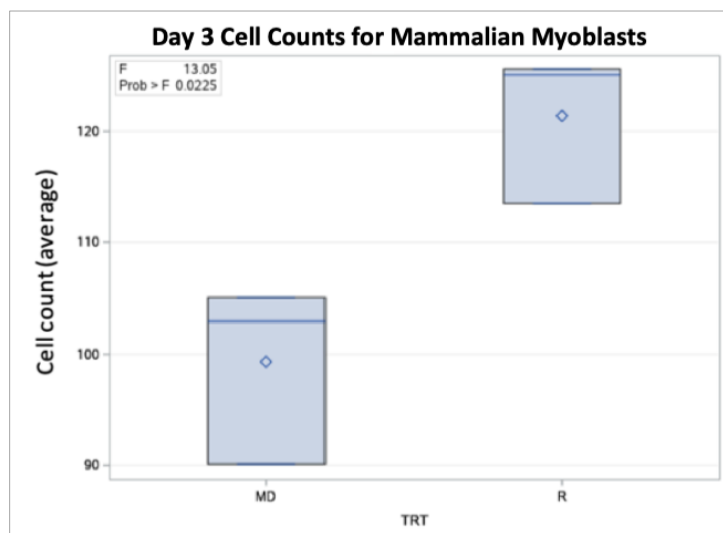


Figure 8. Mammalian myoblast cell count

Proliferation of mammalian C2C12 cell line sourced from frozen stock. Average cell count of myoblasts 72 hours post-inoculation. Comparing cell counts from myoblasts treated with RPMI (R, 15 mg/L methionine) and MdRPMI (MD, methionine deficient).

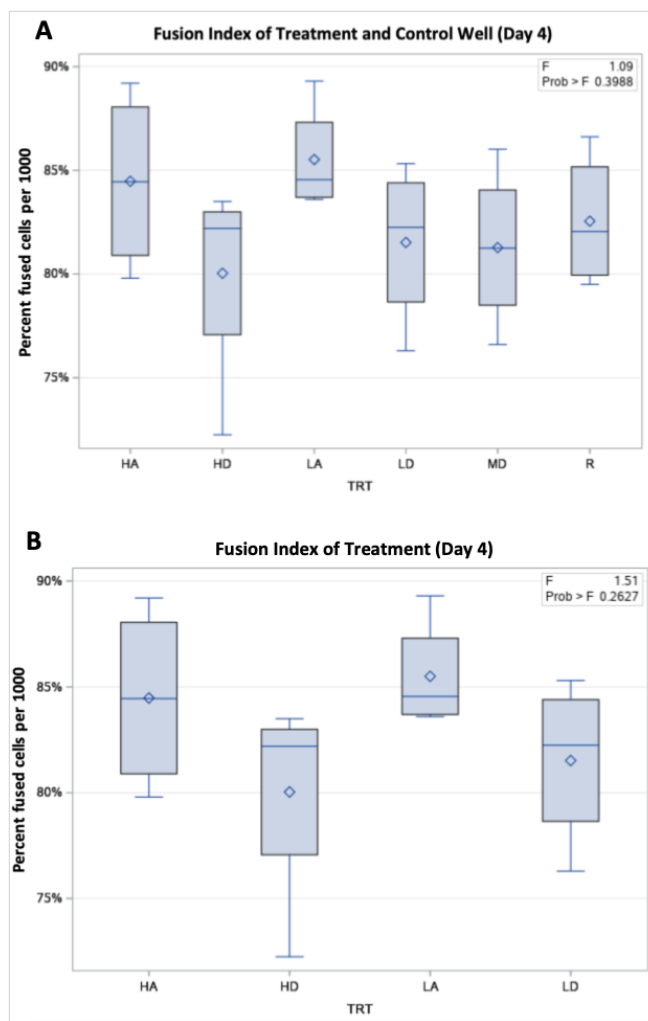


Figure 9. Avian myoblast fusion index

Percent of fused myoblasts per 1000 cells counted separated by treatment. Data includes fusion index from the day 4 plate of the myotube differentiation assay. (A) Fusion indices separated by treatment with 1.125 mg/mL Alimet (HA), 1.28 mg/mL DLM (HD), 0.64 mg/mL Alimet (LA), 0.56 mg/mL DLM (LD), negative control methionine deficient RPMI (MD), and positive control LM RPMI (R). (B) Fusion indices comparing only treatment with 1.125 mg/mL Alimet (HA), 1.28 mg/mL DLM (HD), 0.64 mg/mL Alimet (LA), and 0.56 mg/mL DLM (LD). Statistical significance is indicated by Prob > F ($\alpha < .05$). Mean, upper quartile, and lower quartile of cell counts were calculated for each treatment. Standard errors and outliers included. Proc GLM 1-way ANOVA was conducted using SAS Studio 3.8 (SAS Institute Inc, Cary, NC).

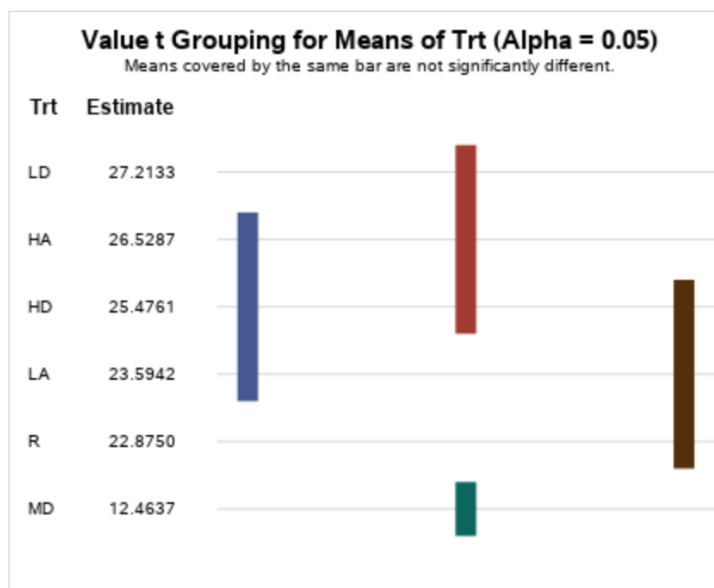


Figure 10. Avian myoblast myotube diameter

Average myotube diameter averaged from three randomly selected myotubes. Myotubes were selected from the day four and day five plates of the differentiation assay. Measurements were taken from positive control RPMI + LM (R), methionine deficient negative control (MD), and wells treated with 1.125 mg/mL Alimet (HA), 1.28 mg/mL DLM (HD), 0.64 mg/mL Alimet (LA), and 0.56 mg/mL DLM (LD). Estimates represent average myotube diameter in μm from each treatment and control group. Treatments covered by the same color line represent myotube diameters that are not significantly different while treatments covered with bars of different colors are myotube diameters that are significantly different ($\alpha < .05$). A least significant difference test was conducted using SAS Studio 3.8 (SAS Institute Inc, Cary, NC).

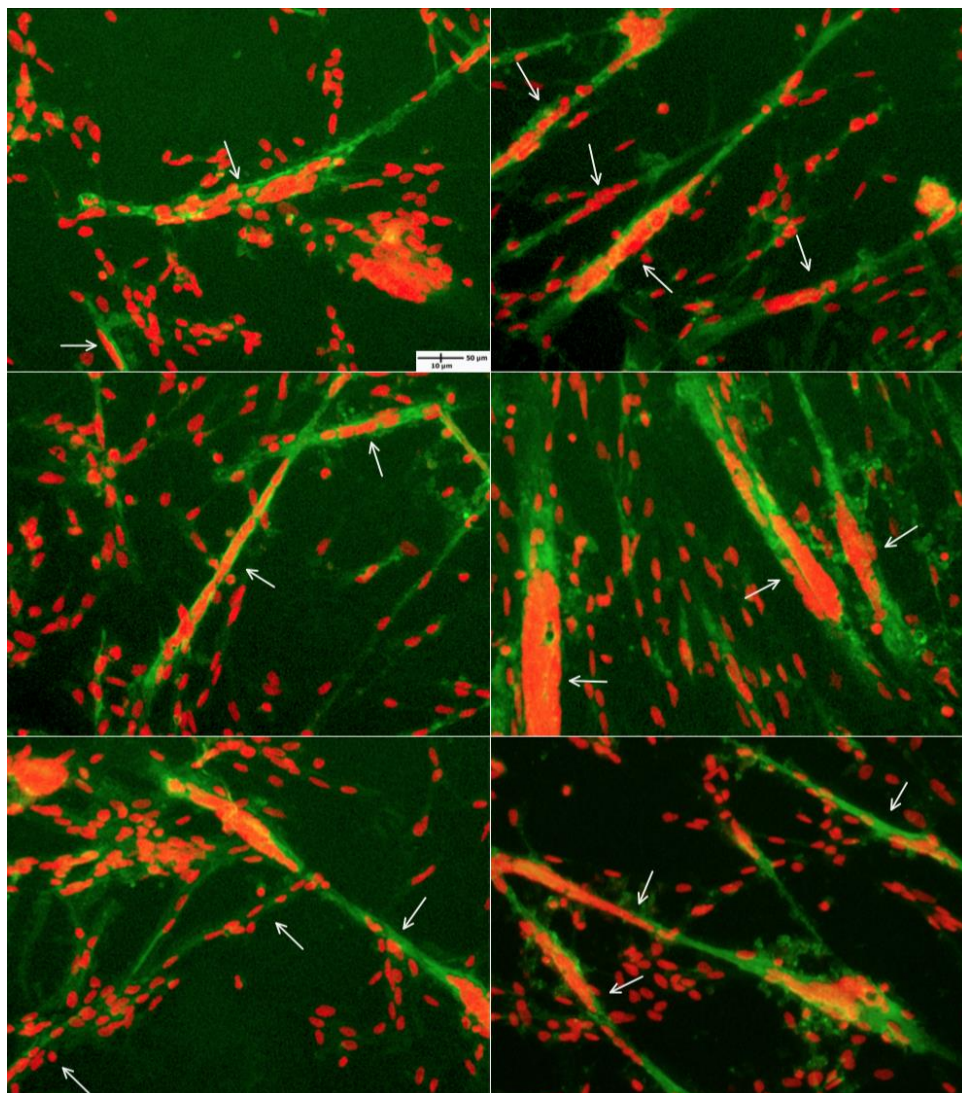


Figure 11. MF-20c stained avian myotubes

Images of differentiated myotubes formed from myoblasts isolated from the *pectoralis major* of embryonic day 21 turkey embryos. Images taken from day 4 plate of the differentiation assay using an inverted microscope under a 20x objective lens using SPOT imaging software (SPOT imaging, Sterling Heights, MI) Myotubes were incubated with MF 20-c myosin heavy chain antibody, IgG Goat anti-mouse secondary antibody conjugated with FITC, and counterstained with propidium iodide. Images taken from each of the six control and six treatment columns: (A) positive control of RPMI with LM, (B) methionine deficient negative control, (C) 1.28 mg/mL DLM, (D) 0.56 mg/mL DLM (E) 1.125 mg/mL Alimet, (F) 0.64 mg/mL Alimet.

Summary

Methionine's role in cells and proteins has continually become more nuanced with the discovery of its expanded capabilities due to the unique sulfur-containing structure. The formation of s-aromatic motifs by methionine provides stability to proteins while still allowing a range of flexibility. These motifs have been found to be quite common within proteins and are also strengthened through the oxidation of methionine rather than weakened which makes these interactions potentially quite significant. Methionine functioning as an endogenous antioxidant has been known for some time, however, what is known of how dependent cells and proteins have become on this property is of interest. It has been discovered that proteins have incorporated an increased number of methionine residues near their active sites during times of high oxidative stress within cells. What this implies is that proteins have adapted to utilize the antioxidant function of methionine as a protection mechanism against oxidation rather than it just being a secondary benefit. The reversible nature of methionine's oxidative status by methionine sulfoxide reductases means that each methionine residue has the potential to repeatedly serve as an antioxidant. This reversible oxidative state has led to the discovery of activity modulation of proteins and cells through the redox state of methionine. Methionine oxidation has been found to modulate the activity of cellular actin cytoskeletons, ion channels, and calmodulin signaling which may serve as a protection mechanism for cells in times of high oxidative stress. In other examples, the oxidation of methionine has reduced enzyme activity to prevent the production of reactive oxidative species as a built-in negative feedback mechanism.

Though methionine does provide a number of important functions within cells and proteins, it is possible that cysteine may be able to replace methionine in these positions. Because cysteine also

includes a sulfur atom in its structure, it is able to form similar s-aromatic motifs and after the discovery of cysteine's polar nature, it is likely that it may be able to form these motifs at similar locations within and on proteins as methionine. Cysteine also provides a significant antioxidant function through the inclusion of thiol groups that may be reversibly oxidized by thioredoxin/glutaredoxin. Additionally, cysteine is crucial for the formation of glutathione which is a powerful free radical scavenger suggesting that cysteine's antioxidant function may be even more powerful than methionine's. In the modulation of cell and protein activity, cysteine has been found to similarly regulate ion channels to prolong or inhibit cellular activation as well as being able to modulate enzyme activity through the formation of disulfide bonds to prevent the production of additional ROS in times of elevated oxidative stress.

Novel evidence of the initiation of protein synthesis via alternative pathways gives further credence to the idea of continued cellular function in the absence of methionine. The use of alternative amino acids has been found to be possible in both prokaryotes and eukaryotes through the use of mis-acetylated formyl methionine tRNAs and other mutant initiator tRNAs. Additional alternative translational initiation mechanisms include the deactivation of ribosome recycling factor and/or internal ribosome entry sites. The deactivation of ribosome recycling factors allows translational machinery to remain attached beyond the termination codon and continue protein synthesis of downstream mRNA regions. Internal ribosome entry sites are mRNA motifs that allow for the attachment of ribosomal translational machinery at internal coding regions that bypasses the traditional scanning and initiation phase of translation.

Data collected from the myoblast proliferation and differentiation assays completed suggest that methionine availability has a significant impact on protein synthesis levels in myotubes but not on the ability of myoblasts to proliferate. If cysteine is potentially assuming the roles of methionine as they relate to cellular activity modulation, protein stabilization, and antioxidant protection, methionine's role as a protein synthesis initiator is the only essential function that would be hindered by a lack of methionine supplementation. By potentially utilizing one or a combination of multiple of the mechanisms for bypassing traditional protein synthesis initiation, cells may be able to maintain a stable level of proliferation in a methionine deficient environment. Furthermore, upon differentiation, the lack of methionine was shown to result in significantly smaller myotube diameters which suggests that there is a correlation to decreased hypertrophy in the absence of methionine. What this all implies is that myoblasts may be able to utilize alternative methods to initiate protein synthesis in a methionine deficient environment for cell survival, but methionine is essential for protein synthesis to occur at a rate necessary for normal hypertrophy. Further research into which, if any, of the methods may be used by avian myoblasts to support proliferation in a methionine deficient environment is warranted as it could change much of what is known about methionine's essential nature and protein synthesis.