The Journal of Toxicological Education **1:** 54-65 (2013)
http://www.jtoxed.org

JToxEd
The Journal of Toxicological Education

Introducing Toxicology into the Biochemistry Curricula: Using Cytochrome *c* (Cyt*c*) Functionalities as a Model.

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Running Title: Cyt*c* as a Toxicology Model in Biochemistry

Abstract

The electron transport chain (ETC) is a keystone topic of all biochemistry courses at the undergraduate level. Many ETC components, especially cytochrome c (Cyt*c*), are also important to the field of toxicology. Unfortunately, many primarily undergraduate institutions (PUIs) are unable to offer dedicated toxicology courses and laboratories due to faculty expertise and/or enrollment requirements. In an effort to provide chemistry and biology undergraduates with toxicology perspective and experience, I have integrated Cyt*c* toxicology, and its role in apoptosis, into my Biochemistry I and II curriculum. This approach fulfills two goals: 1) integration of toxicology concepts into the biochemistry curriculum and 2) validation of fundamental biochemistry principles through demonstration of "real world" relevance in the field of toxicology. These concepts include Cyt*c* "leakage" to the cytosol, activation of the apoptotic signaling cascade, Cyt*c*/membrane interactions, the modulation of apoptosis by Cyt*c* phosphorylation, and chemical/environmental toxicants that activate this function of Cyt*c*. I conclude with a discussion of student assessment in relation to this methodology. Overall, these materials provide biochemistry instructors with a primer to introduce toxicology concepts in the greater biochemistry curricula or a means for toxicology faculty to validate key biochemistry principles within their classroom.

Keywords

Cytochrome *c*, toxicology; biochemistry, undergraduate education

Introduction

Many primarily undergraduate institutions (PUIs) are limited in their capabilities to incorporate specialized courses, such as toxicology and pharmacology, into the curriculum due to scheduling restraints, course enrollment minima, or faculty expertise. This should by no means preclude students from gaining valuable training and knowledge in these fields. Faculty must therefore develop methodologies to introduce toxicology concepts without compromising the necessary biochemistry course content. A vital component of undergraduate biochemistry curricula is the mitochondrial electron transport chain (ETC), and this topic typically includes discussion of mitochondrial structure, redox chemistry, electron-transporting molecules, chemiosmotic theory, oxidative phosphorylation, and reactive oxygen species (ROS). Many of these topics are also central themes in toxicology and allow for blended topic integration.

One ETC component that easily bridges disciplines is cytochrome c (Cyt*c*). This highly conserved protein has evolved two distinct and vital functions that should be discussed in biochemistry courses: 1) mobile electron carrier in ETC and 2) initiator of programmed cell death (apoptosis)[1]. Cyt*c* is of great importance to the field of toxicology as this molecule can serve as marker of toxicant exposure, as a factor in the generation of reactive oxygen species, and as a component of the apoptotic machinery. It is thus important that biochemistry students understand the multi-functional intricacies of Cyt*c* and re-assess the central dogma of “one protein with one function”.

Undergraduate biochemistry texts generally devote a page or less to the concept of apoptosis and the role of Cyt*c* in this process, often relying on molecular biology texts to introduce this topic. As biochemistry courses are often the sole source for chemistry majors to learn higher level biological processes, it is important that students gain insight and appreciation for biochemical principles in relation to cellular viability and toxicity. Currently, my course teaches the topic of the ETC during the final few weeks of my Biochemistry I course, and the ETC is briefly re-visited during the first few lectures of the Biochemistry II course. The Biochemistry I and II student demographics include three important groups: 1) Chemistry majors who likely will not take a molecular biology (Mol. Biol.) course, 2) Chemistry majors (with Biochemistry option) who may or may not have yet taken a Mol. Biol. course, and 3.) Pre-professional students who may or may not have yet taken a Mol. Biol. course. It is thus imperative that this course cover some basic Mol. Biol. concepts to add biological context to the material and enhance the biochemistry learning objectives for all.

To add additional breadth to the ETC discussion in Biochemistry I and re-engage Biochemistry II students at the beginning of the semester, I introduce students to simplified concepts of toxicology and apoptosis using Cyt*c* as the sample molecule. This discussion blends toxicology with key biochemistry concepts and mechanisms for this diverse student clientele. Notably, most students in Biochemistry II are generally bound for various Pre-professional programs or graduate school and are more interested in the additional academic rigor. The information below elaborates on the specific discussion topics, figures, and assessment I currently use to integrate toxicology concepts, via Cyt*c,* into the greater biochemistry curricula.

Basic Function of the Mitochondria and the role of Cyt*c* in Bioenergetics

The mitochondria are uniquely structured organelles that satisfy most of the energy demands for the cell. Structurally, these organelles possess a double membrane system that defines four distinct regions: the outer membrane, the inner membrane space, the inner membrane, and the matrix (Figure 1)[2]. The outer membrane is permeable to ion flow with the cytosol, thereby maintaining pH similarity (pH ~ 7.0). In contrast, the inner membrane is not permeable to ions, and this property is harnessed to generate cellular energy in the form of adenosine triphosphate (ATP)[3]. Although originally hypothesized to be distinct entities, the inner and outer mitochondrial membranes are now known to converge at contact points to facilitate lipid mobilization and transfer[4]. The energy-generating components of the mitochondria reside within the inner membrane and include Complexes I-IV and ATP Synthase. Through a series of electron transfers, protons are pumped out of the mitochondrial matrix and diatomic oxygen is reduced to water. These events generate a pH and charge gradient that is harnessed by ATP Synthase to physically generate ATP from adenosine diphosphate (ADP) and inorganic phosphate (P_i) (Figure 1)[5].

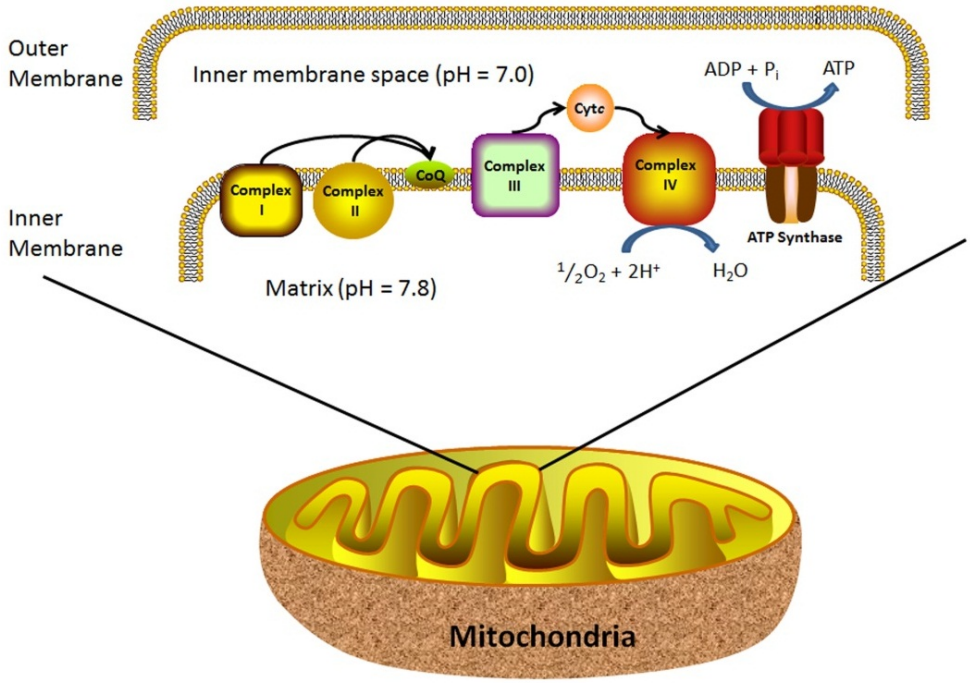

Figure 1. Schematic of Mitochondrial Organization. The mitochondria are composed of four definable regions: the outer membrane, the inner membrane space, the inner membrane, and the matrix. The pH of the inner membrane is equivalent to the cytosol (pH = 7.0), whereas the matrix has a higher pH (7.8). The components of the ETC reside within the inner membrane and include Complexes I-IV and ATP Synthase. Both coenzyme Q (CoQ) and Cyt*c* serve as electron shuttles between the complexes. The electrons are utilized to reduce diatomic oxygen to water and produce both pH and charge gradient across the inner membrane, which is harnessed by ATP Synthase to physically generate ATP.

Cyt*c* plays a vital role in the ETC by serving as a membrane-associated electron transporter between the cytochrome bc_1 complex (Complex III) and cytochrome *c* oxidase (Complex IV). Cyt*c* is the mobile electron carrier prior to the final reduction of water (via Complex IV), representing a highly exergonic ($\Delta G^{\circ\prime}$ = -100 kJ/mole) electron transfer potential between these molecules[6]. The approximately 12.5 kilodalton (kDa) Cyt*c* protein is positively charged due to its high isoelectric point (pI = 9.6) compared to the pH of the inner membrane space (pH ~ 7.0)[7]. A heme prosthetic group anchored to Cyt*c* by two thioether bonds bears the electron-carrying capacity of this protein. The active site heme iron cycles between the ferrous (Fe^{2+}) or ferric (Fe^{3+}) states depending on whether a single electron is being transported or not. Interestingly, it has been postulated that ferric state Cyt*c* predominates during apoptosis[8], despite the well-characterized reductant-rich cytosolic environment[9]. This phenomenon may be a result of multiple factors that include increased reactive oxygen species generation during cellular distress, accessibility of Cyt*c* iron to cytoplasmic reductants, or the predominance of stronger electron acceptors during mitochondrial Cyt*c* release[9].

Programmed Cell Death (Apoptosis) and the role of cytoplasmic Cyt*c*

The process of programmed cell death, more frequently referred to as apoptosis, is a highly regulated biological process of cellular suicide that includes plasma membrane blebbing and nuclear fragmentation[4]. This biological process plays a crucial role in normal developmental, cancer initiation, tumor progression, and toxicology. The "executioners" involved in this process are the cytoplasmic cysteine-dependent aspartate-directed proteases (caspases)[10]. These proteases are maintained in the inactive pro-caspase zymogen form to prevent undesirable caspase activation and cell death. The cascade begins with activation of the initiator caspase (caspase 9 in humans), followed by further proteolytic cleavage of the effector caspases 3 and 7. The activating agent of pro-caspase 9 is a large (1.4 MDa) multimeric complex known as the apoptosome, which is an ATP-dependent heptamer of Cyt*c* and apoptotic protease-activating factor 1 (Apaf1) [11, 12]. The formation of the apoptosome and activation of caspases thus commits the cell to the apoptotic process (Figure 2)[11, 12].

The fact that Cyt*c* is required for the formation of the cytoplasmic apoptosome lends to a topical review of subcellular localization and membrane permeability. Efficient energy production through the ETC necessitates that Cyt*c* remain mobile, yet in proximity to, the mitochondrial inner membrane. The "leakage" of Cyt*c* into the cytosol requires that the integrity of the mitochondrial membrane be compromised, and this active process is termed mitochondrial permeability transition (MPT)[3]. Although several mechanisms have been proposed for this event, the active formation of multi-component MPT pores along the outer mitochondrial membrane causes mitochondrial osmolarity changes, swelling, and eventual membrane rupture[3]. Cyt*c* is thereby liberated from the mitochondria and can associate with Apaf1 and ATP to form the apoptosome platform used in the activation of the caspase signaling cascade (Figure 2).

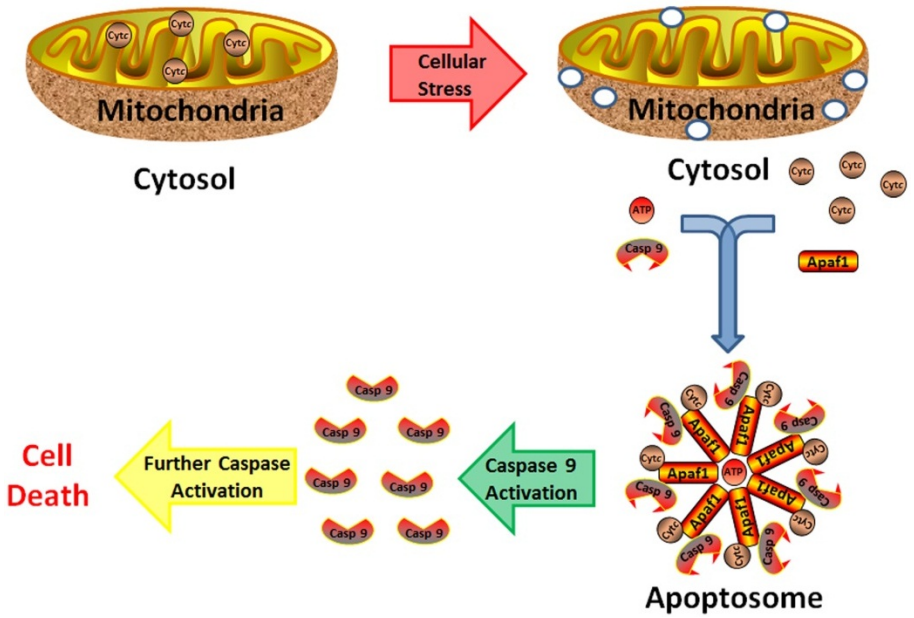

Figure 2. Schematic of Cyt*c*-dependent apoptosis. Cyt*c* remains localized at the inner mitochondrial membrane during normal homeostasis. Cellular distress initiates the apoptotic signaling cascade by formation of the MPT pores. This results in permeability of the outer mitochondrial membrane and release of Cyt*c* to the cytosol. In the presence of ATP, a heptameric complex of Cyt*c* and Apaf1 forms the apoptosome. Proteolytic activation of caspase 9 initiates the caspase cascade that results in nuclear fragmentation, plasma membrane blebbing, and eventual cell death.

Cyt*c* and Lipid Interactions Facilitating Apoptosis

Cyt*c* has been classically characterized as a protein associated with the mitochondrial inner membrane. In reality, there is an equilibrium state of free and membrane-associated protein, and it has been reported that each state possesses distinct functionalities within the mitochondria[8]. The “tethering” of Cyt*c* to the membrane (lipids) occurs via two biochemical interactions: 1) electrostatic interactions with phospholipids and 2) lipid insertion into the Cyt*c* hydrophobic heme tunnel. It has been reported that lipid insertion ablates the electron-carrying function of Cyt*c* by blocking heme accessibility to ETC complexes III and IV[1]. The lipid composition of the mitochondrial membranes therefore plays a crucial regulatory role on the ETC. The inner mitochondrial membrane is uniquely composed of cardiolipin, a diphosphatidylglycerol with high affinity for both Cyt*c* and ETC complexes I-V. This highly symmetrical molecule is composed of two phosphatidylglycerols connected to a central glycerol molecule, which yields four acyl tails available for interaction (Figure 3A). Interestingly, it has reported that up to twenty percent of the Cyt*c* pool is associated with cardiolipin within the mitochondrial inner membrane and is unavailable to participate in the ETC[1].

Beyond the associations of cardiolipin with ETC complexes and Cyt*c*, this membrane lipid also plays a direct role in the apoptotic process. Cardiolipin is predominantly found in the mitochondrial inner membrane, and early apoptotic signaling leads to a redistribution of cardiolipin to the outer mitochondrial membrane via membrane contacts points. This membrane redistribution enhances the heme-dependent peroxidase activity of cardiolipin-associated Cyt*c*, thus leading to the peroxidation of unsaturated acyl chains on cardiolipin (Figure 3B). The addition of a charged, polar substituent to the acyl tail greatly reduces the binding affinity between Cyt*c* and cardiolipin. The amount of free mitochondrial Cyt*c* then increases prior to mitochondrial permeabilization, thereby presenting the "critical mass" of Cyt*c* necessary to initiate apoptosome formation in the cytosol[1, 13].

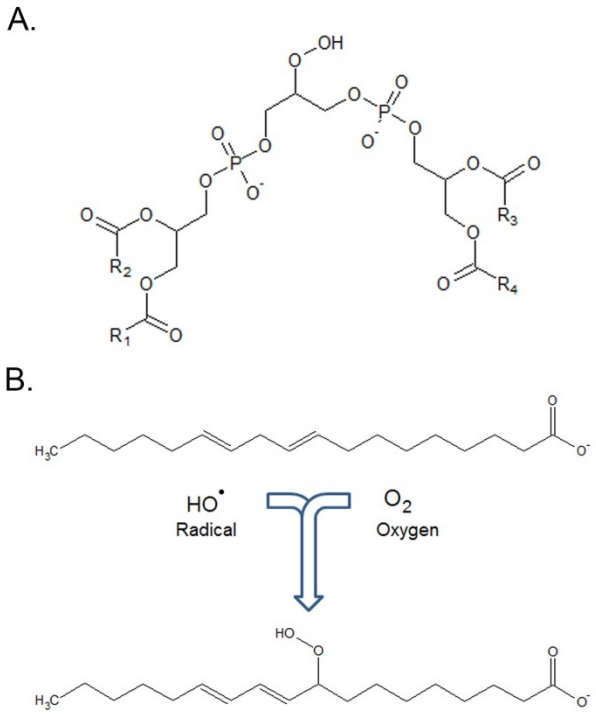

Figure 3: Cardiolipin structure and the peroxidation that contributes to Cyt*c* release. (A) Generalized structure of cardiolipin. Two phosphatidylglycerol molecules are attached to a central glycerol molecule. Four acyl chains (R1-4) are available for hydrophobic interactions. (B) Representative lipid peroxidation reaction. Unsaturated fatty acids can undergo lipid peroxidation in the presence of free radicals and oxygen in a Cyt*c*-dependent manner. The chemical modification alters the hydrophobic character of the lipid and alters the ability of cardiolipin and Cyt*c* to remain associated.

Modulation of Apoptosis by Cytc Post-translational Phosphorylation

The simplified concept of apoptosis relies upon the presence of Cyt*c* in the cytoplasm to initiate apoptosome formation and activation of the caspases. A key regulatory mechanism for protein function is post-translational modifications, such as

phosphorylation of alcohol-containing amino acid R groups. Despite the relatively small size of Cyt*c* (105 amino acids)[1], both the ETC and apoptotic functions of this protein appear to be regulated by phosphorylation. Analysis of the Cyt*c* sequence yields fourteen potential phosphorylation sites: two serine, seven threonine, and five tyrosine residues[14]. Recent advances in the generation of phospho-specific antibodies, protein isolation techniques, and mutational analysis have indeed proven that at least four of these sites are necessary for protein function, with two of these sites being essential for the activation of apoptosis[1].

The Toxicant Connection to Cyt*c* and the Mitochondria

The mitochondrial ETC and Cyt*c* have proven to be vital diagnostic biological tools in the understanding of chemical and environmental toxicants. The necessity of ETC function has revealed that inhibitors of ETC complexes can be potent toxicants. Most Biochemistry textbooks will briefly note inhibitors of ETC; however, there is a lack of physiological significance or toxicology relevance in these texts. Although a number of ETC toxins and specific mechanisms have been elucidated, the identification of specific Cyt*c* inhibitors remains non-existent. This is likely due to the small size and mobile nature of the molecule[7].

To further the complement the toxicology perspective in the absence of Cyt*c* inhibitors/toxins, two specific ETC inhibitors with relevance to toxicology are discussed: rotenone and cyanides. Considering the complex physiological and toxicological etiologies associated with these molecules, this manuscript focuses on the biochemical mechanisms of ETC inhibition. Rotenone is a non-competitive Complex I inhibitor that blocks the flow of electrons to CoQ[15, 16]. This blockage of electron flow reduces ATP production, increases the generation of ROS, and initiates Cyt*c*-dependent apoptosis[15]. It has also been reported that Complex I and III are the predominant sieves in the ETC that result in electron loss and ROS generation[3]. It is also of interest to note that rotenone is utilized in experimental neurodegeneration disease models of persistent ROS generation and cell death, which includes Parkinson's disease[15]. In contrast to rotenone, cyanides inhibit the ETC at Complex IV[17]. Mechanistically, cyanides have a high affinity for an oxidized heme in Complex IV and prevent the flow of electrons to molecular oxygen. Cyanides are also non-competitive inhibitors[17], which allow this discussion of inhibitors to accompany lessons on kinetic mechanisms that are also discussed in the Biochemistry courses. It is noteworthy that more in-depth discussion of specific physiology, toxicity, antidotes, etc. could be included with this discussion. The author also acknowledges that other ETC inhibitors, including paraquat, carbon monoxide, sodium azide, and sulfide, could be included to the discussion or used as term paper topics.

Learning Objectives and Student Assessment

This supplemental instruction approach to the Biochemistry I and II curriculum is designed to increase student engagement with the goal to enhance student comprehension surrounding the topics of electron transport chain and oxidative phosphorylation. The inclusion of toxicology concepts provides "real world" application

to complicated biological systems. Overall, the learning objectives (Table 1) for this toxicology-based biochemistry approach expose students to the field of toxicology in the context of biochemistry principles, thereby complementing the current curriculum of Biochemistry I and II.

Table 1: Learning Objectives for Cyt*c* Functionalities

1. Students will understand the dual role of Cyt*c* in bioenergetics, toxicology, and programmed cell death.
2. Students will describe how cellular compartmentalization alters the activity of biological processes and how compartmentalization can be compromised.
3. Students will be able to compare and predict how perturbation of ETC at specific sites alters Cyt*c* redox states and functions.
4. Students will be able to predict how post-translational modifications or mutagenesis may alter biochemical functions and bio-molecular interactions.
5. Students will understand how toxins can be used in experimental models to determine the specific functions of a protein and/or biological process.

In the Biochemistry I and II courses, student comprehension is assessed by in-class “clicker” or written quizzes and essay-based exams. Quiz questions are more direct and ask specific details while exam questions apply student comprehension to assess a biological situation and/or predict outcomes. The questions below are a sampling of exam questions designed to assess the biochemical context and mechanisms related to Cyt*c*-based toxicology:

1.) Predict how changing the cardiolipin R groups from unsaturated to saturated chains would alter the Cyt*c*/cardiolipin interaction in apoptosis.

2.) Explain how Cyt*c* is able to migrate from the mitochondria to the cytosol. Be sure to explain the biochemical interactions facilitating this transition.

3.) Compare and contrast how DTT (a reductant) and H_2O_2 (an oxidant) will alter Cyt*c* in apoptosis.

4.) Phosphorylation of Cyt*c* Ser48 is critical for the formation of the apoptosome. Predict how apoptosis would be altered in Ser48 is mutated to a threonine or a glycine.

5.) Compare and contrast how toxicants that inhibit Complex I and IV, respectively, will alter the Cyt*c* redox state.

6.) Explain how a toxin could be used to validate that an electrochemical gradient exists across the mitochondrial inner membrane.

Conclusions

The preceding information documents how I have integrated concepts of toxicology and apoptosis into the broader biochemistry curriculum at Bloomsburg University of Pennsylvania. Preliminary assessments and verbal student feedback have been very positive, and students have requested expanded use of this integrated toxicology methodology. Both biology and chemistry majors who move onto the Biochemistry II course tend to be the high achievers and appreciate the emphasis on "real world" application of biochemical mechanisms and functions. I am presently expanding this methodology to include more toxicology themes, as relevant to the current curriculum. I have hopes that this integrated approach will garner faculty interest and enthusiasm for the field of toxicology that will filter to the study body. This approach can hopefully be an effective toxicology recruiting tool for undergraduate universities that cannot offer such specialized courses or lack faculty expertise in toxicology.

Notes

The author declares no competing financial interest.

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