Apoptosis Inducing Factor: Determining Optimal pH of Enzymatic Function

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Introduction
Approximately 12 million people in the United States are living with or had cancer in the past year with an estimated 1.2 million new cases projected in 2012. Cancer is the uncontrollable growth and/or division of cells in the body. This cellular homeostasis is tightly regulated by a series of events leading to programmed cell death, or apoptosis. Apoptosis is a necessary aspect for early embryonic development, inflammatory responses, as well as overall tissue homeostasis during an organism’s life. While apoptosis is essential, if it becomes hyperactive, it can lead to multiple degenerative diseases as well as a suppressed immune response. One of the key players in the regulation of this multifaceted process is the mitochondrial protein, apoptosis inducing factor (AIF).

Apoptosis inducing factor (AIF) is a flavoprotein that resides tethered to the mitochondrial inner membrane with the majority of the protein located in the inner membrane space. In healthy cells, AIF is needed for proper endonuclease activity, it can act as an NADH-oxidase, and is embryonic lethal if removed. During an apoptotic event, AIF is cleaved and translocates to the nucleus, inducing DNA degradation. While AIF does not have endonuclease activity, it can act as an NADH-oxidase, however this cellular function is currently unknown.

Our goal for this study is to determine the optimal physiological pH at which AIF NADH oxidase activity occurs. We hope that this data will provide further insight into the complete cellular function of AIF.

Methods
Recombinant AIF: AIF protein was generously given by Dr. John C. Wilkinson (Wake Forest University School of Medicine). Briefly, recombinant His-tagged AIF protein was expressed in Escherichia coli and purified by Ni-NTA precipitation. Final protein concentration was measured using Bradford analysis and found to be 75 mg/mL. Protein was stored at -80°C for 15 months, and kept at -20°C during the research period.

SOS-PAGE and Immunoblot Analysis: SDS-PAGE was performed as previously described.[4] Briefly, recombinant AIF was diluted to 0.25 mg/mL in 1X LDS sample buffer and incubated at room temperature for 30 minutes. Samples destined for total protein visualization were stained using commassie blue. Samples destined for immunoblot analysis were transferred to nitrocellulose at 30V for 45 minutes then blocked in 1% BSA with 5% milk. Blots were subjected to mouse monoclonal and AIF primary antibody followed by alkaline phosphatase conjugated goat anti-mouse secondary, and developed in 0.1% NBST and 0.05% milk. AIF protein was visualized using a EUROE instant developer.

NADH Oxidation Assay: NADH oxidation assay was performed as previously described.[4] Briefly, 250 µM ovalbumin or recombinant AIF was incubated with 150mM KH2PO4 (pH 6.8 or 7.5 as indicated) in the presence of 10 µM NADH. Change in absorbance of NADH at 320 nm was measured over the indicated time points using Shimadzu Model UV-1601 spectrophotometer.

Results

AIF Activity at Physiological pH

![AIF Activity at Physiological pH](image)

Figure 1. SDS-PAGE and Immunoblot Analysis of Recombinant AIF (A) Gel showing 5 - 10 µg of recombinant AIF were subjected to SDS-PAGE. In (B) Immunoblot analysis was performed as described in the methods and AIF was confirmed using the monoclonal antibody (Santa Cruz Biotechnology).

![NADH Absorbance @ 320nm](image)

Figure 2. Absorbance of NADH at 320 nm was measured at pH 7.5 and normalized over the indicated time points.  NADH absorbance is indicative of AIF presence.  The absorbance of 0.5 µM NADH at pH 7.5 was used to calculate the change in absorbance at 320 nm.

![Enzymatic Rate of AIF](image)

Figure 3. The rate of change in absorbance of NADH at 320 nm was measured in the presence of recombinant AIF (3x) at pH 6.8 (red squares) and pH 7.5 (purple diamonds). The black bars indicate standard deviation (n=3).

Discussion

As with many cellular compartments, the environmental pH of AIF differs with its location. By determining the optimal enzymatic pH, we can suggest the cellular location at which AIF activity occurs thus allowing to cellular function. Recombinant AIF subjected to SDS-PAGE analysis (Fig.1) revealed bands at 54 kDa, indicating the presence of protein at the site expected for AIF. Several lower molecular weight bands also surfaced at 37 kDa, which lead us to believe degradation occurred, however immunoblot analysis confirmed the presence of the recombinant AIF suggesting that the lower bands were not indicative of degradation.

AIF enzymatic activity was measured by the reduction in absorbance of NADH (oxidation) at 320nm. Figure 2 illustrates the normalized change in absorbance of NADH by ovalbumin or AIF, demonstrating that this reaction is both NADH and AIF dependent. Furthermore, this data indicates that the recombinant AIF is enzymatically active and successfully oxidizing NADH. The addition of extra NADH to the AIF sample demonstrates that the enzymatic activity of the recombinant AIF is not pH limiting factor.

Next, we examined the enzymatic rate of AIF over a physiological pH range which resulted in a distinct peak between pH 6.5 and 7.0 (Fig. 3). To further explore these results, several NADH oxidation trials (n=3) were performed at pH 6.8 and 7.5 reflecting the mitochondrial inner membrane space and cellular cytosolic/nuclear pH respectively (Fig. 3). Finally, initial velocities were calculated for oxidation reactions involving no protein, ovalbumin, and AIF at pH 6.8 and 7.5 and compared to the rate of change in NADH concentration (µM/min) using Beer’s Law (Fig. 5). Statistical analysis revealed that the enzymatic rate of AIF was significantly higher (~2-fold) at pH 6.8 when compared to pH 7.5. This allows us to suggest that AIF is increasingly more active in a physiological environment with pH 6.8 rather than 7.5.

Conclusion

This study aimed to delineate the optimal physiological pH at which AIF enzymatic activity occurs. By utilizing an in vitro NADH oxidase system, we determined that AIF is most active at a pH reflective of the environment in the mitochondrial inner membrane space rather than the cellular cytosol and nucleus. This observation suggests that AIF activity is more likely to occur during healthy cell function rather than during an apoptotic event, shedding light on the complex duality of apoptosis inducing factor.

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Life and Death Functions of AIF

Healthy Mitochondria

Cell Death

References