2023

The Effect of S-glutahionylation of KEAP-1/NRF-2 and Caspase 3

Claire Greenless

The Effect of S-glutahionylation of KEAP-1/NRF-2 and Caspase 3

Claire Greenlees[1] and Yuh-Cherng Chai, Ph.D.[1]

[1]Department of Chemistry, John Carroll University, University Heights, OH, 44118;

Abstract

S-glutathionylation is a form of post-translational modification where glutathione forms disulfide bonds with protein cysteine residues under oxidative conditions. The process of S-glutathionylation is important because it is a reversible process, regulates enzyme activity, and may link to disease development. In this work, under physiological conditions, KEAP-1 and NRF-2 bind together as a complex. Upon oxidative stress, NRF-2 dissociates from KEAP-1 and triggers an antioxidant response. However, the dissociation mechanism was not well studied. S-glutathionylation of KEAP-1 causes dissociation of NRF-2. This result demonstrates a possible dissociation mechanism of NRF-2. Caspase 3 is a cysteine containing protein and a key enzyme in executing apoptosis in cells. The cysteine residue is required for its activity. Our results show that S-glutathionylation of the cysteine inhibits caspase 3 activity and that inhibition can be reversed by the addition of dithiothreitol (DTT). The inhibition of caspase 3 activity with GSSG and reactivation of caspase 3 activity with DTT showed a concentration dependent manner. Our work shows that the process of protein S-glutathionylation can affect cellular protein physiological functions under oxidative stress.

Introduction

KEAP-1/NRF-2

The exact mechanism of NRF-2 dissociating from a KEAP-1 complex is unknown and not well studied under physiological conditions. When NRF-2 dissociates from KEAP-1, it travels from the cytoplasm into the nucleus where it turns on transcription by triggering the antioxidant response element of the cell. To study the dissociation, glutathione, the most abundant non-protein antioxidant molecule in cells, was utilized. The glutathione was used to modify proteins with its –SH group which in turn forms disulfide bonds that are present in many physiological diseases. Thus, the mechanism that causes the dissociation of NRF-2 from KEAP-1 under conditions of oxidative stress was explored. Additionally, S-glutathionylation of KEAP-1 was studied as a possible stimulant for the dissociation of NRF-2.

Caspase 3

Caspase 3 is a protein belonging to a family of cysteine proteases that are found in the cytoplasm of cells, and its activity can be activated or deactivated. Caspase 3 in native form is active and able to cleave many key cellular proteins resulting in the condensing and blebbing of cells that eventually leads to cell death- apoptosis. Caspase 3 was reacted with glutathione disulfide (GSSG) which resulted in a protein-SSG complex as a result of oxidative protein modification and the formation of disulfide bonds. We also studied the reverse reaction of the modified caspase 3 with dithiothreitol (DTT).

Materials & Methods

SDS-PAGE and Western Blot:

- SDS-PAGE systems are used to separate the protein of interest based on its molecular weight. Once separated based on size, proteins are transferred from the gel which is translucent to a membrane. After the protein is transferred, the membrane is blocked with milk to prevent non-specific staining. Lastly, the membrane is incubated in primary and secondary antibodies.

S-glutathionylation of KEAP-1

- KEAP-1 was incubated with various concentrations of GSSG (0, 10, 25, 50mM) at 30°C for 30 minutes. The samples were loaded into the electrophoresis apparatus and the SDS-PAGE was run. The membrane was blocked overnight and stained with the anti-glutathione (mouse) and anti-mouse HRP antibodies.

Electrophoresis Mobility Shift Assay (EMSA)

- SDS is an anionic detergent that denatures and unfolds proteins. Therefore, it was removed from the gel system entirely. The samples of KEAP-1 and NRF-2 were combined and put on ice for 5 minutes. The cold solutions were loaded directly into the wells for completion of the EMSA. Once the electrophoresis was complete, the gel was stained with Coomassie blue.

Materials & Methods (continued)

Activity Assay:

- The activity assay samples of caspase 3 were mixed with an assay buffer, deionized water, substrate, GSSG, and DTT.

Deactivation and Reactivation of Caspase 3

- Modified caspase 3 activity assays were preformed with different concentrations of GSSG. Samples of modified caspase 3 with GSSG were incubated at 37°C for 30 minutes. Some modified caspase 3 samples were incubated with DTT at 37°C for an additional 30 minutes. Both sets of samples were incubated with substrate at 37°C for 1 hour.
- Post incubation samples were transferred into a 100 μL cuvette for spectrophotometer analysis. Using the assay buffer as a blank, each sample was tested at 405 nm and the corresponding absorbance values were recorded.

Results

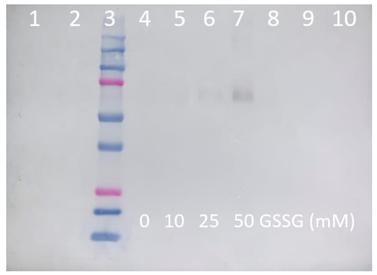


Figure 1: S-glutathionylation of KEAP-1
In order to assess the separation of NRF-2 from the KEAP-1 complex, the same amount of KEAP-1 was modified with different concentrations of GSSG (determine optimum GSSG conc.). Lane 3 is the molecular weight standard, lane 4 is the 0 mM GSSG, lane 5 is the 10 mM GSSG, lane 6 is the 25mM GSSG, and lane 7 is the 50 mM GSSG. There was a significant protein band observed at 70 kDa. This result showed that KEAP-1 could be modified.

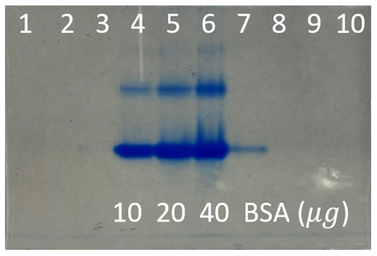


Figure 2: Electrophoresis Mobility Shift Assay (EMSA)
As the goal was to study the proteins under native conditions, the SDS (a detergent) had to be removed from the gel. To save protein bovine serum albumin (BSA) was used as an alternative. Lane 4 is the 10 μg BSA, lane 5 is the 20 μg BSA, and lane 6 is the 40 μg BSA. The molecular weight standard also contained SDS so it could no longer be used. The intensity of the bands demonstrate concentration dependence, and the upper bands show a possible dimer.

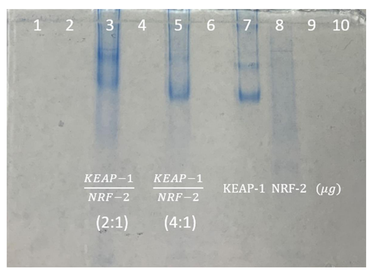
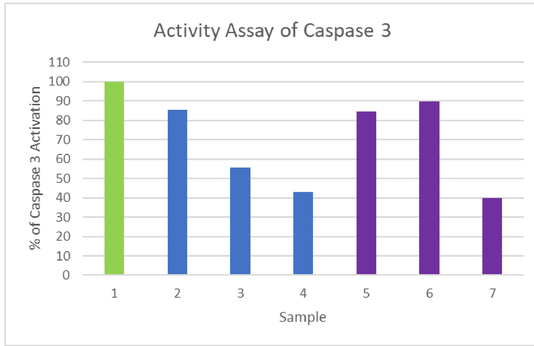


Figure 3: Formation of Complexes by EMSA
Ratios of KEAP-1:NRF-2 were determined from a previous study. Lane 3 is a 2:1 ratio (KEAP-1:NRF-2), lane 5 is a 4:1 ratio (KEAP-1:NRF-2), lane 7 is KEAP-1, and lane 8 is NRF-2. KEAP-1 was not modified with GSSG, rather it was in its natural state. The unmodified KEAP-1 bound to NRF-2 as to be expected. The native gel was successful in creating a physiological system through which the proteins could bind properly.

Results (continued)



Sample Key:
1) Caspase 3
2) Caspase 3 + 2.5 mM GSSG
3) Caspase 3 + 5.0 mM GSSG
4) Caspase 3 + 10.0 mM GSSG
5) Caspase 3 + 2.5 mM GSSG + 100 mM DTT
6) Caspase 3 + 5.0 mM GSSG + 100 mM DTT
7) Caspase 3 + 10.0 mM GSSG + 100 mM DTT

Figure 4 (Representative): Deactivation and Activation of Caspase 3
Sample 1 (in green) containing the unmodified caspase 3 was the reference as it contained 100% activity. Samples 2-4 (in blue) contained the same amount of enzyme with increasing amounts of GSSG. Samples 5-7 (in purple) also contained the same amount of enzyme and amounts of GSSG with the only differentiation being the addition of DTT. As the absorbances of samples 2-4 were lower than the reference sample, the data showed that the higher the concentration of GSSG present, the lower the absorbance and corresponding activation of the protein. Moreover, the DTT was able to reactivate the protein as shown in samples 5-7.

Conclusions

KEAP-1/NRF-2

- The molecular weight of KEAP-1 is approximately 70 kDa. The first pink band denoted in Figure 3 lane 3 indicates a molecular weight of 75 kDa. While the modified KEAP-1 was not as prevalent in Figure 3 lanes 5&6, it was clear that KEAP-1 was modified in lane 7 as shown by the grey band. The higher the concentration of GSSG, the more visible the modified KEAP-1 band. The results indicate that KEAP-1 could be modified via GSSG and that it could be a contributor to the dissociation of NRF-2.
- In order to study protein bindings, native state conditions were achieved via EMSA. The new system was able to properly separate protein (BSA) with a clear resolution of the bands. Additionally, there was a clear indication in differentiation between the concentrations of proteins used in each well.
- Using the native method, a proper analysis of KEAP-1/NRF-2 binding was conducted. As shown in Figure 3, unmodified KEAP-1 bound to NRF-2 forming complexes as predicted even though the bands were quite faint. The data in Figure 3 also indicated that NRF-2 is barely visible as an individual protein at low concentrations.
- In an additional trial KEAP-1 that had been modified with GSSG was combined with NRF-2, but the resolution of the bands were too poor to make any concrete observations. The current theory is that the high concentrations of GSSG interfered with the binding therefore future samples could be desalted and stained with silver for better resolution.

Caspase 3

- After conducting multiple trials, it was determined that GSSG inhibits caspase 3 in a concentration dependent manner. Thus, the higher the concentration of GSSG present, the lower the activity when comparing the same amount of enzyme. It was also found that DTT reactivates caspase 3 after being modified and deactivated by GSSG.
- Although reactivation of caspase 3 was not up to 100% recovery, the results indicated that deactivation was reversible to some degree which could help the regulatory functions of caspase 3 in relation to apoptosis.

Acknowledgements

- Funded by the Colleran Weaver Summer Undergraduate Research Fellowship (2022).