

RESEARCH ARTICLE

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Photo-illuminated Glutathione Inactivates Alpha-2-macroglobulin: Spectroscopic and Thermodynamic Studies

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Background: Glutathione (GSH) is a principle thiol-containing tripeptide (cysteine, glutamic acid and glycine) antioxidant against free radicals and other harmful oxidants in cellular defence. The alpha-2-macroglobulin (α 2M) is large tetrameric zinc-binding glycoprotein which inhibits proteinases regardless of their specificity and catalytic mechanism.

Materials and Methods: The interaction of GSH with α 2M including the structural and functional alterations were analyzed using various biochemical and biophysical methods. UV-visible and fluorescence spectroscopy were used to study the binding of α 2M with GSH and Fourier transform infrared (FT-IR) spectroscopy was explored to study the structural change induced in α 2M.

Results: The results suggest that exposure of α 2M to GSH decreases the antiproteolytic potential as suggested by the amidase assay. The UV-spectroscopic study showed the formation of α 2M-GSH complex and fluorescence analysis showed significant quenching in fluorescence intensity of α 2M suggesting GSH binding and structural alteration in the protein. FT-IR spectroscopy was explored to study the structural change induced in α 2M which suggest that the secondary structure of α 2M changes upon complex formation.

Conclusion: Our studies show that interaction of α 2M with photoilluminated GSH results in functional and conformational changes in the protein.

Keywords: glutathione, GSH, alpha-2-macroglobulin, photo-illumination, ITC, FTIR

Introduction

Glutathione (GSH) is a low molecular weight cellular antioxidant and has various enzymatic and nonenzymatic properties.¹ In the human body, GSH is present as ubiquitous molecule *i.e.*, produced in all organs. The main function of the GSH in the human body is to maintain the integrity of

red blood cells (RBCs). It is a tripeptide antioxidant mainly found in two forms: reduced GSH and oxidized glutathione (GSSG).² Among the two forms, GSH is mainly found in healthy living cells (90%) while GSSG occurs in minor amount (10%) in cells.³ It is mainly found in millimolar (mM) concentration inside the cells, while the plasma contains lower amount of GSH.^{4,5} In cells, GSH may be found

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freely or bound to proteins. As a radical scavenger, GSH plays a major role in the detoxification of reactive oxygen species (ROS) and drug metabolites.⁵ When oxidative stress increases, GSH converts into GSSG *i.e.*, it gets oxidized.⁴ In this process, two molecules of GSH form a molecule of GSSG with the formation of double-sulfur bond.⁵ GSH is converted to GSSG under oxidative stress and can be reverted back to reduced form by the action of the enzyme glutathione reductase (GRx).⁶ It is well known that under oxidative stress, GSH can be converted to GSSG, which results in the impairment of radical scavenging activity and hence, loss of antioxidant activity.^{6,7} GSH plays a major role in the protection of organs such as liver, intestine, lungs and kidney when they are exposed to xenobiotics.⁷⁻⁹ The level of GSH is decreased in many diseases such as alcoholic liver injury, diabetes mellitus, hemolysis and cancer.^{8,9} It is also used for the treatment of many diseased conditions such as chronic renal failure, diabetes, lung inflammation and malnutrition. It is found in blood plasma, produced mainly in the liver in humans, and its concentration is about 2 mg/mL in plasma.^{10,11}

Sheep alpha-2-macroglobulin (α 2M) is a large glycoprotein of 630 kDa found in the plasma and a homologue of human α 2M. Like its human counterpart, it is a tetrameric glycoprotein consisting of four identical subunits. The α 2M captures a variety of proteinases and is considered as a major proteinase inhibitor in the blood.^{12,13} The α 2M plays an important role in innate immunity and is highly conserved throughout evolution.¹⁴ The α 2M possesses a unique trapping mechanism and captures a variety of proteinases both *in vitro* and *in vivo* and is considered as a major proteinase inhibitor. This protein traps the proteinases released by cells during inflammation and hence regulates the extracellular proteolytic activity resulting from fibrinolysis and clotting.¹⁵ It inhibits the proteinases present in the plasma and also protect cells against pathogens since it can also trap proteinases from non-human origin.

In the present study, we explored the interaction of GSH with α 2M in the presence of fluorescent light (as well as dark). In this study, our aim was to study the interaction of GSH with α 2M and how it affects the anti-proteinase and conformational status of the protein. The binding interaction was explored by using ultraviolet (UV), and fluorescence spectroscopy and isothermal titration calorimetry (ITC). The changes in the secondary structure were analyzed by Fourier-transform infrared spectroscopy (FT-IR).

Materials and methods

Isolation of α 2M

Fresh sheep blood was collected from the slaughter house (within minutes of slaughter of animal) for the isolation of α 2M from plasma. Sheep α 2M was purified by simple two step procedure *i.e.*, ammonium sulphate fractionation and Sephacryl 300HR (Sigma-Aldrich, St. Louis, MO, USA) gel filtration chromatography as reported previously.¹⁶

Antiproteinase Activity Assay of α 2M

Activity assay of α 2M was performed in order to determine the change in the function of native and treated protein as reported previously.¹² α 2M was incubated with varying concentrations of GSH (20-100 μ M) in the presence and absence of light. A final mixture of 1 mL was prepared with 50 mM sodium phosphate buffer (pH 7.4) at $22\pm 1^\circ\text{C}$. After 1 hr of incubation at room temperature, 100 μ L of trypsin was added in the reaction mixture for 15 min at 37°C . Following incubation, 100 μ L of soybean trypsin inhibitor (STI) was added for 15 min at 37°C . Finally, 2 mL of N-Benzoyl-DL-arginine-p-nitroanilide (BAPNA) was added for 30 min and absorbance was recorded at 410 nm. GSH, trypsin, STI and BAPNA were obtained from Sigma-Aldrich.

UV-visible Absorption Spectroscopy

To analyze the changes in the native conformation of protein, the UV absorbance spectra were recorded.¹⁷ Ten μ M of α 2M was incubated with varying concentration of glutathione (20-100 μ M) in absence and presence of light. The absorption spectrum was recorded in the range of 250-350 nm on a Shimadzu UV mini visible spectrophotometer UV-1700.

Fluorescence Spectroscopy

Intrinsic fluorescence was performed to analyze the change in the structure of α 2M after its interaction with glutathione. α 2M was incubated with increasing concentrations of glutathione (20-100 μ M) in dark as well as in the presence of light and were recorded on a Shimadzu RF-5301 spectrofluorophotometer (Shimadzu, Tokyo, Japan) in the range of 300-400 nm in a quartz cell of path length 10 nm. The final concentration of protein was 10 μ M.^{15,17}

FT-IR Spectroscopy

Another technique which was used to study the secondary structural changes in the native structure of protein was FT-IR.^{18,19} FT-IR spectra were recorded in the range of 1600-1700 cm^{-1} at room temperature ($22\pm 1^\circ\text{C}$) sodium

phosphate buffer (pH 7.4). Ten μM of $\alpha 2M$ was incubated with increasing concentrations of glutathione (50-100 μM) and were recorded on a Perkin Elmer FT-IR Spectrum 100 (Perkin Elmer, Waltham, MA, USA).

Isothermal Titration Calorimetry (ITC)

To study the thermodynamics signatures for binding of GSH with the protein, isothermal titration calorimetry (ITC) was performed. Enthalpy, entropy, binding sites and Gibbs free energy of $\alpha 2M$ -glutathione interaction were determined on a VP-ITC titration microcalorimetry system (MicroCal, Northampton, MA, USA) as reported previously.¹⁹ Previous studies have shown that the change in enthalpy and entropy for protein-ligand interaction may reveal the binding forces involved in the interaction.²⁰⁻²² If the value of ΔS and ΔH are negative, then Van der Waals and hydrogen bond formation are involved in the interaction and if both are found to be positive, it suggests hydrophobic interaction.²² The concentration of the $\alpha 2M$ in the sample cell was 10 μM , and the syringe was loaded with 20 μM GSH, while the reference cell was filled with 50 mM sodium phosphate buffer (pH 7.4). The sample was continuously stirred at 307 rpm and reference power was kept at 16 $\mu\text{cal sec}^{-1}$. The heat of dilution was determined by control experiments and subtracted from the integrated data before curve fitting. The ORIGIN® software (OriginLab Corporation, Northampton, MA, USA) was used to analyze the titration curves.²³

Results

The Antiproteinase Activity of $\alpha 2M$

Activity assay was performed to study the functional change in the native protein. Figure 1A showed the activity profile of treated $\alpha 2M$ in dark and Figure 1B depicts antiproteinase activity in the presence of light. Loss in antiproteinase activity at 100 μM GSH was 18% under dark and 38% when it was photoilluminated either due to change in the conformational status of the protein or oxidative damage to the bait region amino acids.

UV-visible Absorption Spectroscopy Results

UV-visible spectra were analyzed in order to check the possible change in the conformational status of protein as well possible complex formation between protein and ligand. $\alpha 2M$ showed a peak at around 278 nm which was due to the presence of three aromatic amino acid residues (tryptophan, tyrosine and phenylalanine). Figure 2A showed

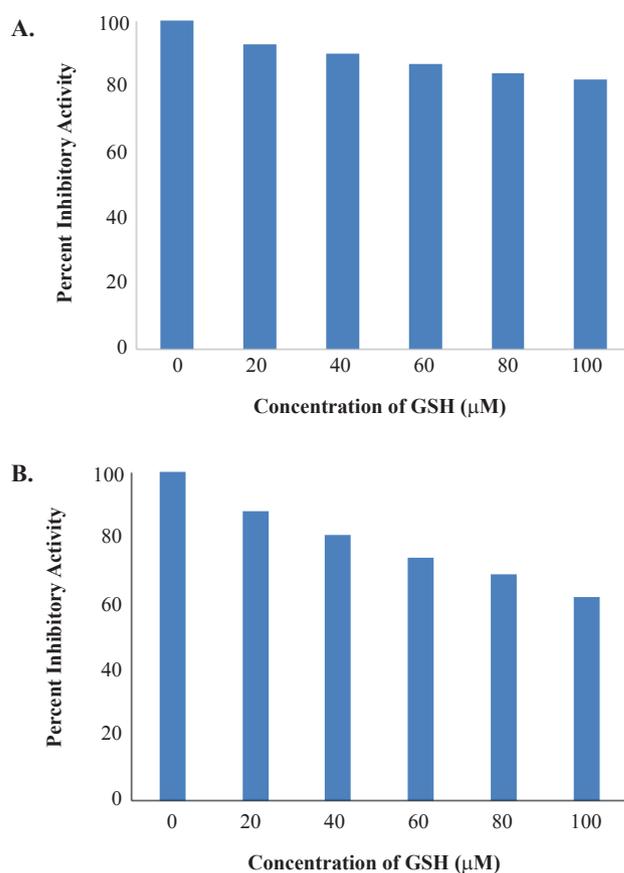


Figure 1. Percent inhibitory activity of $\alpha 2M$. A: $\alpha 2M$ was incubated with increasing concentration of GSH in dark then assayed for antiproteolytic activity; B: $\alpha 2M$ was incubated with increasing concentration of GSH in visible light then assayed for antiproteolytic activity.

the UV visible spectra of $\alpha 2M$ with increasing concentration of GSH in dark. Increasing the concentration of GSH leads to an increase in UV absorbance which suggests the possibility of complex formation between GSH and $\alpha 2M$. The interaction of $\alpha 2M$ was also analyzed with GSH in the presence of white fluorescent light. As shown in Figure 2B, GSH causes a dose dependent decrease in absorbance and the peak shifts to higher wavelength in presence of light. At the highest GSH concentration (100 μM), there was a hyperchromic shift (about 2%) in dark and a hypochromic shift (about 2%) in the presence of light suggesting a change in the polarity around tryptophan residues and hydrophobicity thereby inferring that GSH induces a conformational change in the native protein. Figure 2C showed the comparative UV absorption spectra of $\alpha 2M$ alone and with GSH in the presence of white light and dark at 100 μM .

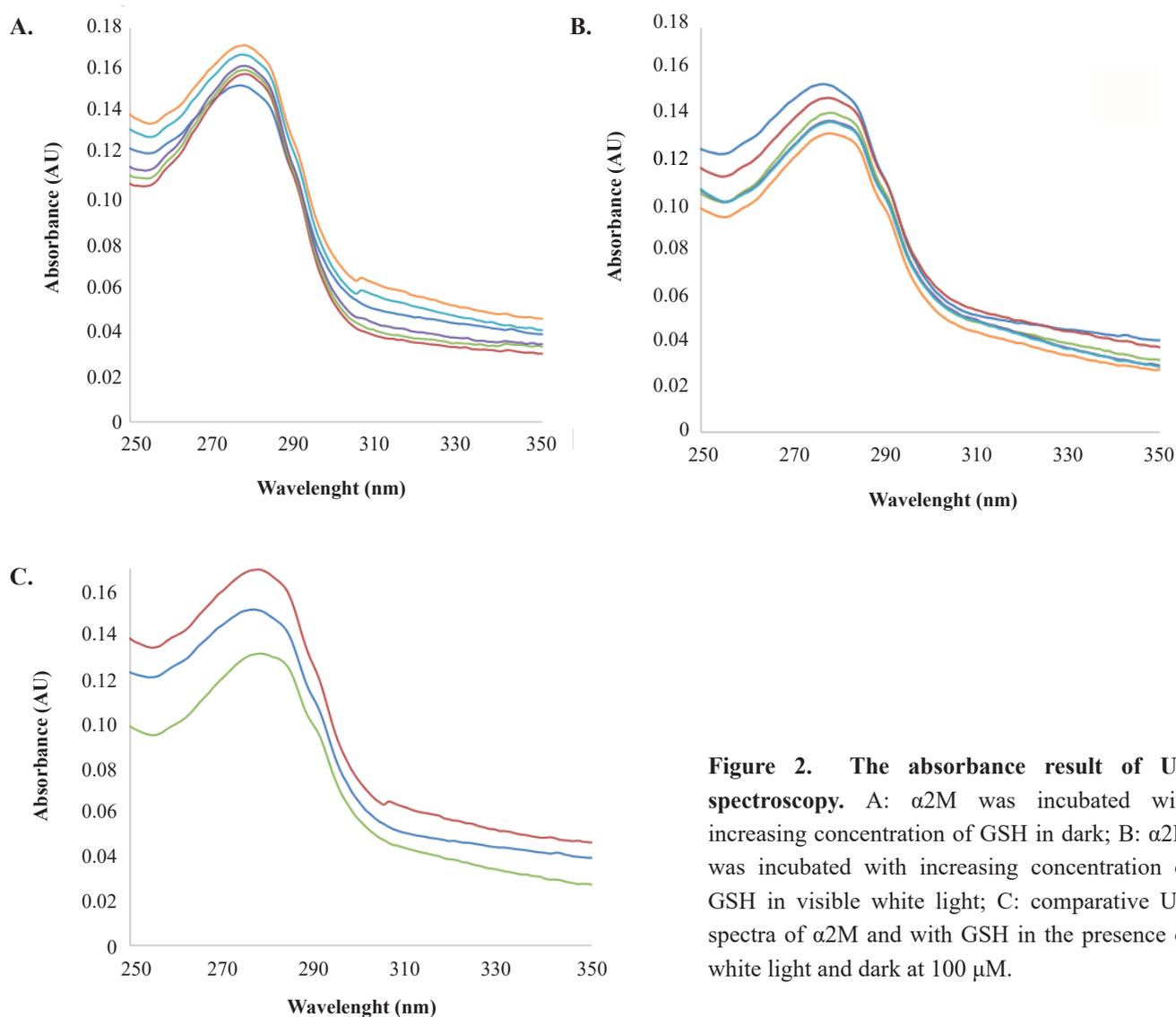


Figure 2. The absorbance result of UV spectroscopy. A: α 2M was incubated with increasing concentration of GSH in dark; B: α 2M was incubated with increasing concentration of GSH in visible white light; C: comparative UV spectra of α 2M and with GSH in the presence of white light and dark at 100 μ M.

Intrinsic Fluorescence Measurements

Intrinsic fluorescence was a useful technique utilized to identify the conformational changes in the native structure of proteins. Intrinsic fluorescence of the protein was mainly depend upon the aromatic amino acid residues *i.e.*, tyrosine, phenylalanine and tryptophan but in α 2M, tryptophan played a major role. Upon increasing the concentration of ligand, fluorescence of native protein gradually decreased. Figure 3A showed the intrinsic fluorescence pattern of α 2M with varying concentration of GSH (20-100 μ M) in dark. Native α 2M showed a peak at 329 nm and the intensity decreasesd upon increasing the dose of GSH. At 100 μ M, there was maximum fluorescence quenching with a shift of peak (1 nm) suggesting a possible change in the microenvironment

around the chromophore of α 2M. Similar results were obtained when photo-illuminated GSH was incubated with α 2M, although there was a minor increase in quenching in the presence of light (Figure 3B and Figure 3C).

FT-IR Spectroscopy Results

FT-IR spectroscopy was employed to study the change in the secondary structure of protein. Amide I band was analysed as it was the most important band in protein used for the analysis of the possible structural change. Amide I band displayed in the wave number range 1600-1700 cm^{-1} which was governed by the stretching of the C=O (70-85%) and minor contribution of C-N groups (10-20%). Amide I band for native α 2M was centered at around 1635 cm^{-1} which was characteristic of β -sheet structure. FT-IR spectroscopy was

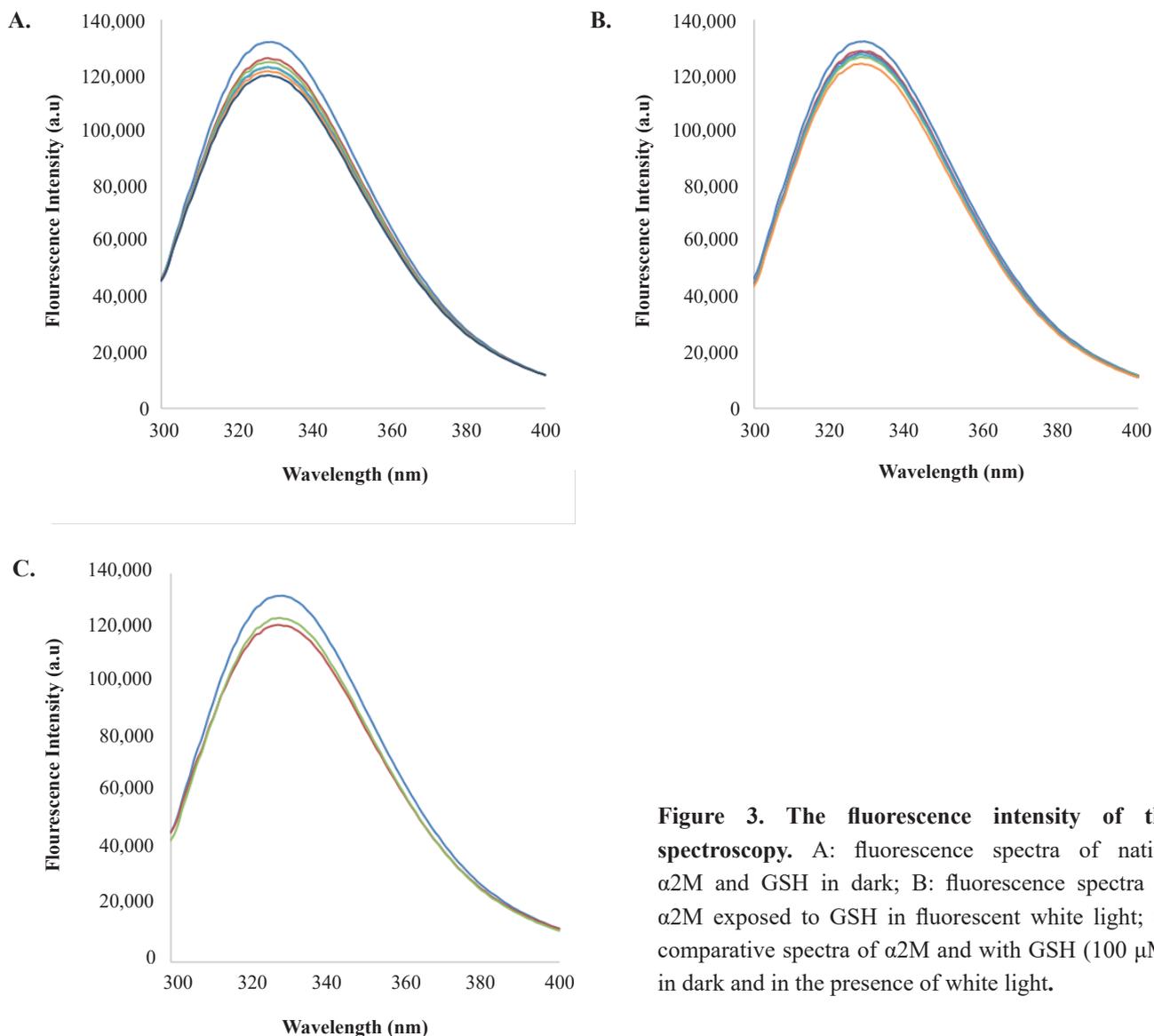


Figure 3. The fluorescence intensity of the spectroscopy. A: fluorescence spectra of native $\alpha 2M$ and GSH in dark; B: fluorescence spectra of $\alpha 2M$ exposed to GSH in fluorescent white light; C: comparative spectra of $\alpha 2M$ and with GSH (100 μM) in dark and in the presence of white light.

utilized to study the structural changes in the protein after its interaction with GSH interaction in dark and light. FT-IR spectra for native $\alpha 2M$ and co-incubation with varying concentration of GSH (50-100 μM) were recorded in the range of 1600-1700 cm^{-1} . Figure 4A showed FT-IR spectra of native $\alpha 2M$, the amide I band was located at 1635 cm^{-1} which confirmed the presence of predominant β -sheet structure in $\alpha 2M$. At 50 μM GSH, there was a slight decrease in intensity which was more pronounced at 100 μM GSH concentration suggesting loss in the β -sheet content of the protein. Figure 4B depicted the $\alpha 2M$ -glutathione interaction curve in the presence of visible fluorescent light. At 50 μM of GSH, a band observed at 1635 cm^{-1} confirmed the decrease in the β -sheet content of $\alpha 2M$ as in the previous case. At

100 μM GSH, there was a shift of peak and new band was observed at 1640 cm^{-1} which suggests subtle perturbation in the secondary structure of the protein upon its exposure to photo-illuminated GSH, not observed in dark.

ITC Results

To understand the $\alpha 2M$ -glutathione interaction, its thermodynamics parameters were determined by ITC. ITC was used to determine the change in entropy (ΔS), enthalpy (ΔH), stoichiometry of binding, and Gibbs free energy (ΔG). Figure 5 showed the titration profile of $\alpha 2M$ -glutathione binding which suggests the binding was exothermic in nature. The data obtained through ITC showed the change in enthalpy and entropy and was found

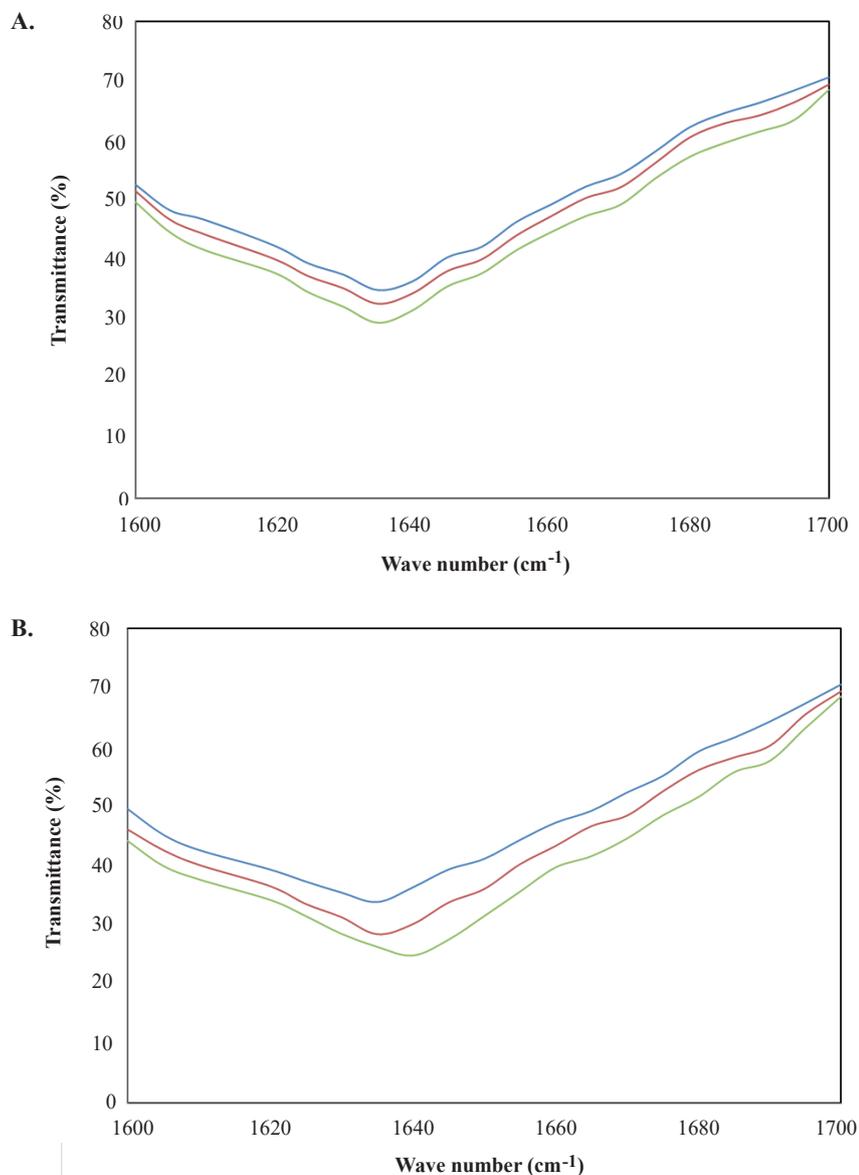


Figure 4. The percentage of transmittance of FT-IR Spectroscopy. A: FT-IR spectra of native α 2M and GSH treated in dark; B: FT-IR spectra of native α 2M and GSH treated in the presence of white light.

to be negative and was shown in Table 1. VPC-ITC Micro Cal was used to determine the binding energetic such as entropy (S), enthalpy (H), Gibbs free energy change (G), stoichiometry of binding sites (N) and binding affinity (Kb) of α 2M-GSH interaction. The negative values of ΔH (-1.594×10^4 joulemol⁻¹) and ΔS (-5.34×10^4 joulemol⁻¹) indicated that the interaction process was exothermic and possibly involved hydrogen bonds. Moreover, the negative value of ΔG (-2.68 kjoulemol⁻¹) signified the reaction to be spontaneous and thermodynamically favourable. The stoichiometry of binding/number of binding sites (N) was found to be around 1.38. The change in enthalpy and entropy for protein-ligand interaction may reveal the binding forces involved in the interaction. If the value of ΔS and ΔH are negative, then Van der Walls and hydrogen bond formation

were involved in the interaction and if both were positive, then it suggested hydrophobic interaction. The negative value for ΔS and ΔH observed in α 2M-GSH interaction suggests it was enthalpy driven and involves hydrogen bonding and Van der Walls forces.

Discussion

This is the first study to explore the interaction of GSH with α 2M, a plasma antiproteinase and major zinc binding protein. The primary aim of this study is to explore the possible interaction between GSH and α 2M. Fluorescence transform infra-red spectroscopy technique was employed to study the secondary structural changes in the native form of protein.^{18,19} FT-IR spectroscopy was utilized to study the

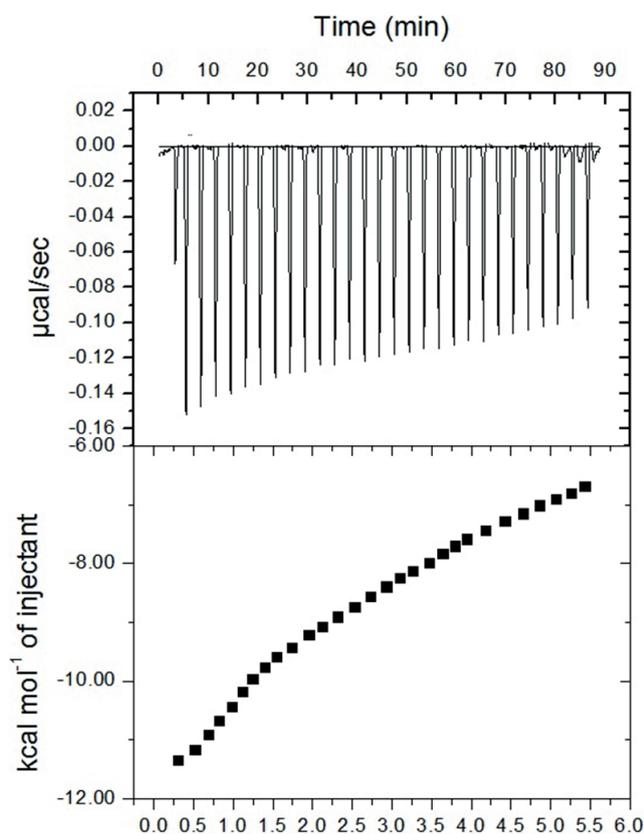


Figure 5. Isothermal titration calorimetry profile of $\alpha 2M$ -glutathione. Titration of $\alpha 2M$ (2 μM) with GSH (20 μM) at pH 7.5 shows calorimetric response as consecutive injection of ligand is added to the sample cell.

structural changes in the protein after its interaction with GSH in dark and light. The FT-IR spectra of native $\alpha 2M$ confirms the presence of predominant β -sheet structure in $\alpha 2M$. At 50 μM GSH, there was a slight decrease in intensity which was more pronounced at 100 μM GSH concentration suggesting loss in the β -sheet content of the protein. At 50 μM GSH, a band observed at 1635 cm^{-1} confirms the decrease in the β -sheet content of $\alpha 2M$ as in the previous case. At 100 μM GSH, there is a shift of peak and new band was observed at 1640 cm^{-1} which suggests subtle perturbations in the secondary structure of the protein due to photo-illumination of GSH, which was not observed in dark. Previous studies have shown that the changes in enthalpy and entropy for protein-ligand interaction may reveal the binding forces involved in the interaction.²⁰⁻²² If the value of ΔS and ΔH are negative, then Van der Waals and hydrogen bond formation are involved in the interaction and if both are found to be positive, it suggests hydrophobic

interaction.²² The negative values for ΔS and ΔH observed in $\alpha 2M$ -GSH interaction suggests it was enthalpy driven and involves hydrogen bonding and Van der Waals forces.^{22,23}

GSH, a principle thiol-containing tripeptide, is an intracellular antioxidant which plays a major role as an antioxidant in cellular defence against free radicals and other harmful oxidants. It is a ubiquitous molecule and provides defence against free radicals, hydroperoxides and other harmful oxidants. However, its production declines with age, and is often given orally or administered intravenously²⁴⁻²⁶, which causes its increased plasma concentration. Our studies show that incubation of $\alpha 2M$ with GSH (20-100 μM) results in loss of functional status of this antiproteinase. This loss in activity is more pronounced when GSH (20-100 μM) is photoilluminated, resulting in secondary structural changes in the protein. This change in conformation of protein might occur due to either perturbation or disturbance induced in the protein by GSH binding or oxidation of some essential amino acids.²⁷ This study assumes importance as long term administration of GSH leads to zinc deficiency since $\alpha 2M$ is a major zinc binding protein in the plasma.^{16,27}

Conclusion

Our studies show that incubation of $\alpha 2M$ with GSH results in loss of functional status of the antiproteinase and is more pronounced when GSH is photoilluminated resulting in secondary structural changes in the protein. This change in conformation of $\alpha 2M$ may be due to either perturbation or damage induced in the protein by GSH binding or oxidation of some essential amino acids.

Table 1. Binding and thermodynamic parameters for the interaction of bilirubin with $\alpha 2M$ obtained by ITC experiment at 37 °C.

Thermodynamic Parameters	Values
N	1.38
$K_b (M^{-1})$	1.42×10^4
$\Delta H (joulemol^{-1})$	-1.594×10^7
$T\Delta S (joulemol^{-1})$	-5.34×10^4
$\Delta G (kjoulemol^{-1})$	-2.68

Note: N is the stoichiometry of binding, K_b is the binding constant, ΔH is the change in enthalpy, ΔS is the change in entropy, and ΔG is the change in Gibbs free energy.

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