AMERICAN JOURNAL OF PHARMTECH RESEARCH

Journal home page: http://www.ajptr.com/

HEMIN DEGRADATION IN PRESENCE OF THIOLS AND TRANSITION METAL IONS.

Ganji Venu[1]*, P.S.Prasanthi[2], Ch.Venkata Ramana Devi[1], N.Lakshmaiah[2].

1. Department of Biochemistry, University College of Science, Osmania University, Hyderabad, India - 500007.
2. National Institute of Nutrition, Hyderabad, India - 500007.

ABSTRACT

Hemin degradation by various thiols including cysteine, homocysteine, N-acetylcysteine, glutamyl-cysteinyl-alanine, mercaptoethanol and dithiothreitol, and also the effect of transition metal ions like cobalt(II), copper(II), manganese(II), zinc(II), chromium(III), iron(II) and iron(III) on this degradation have been investigated. Glutathione (GSH) and other thiols have successfully degraded hemin. Metals like Cobalt(II), Copper(II), Chromium(III) and iron(II) inhibited GSH mediated hemin degradation. However this inhibition was marginally reversed by the addition of metal chelators and diethylenetriaminepentaacetic acid (DTPA). Manganese(II) and zinc(II) were increased the degradation but the DTPA abolished this increase. Iron(III) had no effect on GSH mediated degradation but co-incubation with DTPA resulted a remarkable inhibition. The effect of cobalt(II), manganese(II), Fe(II) and Fe(III) on hemin degradation by homocysteine, dithiothreitol and glutamyl-cysteinyl-alanine showed differences vis-à-vis GSH. In conclusion the results indicate that thiols other than GSH should also be explored for their protective effect on hemin-mediated membrane destabilization by virtue of their hemin degrading ability.

Key Words : Hemin, glutathione, thiols, transition metal ions, RBC membranes.

*Corresponding Author Email: ganjivenu@yahoo.com

Received 26 October 2011, Accepted 9 November 2011

Please cite this article in press as: Venu G *et al.*, Hemin Degradation In Presence Of Thiols And Transition Metal Ions. American Journal of PharmTech Research 2011.

INTRODUCTION

Heme is the prosthetic group of a variety of proteins like hemoglobin, myoglobin, cytochromes, catalase, peroxidase, guanylate cyclase and CooA, which perform diverse physiological functions[1-4]. Hemoglobin contains around 80% of total heme in the body. Endogenous heme is not recycled but undergoes enzymatic degradation by microsomal enzyme heme oxygenase (HO) [5-7] which is present in reticuloendothelial system of liver, spleen and kidney but not in mature RBC and serum[8]. Heme oxygenase requires NADPH-cytochrome P-450 reductase (EC 1.6.2.4), molecular oxygen and NADPH for the degradation of heme[5]. Microsomal NADPH-cytochrome P-450 reductase (cytochrome c-reductase) reduces cytP-450 or molecular oxygen (electron acceptors) giving rise to reduced cytP-450 or superoxide [9,10]. Reduced cytP-450, in turn, can donate electrons to molecular oxygen. The superoxide thus formed dismutates to H_2O_2 which can degrade free heme as well as heme of NADPH cyt P-450 reductase. The degradation can be considered non-enzymatic since H_2O_2 produced in the reaction is ultimately responsible for hemin degradation. Nonenzymatic methods of degrading heme could be useful in addition to HO mediated degradation in pathological conditions.

Heme binds tightly to the hydrophobic region of globin chain, and mediates the normal physiological role of hemoglobin in oxygen transport. However, in hemoglobinopathies like sickle cell anemia and thalassemia or under oxidative stress, the heme gets dissociated from abnormal hemoglobin molecules and exerts various deleterious actions like LDL oxidation, accumulation in RBC membranes causing dissociation of membrane skeletal proteins, increased membrane permeability and eventually red cell hemolysis[11-18]. Normal hemoglobin molecules also release heme when oxidized to methemoglobin form but at a reduced rate. The normal red blood cells have the ability to counter the alterations in physiological processes, but sickle and thalassemic cells are unable to perform such actions. Under such conditions, nonenzymic degradation of heme such as GSH-mediated heme degradation could be useful to remove accumulated heme [19].

Glutathione (GSH) has been shown to bind hemin and protect against hemin mediated hemolysis[14, 20]. GSH and transition metal ions are known to generate superoxide which can dismutate nonenzymically to form H_2O_2 and give rise to highly reactive hydroxyl radical [21,22]. Though the heme degradation by GSH and oxygen has been thoroughly investigated[19], the ability of other thiol compounds to degrade heme and the effect of metal ions on thiol mediated hemin

degradation has not been investigated. Hence, in the present paper, we studied the effect of various thiol compounds (including GSH) along with transition metal ions on hemin degradation.

MATERIALS AND METHODS

Reagents: Hemin, GSH, dithiothreitol (DTT), N-acetylcysteine (NAC), mercaptoethanol (ME), homocysteine (Hcy), cysteine (Cys), diethylenetriaminepentaacetic acid (DTPA), were obtained from Sigma chemicals (Sigma, St. Louis. USA). Glutamyl- cysteinyl-alanine (glu-cys-ala) was synthesized by custom peptide synthesis (Vimta Labs, Hyderabad, India). All other chemicals were of analytical grade (Ashwani chemicals, Hyderabad, India).

Hemin degradation by thiol compounds: Hemin degradation was analyzed by UV absorption at 365 nm[19]. Hemin was prepared fresh at the beginning of each experiment as 1 mM stock in 5 mM NaOH, and stored in dark at 4^0 C. DTPA (20 mM) was prepared as a stock solution in 0.2 M Hepes buffer at pH 7.0 for use as inhibitor of nonspecific oxidation of thiol compounds. Fresh solutions of thiol compounds (GSH, NAC, Cys, Hcy, glu.cys.ala, DTT and ME) were prepared as 0.1 M stock solutions in 0.2 M Hepes buffer at pH 7.0. To evaluate the effect of various thiol compounds, under investigation on hemin degradation, reaction mixtures were prepared with individual thiol compounds and DTPA. Each reaction mixture contained 1mM DTPA, 2mM of individual thiol compounds and 10µM hemin in a final volume of 1ml of 0.2 M Hepes buffer at pH 7.0, pre-warmed to 37^0C. The spectral changes between 300nm and 800nm were measured immediately after gentle mixing on a Hitachi 2000 Spectrophotometer and thereafter at 1 minute intervals till 5 minutes. Decrease in absorbance at 365 nm and a millimolar extinction coefficient of 64.1 were used for calculation of hemin degradation by all the thiols[19].

pH dependence of hemin degradation by thiol compounds: To evaluate the effect of pH on the reactivity of each thiol compound used in the present study, we measured hemin degradation by thiol compounds at different pH's as described in the above method. The reaction mixture contained 1mM DTPA, 2mM indicated individual thiol compound and 10µM hemin in a final volume of 1ml of 0.2M Hepes buffer, adjusted to the desired pH. The pH was determined at the beginning and at the end of the reactions to verify the efficacy of Hepes buffers in the pH range 6.0 - 8.0 and the pH was found unaltered during the reaction.

Degradation of erythrocyte membrane-associated hemin by thiol compounds: Membrane associated heme degradation by thiols was studied using erythrocyte membranes prepared by the method of Hanahan and Ekholm[23]. Erythrocyte membrane ghosts were washed with 10mM Tris-HCl buffer (pH 7.6), suspended in 10mM PBS (phosphate buffered saline) pH 7.4 and incubated

with 30µM hemin for 60 minutes at 37^0 C. The membrane ghosts were then washed in 10 mM Tris-HCl buffer (pH 7.6) and finally resuspended in 0.2 M Hepes buffer (pH 7.0) to attain concentration of 3.0 mg of membrane protein/mL (protein by Lowry's method) [24]. The hemin-loaded ghost suspension (2mL) was mixed with 1 mL Hepes buffer (pH 7.0) containing the thiol agents and incubated for 3 hours at 37^0 C. The membrane ghosts were dissolved by adding 10µL of 10% TritonX-100. The hemin concentration was determined by measuring the UV absorbance at 399nm and calculated using millimolar extinction coefficient of 83.5[19]. All the thiols tested, degraded hemin associated with RBC ghost membranes, showing similarity to GSH.

Effect of transition metal ions on thiol mediated hemin degradation: Stock solutions (0.1 M) of Co (II), Mn(II), Zn(II), Cu(II), Cr(III), Fe(III) and Fe(II) salts were prepared in milli-Q water. Reaction mixtures for hemin degradation were prepared by adding specific thiols (2mM), metal ions and hemin (10µM). The spectral changes between 300nm and 700nm were measured immediately and thereafter at 60 second intervals for 5 minutes. The percentage of hemin degradation was measured by calculating the decrease in absorbance at 365 nm and a millimolar extinction coefficient of 64.1 was used for calculations[19].

RESULTS AND DISCUSSION

Heme released from hemoglogin in hemoglobinopathies like beta thalassemia and sickle cell anaemia[25] gets incorporated into the membranes of abnormal RBCs and destabilizes the membrane structure causing hemolysis[11]. Red cells contain several antioxidants which protect against oxidative damage and hemolysis[14]. GSH has been reported to degrade hemin in vitro, indicating a possible role for GSH in the protection of red cell membranes against hemin mediated damage[14]. In this study we investigated the role of several thiols in the degradation of hemin and the effect of transition metal ions on the degradation.

Time course of hemin degradation by different thiols showed considerable differences in degradation rates (Figure 1). Hemin degradation also showed concentration dependence with respect to different thiols at fixed hemin concentration (10 µM, Table 1a) and with respect to hemin at fixed thiol concentration (2 mM, Table 1b). The pH optimization showed some differences but, qualitatively, all thiols showed good activity at pH 7.0 and slightly better activity was seen with NAC and glu-cys-ala at pH 8.0 (Figure 2). Low reactivity at pH 6.0 with NAC, glu-cys-ala and GSH and lesser activity at this pH with other thiols is not due to precipitation of hemin at this pH since reaction conditions preclude this possibility. The reactivity of a thiol group may depend on a number of factors such as the steric accessibility, hydrogen bond

formation and the specific microenvironment of a given SH-group. Presence of -SH group in protonated or in thiolate anion form may have bearing on the pH dependence of the degradation. Our study seems to be the first report with these compounds on hemin degradation.

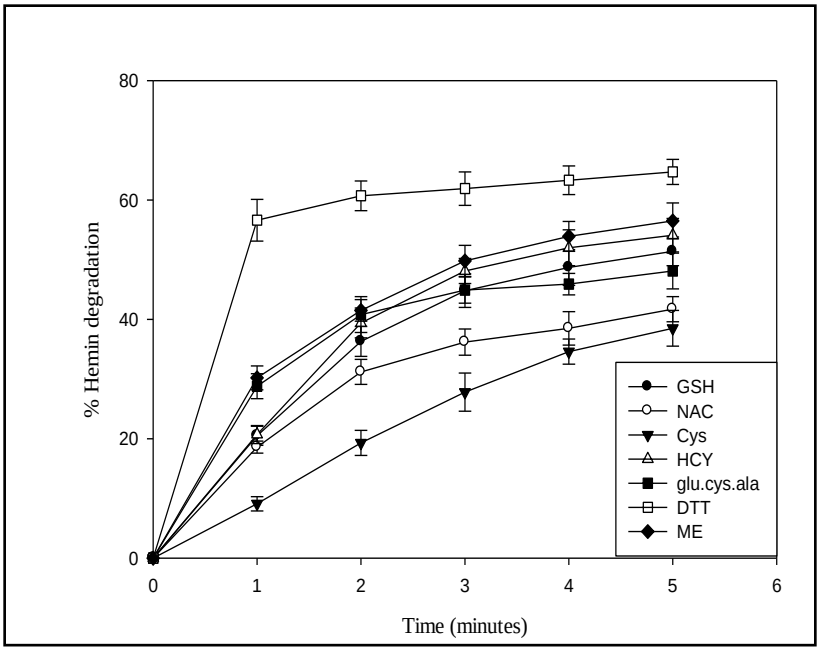

Figure 1: Time course of hemin degradation by different thiol compounds

Reaction mixture containing hemin (10µM), indicated thiol compounds (2mM) and 1mM DTPA in 0.2 M Hepes buffer (pH 7.0) was incubated at 37^0C. Immediately after mixing the samples, hemin degradation was determined by measuring the decrease in absorbance at 365 nm for 5 min in 1 min intervals. Data represent mean ± SD of 3 independent experiments.

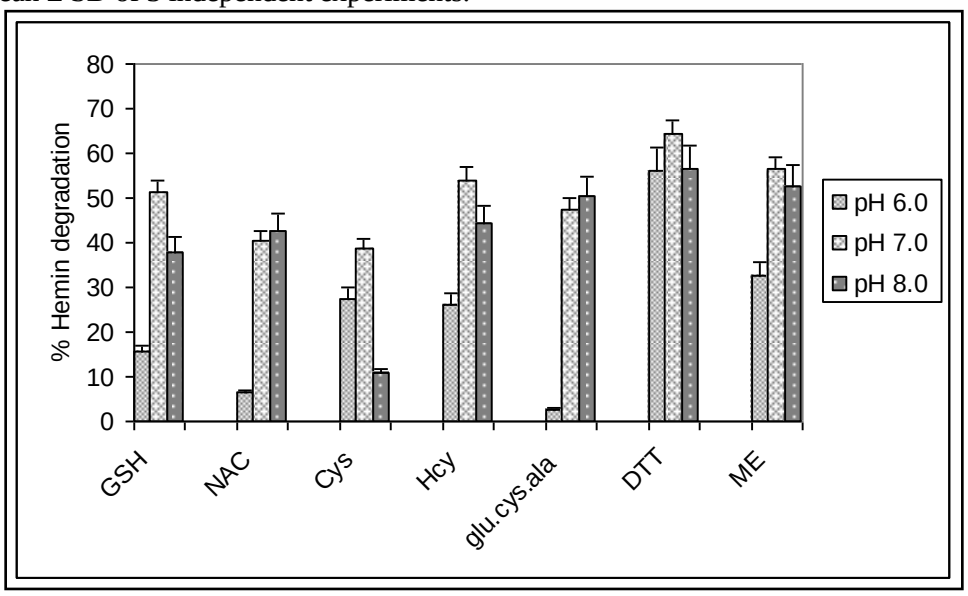

Figure 2: The pH dependence of hemin degradation

Hemin (10µM) was mixed with 2mM thiol (GSH, NAC, Cys, Hcy, glu-cys-ala, DTT and ME) and 1mM DTPA in 1ml of 0.2M Hepes buffer preset to the indicated pH at 370C and hemin degradation was monitored for 5 minutes at 365nm. Data represent mean ± SD of 5 separate experiments.

Table 1a: Dependence of hemin degradation on concentration of the thiols tested.

Parameter→	GSH	NAC	Cys	HCY	glu.cys.ala	DTT	ME
↓Thiol Concentration	% Hemin degradation↓						
0.5 mM	31.3±0.68	22.4±0.40	16.4±0.39	37.2±0.51	26.3 ±0.40	54.6±0.33	43.5±0.38
1 mM	42.2±0.65	34.2±0.51	27.3±0.41	48.8±0.64	38.9 ±0.51	62.4±0.51	52.1±0.42
2 mM	51.1±0.57	42.2±0.68	39.2±0.53	57.7±0.52	47.8 ±0.55	65.0±0.56	59.7±0.48
3 mM	55.4±0.48	46.8±0.65	43.5±0.51	62.5±0.68	52.1±0.58	68.3±0.61	63.7±0.47
4 mM	57.7±0.69	48.5±0.59	45.2±0.60	63.0±0.56	53.8 ±0.63	68.3±0.63	66.0±0.52

The extent of hemin degradation showed concentration dependence with respect to different thiols at fixed concentration of 10 µM hemin. Values are expressed as mean ± SD of five experiments.

Table 1b: Dependence of hemin degradation on the concentration of hemin.

Parameter→	GSH	NAC	Cys	HCY	glu.cys.ala	DTT	ME
↓ Hemin Concentration	µM hemin degraded ↓						
5 µM	2.83±0.02	2.50 ±0.08	2.10±0.05	3.10±0.03	2.80 ±0.03	3.58±0.05	3.29±0.06
10 µM	5.02±0.08	4.20±0.07	3.95±0.04	5.50±0.06	4.80±0.05	6.41±0.07	5.69±0.06
15 µM	6.10±0.08	5.36±0.05	4.90±0.05	7.09±0.08	5.51±0.06	7.71±0.06	7.38±0.07
20 µM	6.64±0.05	5.69 ±0.04	7.67±0.04	5.23±0.09	8.50±0.05	6.06±0.08	8.12±0.09

The extent of hemin degradation by different thiols showed concentration dependence on hemin. Thiol concentrations were 2 mM. Values are expressed as mean ± SD of five experiments.

The effect of metal ions on thiol mediated hemin degradation was not uniform and showed considerable variations (Figure 3, Figure 4). These thiols form chelate complexes with metal ions and reduce higher oxidation states to lower oxidation states such as ferric to ferrous state[26]. GSH mediated degradation was inhibited by Co (II), Cr(III), Cu(II) and Fe(II) but the inhibition was reversed to different extents in presence of DTPA (Figure 3). This could be due to prevention of nonspecific oxidation of GSH by chelating the metal ions. However DTPA markedly inhibited hemin degradation when co-incubated with Fe(III) even at 0.1 mM concentration at which Fe(III) alone had negligible effect on hemin degradation. Fe(III) may undergo reduction to Fe(II) form by GSH easily when chelated to DTPA. Fe(II)-DTPA thus formed could inhibit degradation of hemin (Figure 3) by acting on H_2O_2 produced by GSH and hemin. Such Fenton chemistry generates hydroxyl radicals in solution and depletes H_2O_2 needed for hemin degradation. In the present study, Mn^{++} showed stimulatory effect on GSH-mediated hemin degradation which may be due to H_2O_2 formation. Taher & Lakshmaiah reported folic acid degradation by hemin in presence of H_2O_2 and degradation of heme proceeded concurrently with folic acid degradation in this system[27]. GSH+Mn(II) did not support folic acid degradation by hemin which shows that either H_2O_2 is not formed or is used for other side reactions. However

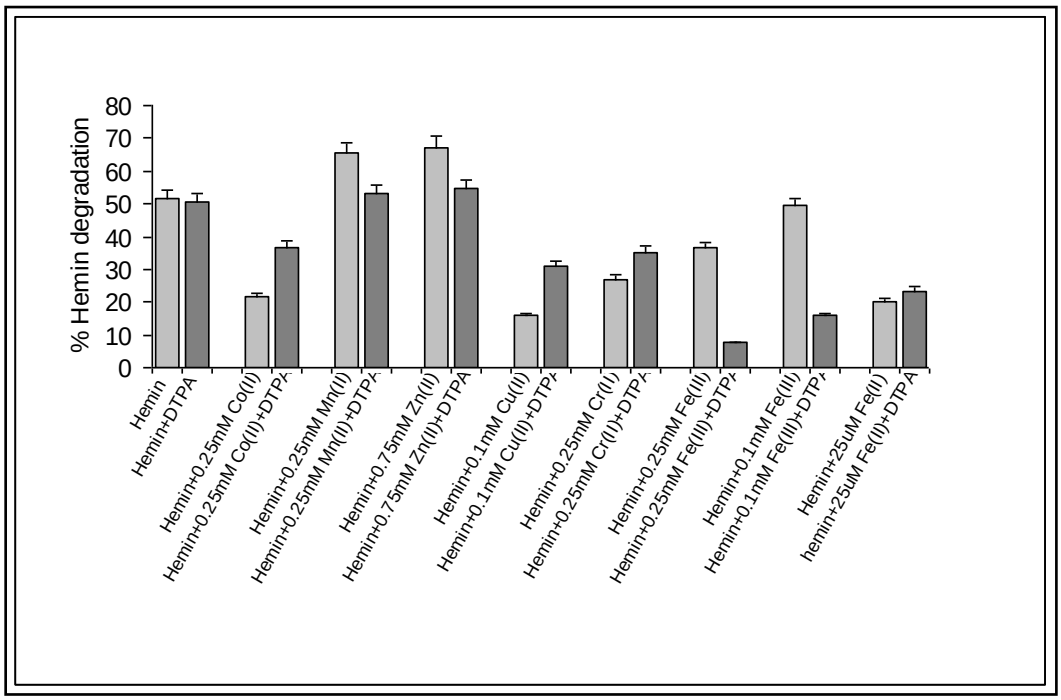

Figure 3: GSH-mediated hemin degradation in presence of transition metal ions

The reaction mixture contained hemin (10µM), 2mM GSH, 1mM DTPA and transition metal ions of indicated concentrations in a total volume of 1ml of 0.2M Hepes buffer, pH 7.0. Reactions were carried out at 37^0C for 5 minutes and hemin degradation was monitored as decrease in absorbance at 365nm. Data represent mean ± SD of 5 separate experiments.

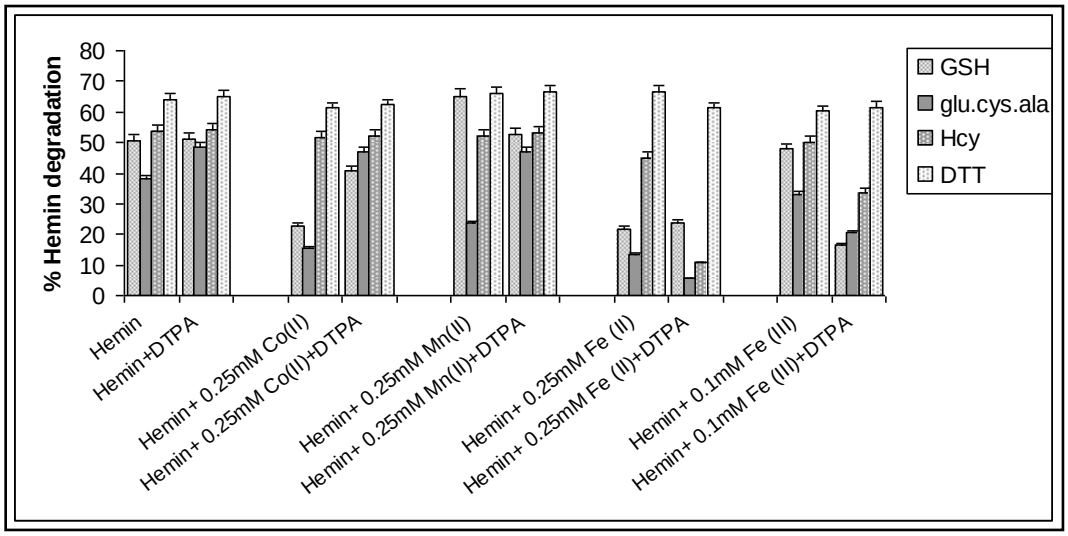

Figure 4: Effect of Co(II), Mn(II), Fe(II) and Fe(III) ions on hemin degradation by Hcy, glu-cys-ala and DTT

The reaction mixture contained Hemin (10µM), indicated thiols 2mM, 1mM DTPA and transition metal ions of indicated concentrations in a total volume of 1ml of 0.2M Hepes buffer, pH 7.0. Reactions were carried out at 37^0C for 5 minutes and hemin degradation was monitored as decrease in absorbance at 365nm. Data represent mean ± SD of 5 separate experiments.

lactoperoxidase, in presence of H_2O_2 or GSH+Mn(II), was also reported to degrade folic acid in vitro[28]. De Toledo et al. did demonstrate the formation of H_2O_2 by GSH+Mn(II) [29]. The effect of Zn(II) on hemin degradation was similar to the effect of Mn (II) which requires further study.

The effects of Co(II), Mn(II), Fe(II) and Fe(III) on heme degradation by Hcy, DTT and glu-cys-ala seem to depend on the nature of thiol agent (Figure 4). The opposite effect of Mn(II) on GSH and glu-cys-ala mediated degradation is noteworthy since both are cysteinyl tripeptides with cysteine in the middle. Thiol peptide-heme complexes are reported to be possible chemical models of P-450 enzymes[30, 31] showing aromatic hydroxylation activities. GSH and glu-cys-ala complexes of hemin used in this study could hydroxylate methylene bridges of heme randomly as a prelude to degradation. Hemin-thiolate complexes as chemical models of cyt P-450 are reported to catalyze strand scission of DNA also, the degradation being dependent on the structure of the thiol ligand (ME, DTT, NAC, Cys, GSH and others) and the ratio of thiol ligand to hemin concentration[32]. Generation of O^{2-} is essential for DNA strand scission by hemin-thiolate complexes and the mechanism has been suggested to be different from that of aromatic hydroxylation by these complexes. Degradation of hemin and DNA by hemin-thiolate complexes may have similarities.

However, the mechanism of heme degradation by the thiols tested remains to be a matter of speculation. Atamna[19] proposed a mechanism for GSH mediated degradation similar to the one suggested by Minotti and Aust for iron mediated lipid peroxidation[33]. The Fe^{3+} of hemin can be reduced by GSH to Fe^{2+} which can lead to the following sequence of reactions.

$$Fe^{2+} + O_2 \leftrightarrow (Fe^{2+}O_2 \leftrightarrow Fe^{3+} + O_2^{-}) \leftrightarrow Fe^{3+} + O_2^{-} \quad 1$$

$$O_2^{-} + H^{+} \leftrightarrow HO_2^{\cdot} \quad 2$$

$$HO_2. + O_2^{-} + H^{+} \rightarrow H_2O_2 + O_2 \quad 3$$

Formation of O^{2-} and its nonenzymatic dismutation can occur at physiological pH. Once O^{2-} and H_2O_2 are present in the system, the following reactions could take place.

$$Fe^{3+} + O_2^{-} \rightarrow Fe^{2+} + O_2 \quad 4$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\cdot} + OH^{-} \quad 5$$

Fe(II) can be oxidized to Fe(III) by H_2O_2 (reaction 5) or O_2 (oxidation as in reaction 1) and Fe(III) can be reduced to Fe(II) by O_2^{-} (reaction 4) or reduced thiols to maintain a suitable proportion of Fe^{2+} and $Fe3^{+}$ of perferryl radical (reaction 1) which is suggested to be responsible for hemin degradation.

In this study, several other thiols were found to catalyze hemin degradation. The common denominator in these compounds is the aliphatic –SH group. Alkyl, alkoxy or ester derivatives of –SH are unlikely to be active. Hence any mechanism proposed has to revolve around the participation of –SH group. Thiols could form several sulphur-centered radicals like RS·, RS·⁻SR and oxyradicals like RSOO· during the course of the reaction and all of them have the potential to attack randomly the methane bridges of hemin.

It has been reported that the RBC membrane-associated hemin is degraded by GSH leading to the release of iron from the membrane[19]. To know whether other thiols also degrade membrane-associated hemin, erythrocyte ghosts were loaded with hemin and incubated with thiols (as described in methods) to test their hemin degrading ability (Table 2). All the thiols tested, degraded hemin associated with RBC ghost membranes and showed similarity to GSH. Hemin incorporated into RBC ghost is expected to be in hydrophobic lipid environment. The thiols tested show time dependent degradation of membrane associated hemin. Such studies will help us understand the role of thiols in hemolytic reactions.

Table 2: Degradation of RBC ghosts-associated hemin by thiol compounds.

Parameter	GSH	NAC	Cys	HCY	glu.cys.ala	DTT	ME
↓Time (minutes)	Hemin (nmol/mg protein) ↓						
0	7.50±0.02	7.50±0.03	7.50±0.03	7.50±0.02	7.50±0.04	7.50±0.03	7.50±0.03
60	6.20±0.04	6.59±0.06	6.98±0.06	5.61±0.05	6.45±0.07	3.96±0.06	4.59±0.06
120	5.12±0.06	5.61±0.07	5.89±0.08	4.42±0.08	5.29±0.07	3.22±0.08	3.85±0.09
180	3.57±0.09	4.38±0.048	4.70±0.06	3.40±0.08	3.92±0.08	2.59±0.09	3.08±0.08

White erythrocyte ghosts (containing 3 mg membrane protein/mL) were loaded at 37^0C for 60 min with 30 µM hemin. After removal of non-associated hemin, the membranes were incubated with 5mM indicated thiols in Hepes buffer (pH 7.0) at 37^0C for 180 min. Hemin content was assayed by dissolving the white ghosts with 30µl of 10% TritonX100. Values are expressed as mean ± SD of five experiments.

CONCLUSION

In conclusion, these studies emphasize the role of several structurally different thiol compounds in the degradation of hemin. GSH mediated hemin degradation has been shown to be physiologically relevant defense mechanism against oxidative damage of free hemin. An understanding of hemin degradation by other thiols is expected to help explore the possibility of using them for therapeutic purposes.

ACKNOWLEDGEMENT

This research was supported by a grant (BMS: 52/05/2002) from the Indian Council of Medical Research (ICMR), New Delhi, India. We express our sincere thanks to Dr. Sesikeran, Director , NIN and Mr. T. Longvah, Scientsit F, NIN, Hyderabad for their support to carry out this work.

REFERENCES

1.Gunsalus IC, Sligar SG, Nordulund T, Frauenfelder H. Sensing heme proteins: monooxygenases, myoglobin and hemoglobin. Adv Exp Med Biol 1977; 78: 37-50.

2.Hasler JA, Estabrook R, Murray M, Pikuleva I et al. Human cytochromes P450. Mol Aspects Med 1999; 20: 1-137.

3.Isaac IS, Dawson JH. Heme iron-containing peroxidases Essays. Biochem 1999; 34: 51-69.

4.Lanzilotta WN, Schuller DJ, Thorstelnsson MV, Kerby RL, Roberts GP, Poulos TL. Structure of CO sensing transcription activator CooA. Nat Struct Biol 2000; 7: 876-880.

5.Ryter SW, Tyrrell RM. The heme synthesis and degradation pathways*:* Free Radic Biol Med 2000: 28: 289-309.

6.Shibahara S, KitamuroT, Takahashi K. Heme degradation and human disease: diversity is the soul of life. Antioxid Redox Signal 2002: 4: 593-602.

7.Tenhunen S, Marver HS, Schmid R. The enzymatic conversion of heme to bilirubin by microsomal heme oxygenese. Proc Natl Acad Sci USA 1968; 61: 748-755.

8.Tenhunen R, Marver HS, Schmid R. The enzymatic catabolism of hemoglobin: stimulation of microsomal heme oxygenase by hemin. J Lab Clin Med 1970; 75: 410-421.

9.Masters BS, Schacter BA. The catalysis of heme degradation by purified NADPH-cytochrome C reductase in the absence of other microsomal proteins. Ann Clin Res 1976; 17: 18-27.

10. Guengerich FP. Destruction of heme and heme proteins mediated by liver microsomal reduced nicotinamide adenine dinucleotide phosphate-cytochrome P-450 reductase. Biochemistry 1978; 17: 3633-3639.

11. Hebbel RP, Eaton JW. Pathophysiology of heme interaction with the erythrocyte membrane. Semin Hematol 1989; 26: 136-149.

12. Rachmilewitz EA. Denaturation of the normal and abnormal hemoglobin molecule. Semin Hematol 1974; 11: 441-462.

13. Kirschner-Zilber I, Rabizadeh E, Shakalai N. The interaction of heme bilirubin with the human red cell membrane. Biochem Biophys Acta 1982; 690: 20-30.

14. Shivro,Y, Shaklai N. Glutathione as a scavenger of free hemin. Biochem Pharmacol 1987; 36: 3801-3807.

15. Liu SC, Zhai S, Lawler J, Palek J. Hemin mediated dissociation of erythrocyte membrane skeletal proteins. J.Biol.Chem 1985; 260: 12234-9.

16. Shakali N, Avissar N, Rabizadeh E, Shaklai M. Disintegration of red cell membrane cytoskeleton by hemin. Biochem Int 1986; 13: 467-77.
17. Fitch CD, Chevli R, Kanjananggulpan P, Dutta P, Chevli K, Chou AC. Intracellular ferriprotoporphyrin IX is lytic agent. Blood 1983; 62: 165-8.
18. Chou AC Fitch CD. Hemolysis of mouse erythrocytes by ferriprotoporphyrin IX and chloroquine chemotherapeutic implications. J Clin Invest 1980; 66: 856-58.
19. Atamna H, Ginsburg H. Heme degradation in the presence of glutathione. J Biol chem 1995; 270: 24876-83.
20. Shu-De LI, Yan-Dan SU, et.al. Hemin-mediated hemolysis in erythrocytes: Acta Biochimica et Biophysica Sinica 2006; 38 (1): 63-69.
21. Misra HP. Generation of superoxide free radical during the autoxidation of thiols. J Biol Chem 1974; 249: 2151-5.
22. Halliwell B, Gutteridge JMC. Role of free radicals and catalytic metal ions in human disease: an overview. Meth Enzymol 1990; 186: 1-85.
23. Hanahan DJ, Ekholam JE. The preparation of red cell ghosts (membranes). Meth Enzymol 1974; 31: 168-72.
24. Lowry OH, Resebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagents. J boil chem 1951; 193: 365-75.
25. Shaklai N, Shviro Y,Rabizadeh E,Kirschner-Zibler I. Accumulation and drainage of hemin in the red cell membrane. Biochim Biophys Acta 1985; 821: 355-366.
26. Hamed MY, Silver J, Wilson MT. Studies of the reactions Ferric Iron with Glutathione and some related Thiols. Inor Chimica Acta 1991; 78: 1-11.
27. Taher MM, Lakshmaiah N. Studies on hydroperoxide-dependent folic acid degradation by hemin. Arch Biochem Biophys 1987; 257: 100-106.
28. Taher MM, Lakshmaiah N. Folic acid stability in hydrogen peroxide-potassium thiocyanate-treated milk. Food Chemistry 1992; 44(5): 343-347.
29. De Toledo SM, Haun M, Bechara EJ, Duran N. Peroxidase and hydrogen peroxide detection by a bioenergized method. Anal Biochem 1980; 105 (1): 36 – 8.
30. Hiromu Sakurai, Shigeru Shimomura, Kazuhiko Ishizu. Glutathione-Hemin complex as a cytochrome P-450 model characterization of the complex and its aromatic oxidation activities. Biochem Biophys Res Commun 1981; 101: 1102-1108.

31. Hiromu Sakurai, Hetsuji Hatayama,Tetsuhiko Yoshimura *et. al.* Thiol-containing peptide- hemin complexes as models of cytochrome P-450. Biochem Biophys Res Commun 1983; 115: 590-597.
32. Hiromu Sakurai, Masayuki Shibuya, Chiharu Shimizu *et.al*. DNA strand scission by hemin- thiolate complexes as models of cyt P-450. Biochem Biophys Res Commun 1986; 136: 645-650.
33. Minotti G, Aust SD. Redox cycling of iron and lipid peroxidation. Lipids 1992; 27(3): 219-226.