1. Introduction

Homocysteine (Hcy) is a sulfur containing non-protein amino acid synthesized from methionine. Elevated level of Hcy is associated with cardiovascular complications and neurodegeneration. Hcy is believed to induce organ damage and apoptosis via oxidative stress. The pro-oxidant nature of Hcy is considered to originate from the metal-induced oxidation of thiol group-containing molecules forming disulfides (Hcy-Hcy, Hcy-cysteine, Hcy-glutathione, etc) or with free cysteine residues of proteins (a process called protein S-homocysteinylation). Formation of such disulfides indeed results in the release of ROS which eventually leads to loss of cellular integrity. In the present manuscript, we performed systematic investigation of the effect of Hcy on iron containing proteins. We discover a novel mechanism of Hcy toxicity wherein Hcy oxidation is linked with the functional loss of the protein with iron as cofactors. Our results indicate that redox regulated heme proteins might be primarily involved in the Hcy toxicity and associated oxidative stress.

Homocysteine (Hcy) is a sulfur containing non-protein toxic amino acid synthesized from methionine. Elevated levels of Hcy range between 5 and 15 μM in healthy individuals. Under high methionine levels, trans-sulfuration pathway plays an important role in converting Hcy to cystathionine with the help of cystathionine β-synthase (CBS). On the other hand, when cellular methionine level is low, Hcy is methylated back to methionine via remethylation pathway with the help of enzyme, methionine synthase (MS) [1]. However, mutational defects in enzymes involved in Hcy metabolism can cause elevated serum Hcy levels ranging from 15 to 20 μM (mild forms) up to 500 μM (severe forms) [2–4]. In addition to the genetic defects, various other factors including prolonged use of anti-inflammatory drugs, smoking, alcoholism, low vitamin B6 and folate intakes also result in elevated Hcy levels in blood. Such condition of elevated serum Hcy levels is known as hyperhomocysteinemia and is associated with various neurodegenerative and cardiovascular complications [5–9].

Oxidative stress is considered to be the major cause of Hcy-induced toxicity [10–17]. Since Hcy has a reactive sulphydryl group, it works as an important pro-oxidant because of formation of disulfides of various nature including, Hcy-Hcy, Hcy-Cys, Hcy-GSH in presence of metals. Formation of such disulfides (in presence of metals) results in the release of reactive oxygen species (ROS) which leads to further downstream reactions with other cellular components [11], and eventually disruption of cellular functions. The pro-oxidant nature of Hcy is not confined to these small pool of disulfides, but Hcy is also known to form stable disulphide linkage with free -SH group of cysteine residues of proteins (a process called “S-homocysteinylation”) [18] rendering proteins and enzymes with impaired functions. Hcy has also been demonstrated to alter cellular signalling by affecting nitric oxide synthase (NOS)/Akt pathway [19] leading to increase oxidative stress and eventually endothelial dysfunction. Despite these progresses, the potential damaging effect of Hcy on protein structure and function has not been completely unveiled. In the present study, we have investigated the effect of Hcy on several protein and enzymes. Our major discovery is that redox regulated heme proteins are susceptible to the Hcy-induced functional loss and could be a novel triggering factor for the generation of Hcy-induced oxidative stress.
2. Methods

**Materials**- Horseradish peroxidase (HRP, from horseradish), Hemoglobin (Hb, from bovine blood), Myoglobin (Myo, from Bovine skeletal muscles), Cytochrome c (Cyt c, from bovine heart), Lysozyme (Lyz, from chicken egg white), Ribonuclease-A (RNase-A, from bovine pancreas) and other chemicals were purchased from Sigma-Aldrich chemical Co. L-homocysteine (Hcy), H₂O₂, 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 5, 5’-Dithiobis (2-nitrobenzoic acid) (DTNB), guaiacol, cytidine 2’-3’ cyclic monophosphate (C > p), M. luteus cells were also obtained from Sigma Chemical Co.

**Preparation of protein stock solutions and determination of concentrations**- Protein solutions were dialyzed extensively against 0.1 M KCl solution at 4 °C and filtered using Millipore syringe filter (0.22 μm). Concentrations of protein solutions were determined using ε, molar extinction coefficient values of 39,000 M⁻¹ cm⁻¹ at 280 nm for Lyz [20], 9800 M⁻¹ cm⁻¹ at 277.5 nm for RNase-A [21], 1,05,000 M⁻¹ cm⁻¹ at 405 nm for HRP [22], 1,09,000 M⁻¹ cm⁻¹ at 409 nm for Cyt c [23], 1,79,000 M⁻¹ cm⁻¹ at 405 nm for Hb [24] and 1,16,000 at 408 nm for Myo [25]. All solutions for optical measurements were prepared in degassed 0.05 M potassium phosphate buffer (pH 7.4) containing 0.1 M KCl.

**Protein modification**- For protein modification with Hcy, proteins were incubated in presence of varying concentrations of Hcy (0–5 mM) in 0.05 M potassium phosphate buffer, pH 7.4 at 37 °C. These Hcy-treated/untreated protein samples were further used for subsequent analyses.

**Enzyme activity measurements**- RNase-A activity was measured using cytidine 2’-3’ cyclic monophosphate (C > p) as a substrate. The concentrations of enzyme and substrate were 0.035 mg ml⁻¹ and 0.4 mg ml⁻¹, respectively and the progress of enzyme catalysed reaction was followed at 292 nm. For measuring Lyz activity, M. luteus cells were used as substrate. Concentrations of enzyme and substrate were 0.015 mg ml⁻¹ and 0.14 mg ml⁻¹, respectively, and the progress of reaction was followed at 450 nm. HRP activity was assayed with ABTS by measuring increase in absorption at 420 nm [26]. Final concentrations of reaction mixtures were 1 μM HRP, 50 μM ABTS, and 300 μM H₂O₂. Cyt c, Hb and Myo peroxidase activities were assayed with guaiacol (1 mM) by measuring absorption at 470 nm for tetraguaiacol formed as product [27]. Final concentrations of reaction mixtures were 1 μM Cyt c, 0.16 μM Hb, 1 μM Myo and 2 mM H₂O₂. Reactions were followed for 1 hr in case of Lyz and RNase-A, 10 min in HRP and 20 min in Cyt c, Hb and Myo. Results for RNase-A, Lyz and HRP were expressed in percent, considering the activity of unmodified protein as 100 %.

**CD Measurements**- CD measurements were made in a Jasco J-810 spectropolarimeter equipped with a Peltier-type temperature controller with at least three accumulations. Protein concentration used for CD measurements were 15–20 μM. Cells of 0.1 and 1.0 cm path lengths were used for the measurements of far- and near-UV CD spectra, respectively. Necessary blanks were subtracted for each measurement.

**Fluorescence Measurements**- Fluorescence spectra were measured in a Perkin Elmer LS 55 Spectrofluorimeter in a 3 mm quartz cell, with excitation and emission slits set at 10 nm. Protein concentrations used were 3 μM. For Trp fluorescence measurements, Lyz, HRP, Hb, Myo and Cyt c were excited at 295 nm, while the emission spectra were recorded from 310 to 450 nm. Necessary blanks were subtracted for each measurement.

**Free sulfhydryl (SH) group estimation using Ellman’s reagent**- SH group estimation was carried out as described by Ellman [28] with some minor modifications. The levels of thiol groups in free Hcy and in presence of proteins were assayed using DTNB. Absorbances were measured at 412 nm, using a 1 cm path-length cuvette and results were expressed as percent, considering the free–SH at zero times incubation as 100 %, Necessary blanks were subtracted for each sample.

**UV-Visible Spectrophotometry**- Absorption spectra of the unmodified/modified proteins were recorded with a Jasco V-660 spectrophotometer equipped with a Peltier-type temperature controller at 37 °C. The protein concentration used was 15 μM for soret band measurements. For absorption measurements for redox state studies, the protein concentration used was 50 μM. Cell of 1.0 cm path lengths were used for all measurements.

**Electron Paramagnetic Resonance (EPR) Measurements**- EPR measurements were carried out in a Bruker EMX MicroX spectrometer. The following conditions were used for the measurements: gain, 1 × 10³; modulation amplitude, 4.0 G; microwave power, 16 mW; conversion time, 20 ms; and time constant, 655 ms. Samples were loaded in sealed quartz capillary tubes and transferred to the EPR cavity to obtain spectra. Protein concentration used for EPR measurements were 150 μM.

**MALDI TOF Mass spectrometry**- Intact masses of native and Hcy-treated HRP samples were determined by an ESI-based MS. Samples were desalted using C18 ZipTip and eluted with 50 % acetonitrile in 0.1 % trifluoroacetic acid. The positive ion mass spectra were acquired on an Applied Biosystems, MDS Sciex (Model 4800 plus MALDI TOF/TOF Analyser), to obtain the final average mass of target protein. Spectra of the proteins were obtained in linear mode.

3. Results

**Hcy induces loss of HRP activity but does not affect non-heme proteins**- First of all, we measured the functional activity of three enzymes [Lyz, RNase-A and HRP], upon overnight incubation with Hcy (see Fig. 1). The concentration chosen was in the range 50 μM to 5 mM which includes pathophysiological (50 μM–500 μM) conditions. Overnight incubation with Hcy led to gradual decrease in functional activity of HRP in a concentration dependent manner. A ~40 % decrease was observed in HRP activity at 1 mM Hcy concentration. However, no significant effects were observed on Lyz and RNase-A upon Hcy treatment.

**Activity loss is unique to heme proteins**- To get a better understanding if heme proteins in general are targeted in a similar manner, we extended our study on few common cellular heme proteins, namely Cyt c, Hb and Myo. The choice of proteins was made keeping in mind that all

Fig. 1. Percent activity Lyz, RNase-A and HRP upon overnight treatment with varying Hcy concentrations.
these proteins have different cysteine contents. Cyt c has two cysteines, both covalently linked to heme centre, whereas Hb has six which are all free and Myo has no cysteine at all. All these proteins are known to exhibit residual peroxidase activity and therefore, we investigated if Hcy treatment could influence their intrinsic peroxidase activity (Fig. 2A).

We observed large decrease in the residual peroxidase activity of all three proteins. The heme environment was further analysed by measuring heme absorption at visible region (Fig. 2B). In Cyt c, there is a steep rise in the 409 nm absorption band with a peculiar red shift. On the other hand, Hb and Myo displayed a decrease in this absorption band suggesting alterations in microenvironment of the heme moieties. Furthermore, absorption spectral analysis of the heme protein also revealed alterations in the redox states (Fig. 2C). For instance, splitting of band at 530 nm into two distinct bands at 520 and 550 nm is a characteristic of reduced (Fe$^{2+}$) state of heme in Cyt c. In case of Hb, there is disappearance of bands at 500 nm and 630 nm and additional appearance of peaks at 540 nm and 575 nm which is also a signature of the reduced state of heme. Similarly, loss of peak at 500 nm and appearance at 550 nm is also a characteristic feature of the Fe$^{2+}$ state of heme. Thus, it is clear that all the heme proteins displayed functional loss and concomitant change in the redox state of heme.

We have also investigated the structural alterations (if any) in these proteins due to Hcy treatment. Fig. S1 shows the far- and near-UV CD spectra of Cyt c, Hb and Myo. Far-UV CD measurements suggest that the spectral behaviours of the Hcy treated proteins remain nearly identical to that of the native proteins at all concentrations of Hcy used in the study. Furthermore, deconvolution of the far-UV CD spectra confirmed that Hcy had no significant effect on different secondary structural components of the proteins (Fig. S2). However, near-UV CD measurements revealed different structural alteration in the three different proteins. In the case of Cyt c there is slight decrease in intensity at around 260 nm (Fig. S1) but no significant effect at 290 nm. Bands at 260 nm and 290 nm are known to originate from the asymmetry of Phe and Trp residues respectively. The results thus indicate that some of the Phe residues are getting exposed to solvent; however this exposition had not affected the micro-environment of Trp residues. In case of Hb, there is an overall increase in the intensities at 260 nm and 290 nms indicating that there is increase in tertiary contacts in terms of Phe and Trp residues. Hcy, on the other hand did not bring about any structural alterations in Myo. Similarly, alteration in the micro-environment of the chromophoric groups (in case of Cyt c and Hb) are also observed in the intrinsic Trp fluorescence spectra as evident from the hyperchromicity accompanied with red shift (Fig. S3) indicating large structural reshuffling in terms of the chromophoric micro-environment. Additionally, we also observed that, even in Myo, there is conformational change in terms of Trp micro-environment (Fig. S3). Since all proteins do not exhibit any significant distortions in the secondary structure, the observed changes in the near and Trp fluorescence spectral properties clearly indicate that Hcy induces structural alterations to the proteins that are not sufficient to affect the secondary structure.

**Activity loss in HRP is not associated with structural changes, but heme micro-environment**

To get a better understanding of the
mechanism for functional loss in HRP, we assessed structural properties of HRP upon overnight incubation with Hcy. Fig. 3 shows the near- and far-UV CD spectra upon overnight incubation with varying concentrations of Hcy. The 208 nm and 222 nm dichroic bands, characteristic of the α-helical structures were unperturbed suggesting no change in the secondary structural content upon treatment with Hcy (Fig. 3A). The percent secondary structural elements of the protein were also found to be unperturbed upon Hcy treatment (Fig. S4). In addition, based on near-UV CD measurement, there was no significant tertiary structural alteration in presence of Hcy as revealed from no significant alteration in the spectral properties (see Fig. 3B). We have further investigated alterations in the heme and Trp environment of HRP using Trp fluorescence and UV–Vis absorption spectral measurements (Fig. 3C and D). A large decrease in the absorption spectra at 401 nm region was observed with a peculiar red shift suggesting alterations in the heme environment. Concomitantly, increase in 280 nm absorption band along with enhanced Trp fluorescence emission, confirms alterations in heme micro-environment. These results provide hint for the loss of heme-Trp distance in HRP upon Hcy treatment.

Determination of Hcy oxidation in presence of HRP and Hemin-
Hcy is highly pro-oxidising in nature and can undergo oxidation at physiological conditions to form Hcy-Hcy dimer (homocystine) with the generation of free radicals (see Scheme 1). Oxidation of Hcy was

Fig. 3. Effects of Hcy on the conformational and heme status of HRP: Far-UV CD (A) near-UV CD (B), Trp fluorescence (C) and UV–Visible absorption (D) spectra of native abd modified HRP. In panel D, the 280 nm absorption band depicts alterations in the Trp environment as a consequence of alterations in the heme environment.

Scheme 1. Representation of Hcy oxidation in physiological conditions with generation of superoxide.

Fig. 4. Free -SH content of Hcy in absence and presence of HRP, Lyz, RNase A and Hemin: Plots of percent free -SH as a function of incubation period. The concentration of Hcy used was 1 mM.
assessed by measuring the amount of free –SH available using Ellman’s assay. Reduced Hcy has a free –SH group which would be blocked upon oxidation and therefore, decrease in magnitude of free –SH is an estimate of total homocysteine (dimer) formation. The amount of free –SH at zero time was taken as 100 %. Fig. 4A shows the percent free –SH in presence and absence of HRP at different time periods. Upon overnight incubation, the free –SH of the control samples (in absence of HRP) was found to be decreased to ~80 %, which further decreased to ~40 % after two days, suggesting auto-oxidation of Hcy. However, a drastic decrease in free –SH contents is observed in presence of HRP under the same experimental conditions as compared to that of Hcy control. The results indicate that HRP induces further oxidation of Hcy. The oxidation behaviour of Hcy were also analysed in the presence of other proteins Lyz and RNase-A (Fig. 4B and C). In contrast to HRP, presence of Lyz or RNase-A did not result in enhancement of Hcy oxidation. Taken together, the results indicate that auto-oxidation of Hcy is exclusively influenced by presence of metalloproteins (with heme moiety).

To further evaluate for the direct involvement of heme in the Hcy oxidation process, we have performed similar experiments with hemin. Fig. S5 shows the free –SH content in Hcy in absence and presence of hemin as a function of incubation periods. Similar to our observations on HRP, a reduction in free –SH is seen in the presence of hemin.

Oxidation of cysteine and glutathione by HRP- Since, HRP could also induce disulfide formation not only to Hcy but also to other sulfur containing molecules, glutathione and cysteine, we have further investigated if HRP also induces disulfide formation of both cysteine and glutathione (Fig. S6). It is seen that HRP induces oxidation of cysteine or glutathione but the magnitude of disulfides forms are very low as compared to Hcy-Hcy.

Hcy does not bind to HRP- To investigate the possibility of Hcy
binding to HRP or disulfide exchange occurring in case of HRP, we have performed MS analysis of the untreated and Hcy-treated HRP samples. Fig. 5 shows the MS spectra of untreated HRP (A) and HRP treated with 1 mM Hcy (B). There was no variation in the intact mass of the Hcy treated HRP as compared to that of the native protein. Our MS study thus confirms that incubation of HRP with Hcy does not lead to any Hcy binding to Cys residues or any possible disulfide exchange in the protein.

**Redox status of HRP upon treatment with Hcy** - To check if the presence of Hcy induces any alteration in the status of heme in HRP, we assessed redox status by measuring visible absorption spectra. Fig. 6A shows the visible absorption spectra of Hcy treated and untreated HRP. As expected, the redox state of heme was found to be perturbed in presence of Hcy. The spectrum of Hcy-modified HRP is in consistent with that of inactive HRP species (compound I) with loss of 500 nm absorption band a concomitant appearance of 660 nm [29]. We further carried out EPR measurement for the Hcy-modified HRP, to confirm the perturbation in the redox state (see Fig. 6B). Oxidized form of HRP is characterised by three distinct bands (g = 6.44, 5.05, 1.96) in the EPR spectrum. We observed complete silence of bands at g = 6.44 and 5.05 but a slight band at 1.96 region. These characteristics are the signature compound I [30]. Thus, it is confirmed that presence of Hcy in solution also affects the heme centre of a protein without direct interaction with the protein.

Similar experiments were also performed with hemin. Hemin, an iron-containing porphyrin, which exists in ferric state (Fe³⁺), is rendered reduced to ferrous form (Fe²⁺) in presence of Hcy. Oxidized hemin displays two major absorption bands regioned around 550 nm and 650 nm in the visible range (see Fig. 6C). These absorption bands were significantly diminished upon Hcy treatment suggesting reduction of the heme centre. Furthermore, free hemin is characterized by a broad absorption band at around 385–390 nm region, which upon treatment with varying concentrations with Hcy, is significantly lost (see Fig. 6D), suggesting heme destruction [31].

**Redox status of Hemin upon treatment with cysteine and glutathione** - To check if the presence of Cys and GSH induces any alteration in the status of heme in Hemin, we assessed redox status by measuring visible absorption spectra. The absorption band at around 650 nm was slightly diminished upon Cys and GSH treatment suggesting reduction of the heme centre in Hemin (Fig. S7A). Furthermore, the characteristic absorption band at around 385–390 nm region was slightly decreased, suggesting destruction of heme moiety upon treatment with Cys and GSH (Fig. S7B).

**Quenching of oxidative stress does not restore the enzyme activity:** Since Hcy oxidation results in generation of O₂⁻ that can affect some of the functionally important or critical Cys residue(s) that eventually leads to the functional suppression of HRP. If this is the case, quenching of oxidative stress should result in retrieval of enzyme function. We assessed the enzyme activity of oxidized HRP after adding varying concentrations of ascorbic acid. There was no gain in enzyme function even after addition of ascorbic acid (see Fig. S8). Thus, our results confirm that quenching of oxidative stress could not restore the activity of oxidized HRP.

4. Discussion

Our results on the activity measurements revealed that heme-containing proteins could be the major target of Hcy. To the best of our knowledge, there has been no systematic *in vitro* report on Hcy-induced protein functional alteration. Among non-heme proteins *in vivo*, two proteins, namely serum albumin and transthyretin, have been reported to be modified by Hcy [32,33]. However, both proteins are believed to be the natural reservoir of Hcy. Although the biological importance and functional status of natural reservoirs is not known, large fraction of Hcy exists in bound form to albumin [16] or transthyretin [33]. At least few proteins have been identified to undergo functional loss upon modification by Hcy under *in vitro* conditions, including LDL, fibronectin, annexin2 and plasminogen [34–37]. Our observed effect on HRP is first of its kind among the functional inactivation of heme-containing proteins. Even at pathophysiological concentration of Hcy (500 μM), there is around 25 % inhibition of HRP activity. Interestingly, many variants of HRP were found to be inhibited by specific substrates. For instance, HRP isozyme A2, Arthromyces ramosus peroxidase, and Phanerochaete chrysosporium lignin peroxidase were inhibited by H2O2 [38]. Irreversible functional inhibition of HRP in presence of the substrate m-Chloroperoxybenzoic has also been reported [39]. Furthermore, we also observed significant decrease in residual peroxidase activity and consequent alteration in redox status in other heme proteins (Cyr c, Hb and Myo). Loss of activity was also accompanied by change in heme micro-environment (Fig. S3). These results led us to believe that all heme proteins could be functionally altered by Hcy.

We were further interested in examining the mechanism of inactivation of HRP by Hcy. It may be possible that Hcy induces certain conformational change in native HRP due to specific or non-specific interactions. First of all, we have assessed the structural alterations in HRP due to Hcy treatment using multiple probes. Conformational analyses by far- and near-UV CD spectral measurements revealed that the secondary structural components as well as the gross tertiary structure remain unperturbed (see Fig. 3A–B and Fig. S4). However, the observed hyperchromicity in the absorption at 280 nm indicates alterations specifically in Trp micro-environment (Fig. 3C) which remain undetectable by the CD measurements (most probable due to low sensitivity). In
native HRP, heme maintains a particular distance with Trp for its functional integrity, the alteration in Trp micro-environment might have affected heme-Trp distance. It is evident in Fig. 3D that native HRP exhibits no net fluorescence as is quenched by the heme moiety. However, the distance has been largely affected by the presence of Hcy (as evident from the observed hyperchromicity and red shift). Consequently, we also observed exposure of heme moiety (Fig. 3C) to the solvent. Thus, we conclude that Hcy induces subtle change in the tertiary structure but that has largely affected the environment of the local chromophoric groups and in particular, heme micro-environment thereby converting native HRP to a “conformationally inactive state” (CIS).

One important mechanism that brings about transition of native HRP to CIS by Hcy is due to covalent modification of unbridged cysteine residues, a process called protein S-homocysteinylination, or by interchanging already formed disulfide bridges (disulfide exchange). In another approach, it has also been shown that redox regulated enzymes undergo sulfheme formation due to binding of homocysteine at the iron centre making the enzyme inactive. Such sulfheme formation mechanism has been specifically identified in catalase [40]. There, Scheme 1 summarizes structural alteration and inactivation of the enzyme might be due to incorporation of Hcy via S-homocysteinylination or sulfheme formation. If any of these mechanisms is involved in inactivation of HRP, the protein should either be covalently modified or exhibit an increase in molecular weight as compared to untreated HRP by Hcy incorporation. Mass spectrometric analyses (Fig. 5) revealed that there is no increase in the intact molecular mass of HRP with Hcy treatment. Thus, it is clear that protein S-homocysteinylation or sulfheme formation is not accountable for the observed transition of HRP to CIS.

It has also been reported that presence of transition metal enhances the oxidation of cysteine or homocysteine [11,41] thereby liberating a free radical in the process as shown in the relation below:

\[ 2\text{RSH} + \text{O}_2 \rightarrow \text{RSSR} + [\text{O}_2^•] \]

Our working hypothesis is that the released free radical [O₂] due to Hcy oxidation in turn acts as a nucleophile thereby affecting the oxidation state of iron in HRP. Iron (being a transition metal) in HRP is also capable of inducing Hcy oxidation. Thus, there is a loop wherein HRP induces Hcy oxidation which in turn inactivates HRP (see Scheme 1). If this is the case, we should observe formation of more Hcy-Hcy dimers in presence of HRP as compared to Hcy control (in absence of HRP) and HRP should render inactive. For this, we have first of all analysed formation of Hcy-Hcy dimers in presence and absence of HRP. It is seen in Fig. 4A that there is formation of more Hcy-Hcy disulfides (due to Hcy oxidation) as compared to the Hcy control (i.e., in absence of HRP). Such oxidation phenomenon of Hcy is not apparent in case of non-heme proteins, Lyz and RNase-A (Fig. 4B and C) indicating that further oxidation of Hcy is due to the presence of heme group with iron centre. We also performed additional experiment wherein Hcy oxidation is allowed in the presence of hemin-chloride and measured the magnitude of the Hcy-Hcy dimers formed. It is evident in Fig. S5 that hemin-chloride treatment also induces formation of more Hcy-Hcy dimers relative to the Hcy control suggesting that the iron centre is responsible for the observed consequences. To prove for the second possibility, we have further analysed HRP samples (co-incubated with Hcy) for any alteration in the oxidation state. Indeed, the spectrum of Hcy-modified HRP is consistent with that of HRP compound I (with half the intensity at ~409 nm, a disappearance of 500 nm absorption band with a concomitant appearance of the 660 nm absorption band), which is an inactive intermediate in the redox cycle of HRP protein [29] (Fig. 6A). Thus, loss of functional activity (and the resultant structural consequences) of HRP is due to conversion into its inactive compound I form by Hcy. EPR measurement further confirmed the redox status of the inactive HRP. Taken together, we conclude that inactivation of HRP by Hcy involves both structural alterations and change in redox state. It has been previously demonstrated by various studies that Histidine axial ligand in HRP also plays a crucial role in mediating the redox state of iron. Indeed, histidine in the distal cavity of the heme forms H-bond with Arginine residue and mutations (or structural alterations) that disrupt these particular H-bond results in the inability of HRP to abstract proton from H₂O₂ [42].

HRP may undergo oxidase (in presence of molecular oxygen) or peroxidase cycle (in presence of H₂O₂) depending on the available substrates. Since our conformational studies and analysis of the redox state or HRP is performed in absence of H₂O₂, it is expected to follow oxidase cycle. Indeed, formation of compound I is possible only with H₂O₂ as substrate (not O₂) (as shown in Scheme 2). Formation of compound I by Hcy treatment in absence of H₂O₂ means that the oxidase cycle is followed by peroxidase cycle. Interestingly, production of O₂ anions under oxidase cycle has been reported on some HRP variants in presence of salicylic acid or indole 3 acetic acid as substrates [43,44]. According to oxidase cycle, using molecular O₂, the ferrous iron (Fe²⁺) is converted to unstable compound III (Fe³⁺) form thereby forming O₂–Heme-Fe³⁺ or O₂–Heme-Fe⁴⁺. O₂–Heme-Fe³⁺ or O₂–Heme-Fe⁴⁺ intermediates with the help of H⁺ regenerate Fe³⁺-iron and subsequent release of OOH⁻. In our system we believe that O₂ generated as a result of Hcy oxidation aids the conversion of Fe³⁺⁻ to Fe⁵⁺⁻. The compound III (O₂–Heme-Fe³⁺ or O₂–Heme-Fe⁴⁺) uses 2H⁺ (instead of H⁺) leading to the generation of H₂O₂. Such a build-up of H₂O₂ in the vicinity of the iron center is sufficient to initiate a peroxidase cycle resulting in the formation of compound I. In a similar manner, certain peroxidases (e.g., myeloperoxidase) induces thiol oxidation without the involvement of exogenous H₂O₂ but with other substrates (methyl and ethyl esters of cysteine and cysteamine) [45]. In another development, it has also been reported that certain peroxidases can undergo oxidation of thiols (SH) with the formation thyl radical (RS⁺). It may however, be noted that such thyl radicals are formed under conventional peroxidase cycle (not under oxidase cycle) (i.e., in the presence of H₂O₂) as indicated

![Scheme 2. Redox cycle of HRP](image-url)
previously \cite{46,47}. Thus, Hcy oxidation may arise via oxidase or peroxidase cycle.

Major fraction of Hcy is present in the serum as disulfides (including Hcy-Hcy, Hcy-cys, Hcy-glutathione and some are bound to proteins). A small fraction (~1\%) exists in free form. We speculate that presence of abundant heme proteins in the human serum is the rationale for the existence of majority of Hcy in its dimeric forms. On the other hand, in the real biological setting glutathione exists several folds higher than the Hcy concentration in serum making it unlikely that Hcy oxidation will be a cause of oxidative stress under hyperhomocysteinemic condition. However, our results (see Fig. S6) show that the ability of HRP to induce oxidation of cysteine or glutathione is far less as compared to the Hcy inferring Hcy a better substrate of HRP for the observed reaction. Furthermore, under hyperhomocysteinemic condition the fraction of Hcy-Glutathione is very small around 1–2\% relative to the spectrum of heterogenous Hcy-disulfides indicating that serum glutathione concentration is not sufficient to reduce Hcy-Hcy disulfides. It has also been well known that Hcy downregulates several anti-oxidant enzymes under hyperhomocysteinemic conditions \cite{48}. Hcy is upregulated in a large number of pathological conditions including cardiovascular disorders, neurodegeneration, cancer, etc. \cite{5–9,49}. Our results highlight possible involvement of redox regulated proteins in these pathological conditions. For instance, cancer cells work under dysregulated/altered redox homeostasis \cite{50}. Cardiovascular diseases and several types of neurodegenerative conditions are believed to be due to increased oxidative stress, wherein Hcy-induced redox dysregulation of various proteins might play a pivotal role.

5. Summary

Hcy is a pro-oxidant and is associated with several human diseases, including cardiovascular complications and neurodegenerative conditions. In the present study, we identified a novel pathway of oxidative stress induced by Hcy via affecting the redox state of iron containing proteins. The common consensus in the literature for the cause of oxidative stress by Hcy is due to production of $O_2^-$ species from its oxidation process. However, it appears that Hcy induces in ROS level at various steps as summarized in Scheme 3. Indeed, the cause of oxidative stress may stem from at least two different pathways. In the first pathway, Hcy oxidation produces $O_2^-$ that in turn can affect the redox status of major cellular anti-oxidant enzymes (including catalases and peroxidases) leading to impairment of anti-oxidant system. In the second pathway, Hcy can also be converted to homocysteine thiolactone (HTL) in an error editing mechanism by methionyl tRNA synthetase. HTL has also been known to covalently modify proteins specifically at the lysine residue and the modification results in the formation of toxic protein oligomers. The toxic protein oligomers may primarily impair mitochondrial function and many other membranous organelles.

Author contributions

LRS designed the experiments. GSS and RB performed the experiments. LRS and GSS analysed the data. LRS and RB wrote the manuscript.

Declaration of competing interest

None.
Acknowledgements

This work is supported partly by R&D Grant and DU-DST Purse Grant, University of Delhi and Department of Biotechnology (Bt/PR17096/NER/95/407/2015). We thank Advanced Instrumentation Research Facility (AIRF), Jawaharlal Nehru University, New Delhi for providing EPR facility and Central Instrumentation Facility (CIF), University of Delhi, South Campus for providing MS facility. We also thank Indian Council of Medical Research (ICMR), New Delhi for providing fellowship (45/06/2019/BIO/BMS) to RB.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2021.102080.

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