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# Characterization of the reaction products of cytochrome *c* with glutathione by mass spectrometry

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**Abstract**

Cytochrome *c* and glutathione (GSH) are two important biomolecules that regulate many cellular processes. The reaction of cytochrome *c* with GSH involves radical oxygen species and exhibits significant complexity. In the present work, the reaction of cytochrome *c* with GSH in water was characterized using mass spectrometry. The results show for the first time that the reaction generates multiple products including apocytochrome *c* in oxidized and reduced forms, glutathionylated apocytochrome *c*, GSH-modified cytochrome *c*, and oxidized and hydroxylated species. The reaction is $O_2$ dependent and is rapid in water at neutral pH and 37 °C. The reaction involves the cleavage of thioether linkages between the heme and apocytochrome *c*. Evidence for the role of $H_2O_2$ and other oxygen radicals in this reaction is also provided.


*Keywords:* Cytochrome *c*; Glutathione; Redox reaction; Mass spectrometry

The eukaryotic C-type cytochromes are small globular heme proteins that have a unique structure in which the vinyl groups of heme are covalently bound to two cysteine residues by thioether linkages [1,2]. The covalent bonding of heme to apocytochrome *c* enhances the thermal and chemical stability of cytochrome *c*. Covalent attachment of heme to apocytochrome *c* in vivo is catalyzed by enzymes such as cytochrome *c* maturation proteins in bacteria or heme lyase in fungi, vertebrates, and invertebrates [3,4]. Cytochrome *c* is also formed in vitro through a non-enzymatic reaction [5,6]. This reaction proceeds faster at lower pH and requires ferrous heme that is formed under reducing conditions. The reducing conditions also ensure that the cysteine residues have free thiol groups. Interestingly, it was also found that thioether bonds were selectively cleaved by reducing reagents such as Raney nickel [7] and by microorganisms [8,9].

Cytochrome *c* is a major component of the electron transport chain in which it accepts an electron from cytochrome *c* reductase and transfers an electron to cytochrome *c* oxidase. The heme iron of cytochrome *c* participates in the cyclic redox reactions, alternating between oxidized ($Fe^{3+}$) and reduced forms. Cytochrome *c* possesses antioxidant activity [10,11]. Low concentrations of horse cytochrome *c* strongly inhibit $H_2O_2$ production under conditions of reverse electron transfer from succinate to $NAD^+$ [12]. Cytochrome *c* at high concentrations works as a peroxidase to eliminate $H_2O_2$ [13]. Cytochrome *c* also mediates a process of superoxide removal, resulting in regeneration of $O_2$ and utilization of the electron involved previously in the $O_2$ reduction [14].

In 1996, a novel function of cytochrome *c* in mediating apoptosis was discovered that revived interest in this mitochondrial hemoprotein [15]. The release of cytochrome *c* from the mitochondria into the cytosol activates caspase proteases and triggers apoptotic cell death. The biochemical mechanisms and regulation of cytochrome *c*-mediated apoptosis have been extensively studied. Two of the postulated mechanisms suggest that redox regulation plays an important role for induction of cytochrome *c* release [16,17]. This has linked the cytochrome *c* mediated

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apoptosis with the cellular redox states. A key regulator of the cellular redox states is glutathione (L-γ-glutamyl-L-cysteinyl-glycine), a tripeptide. Indeed, it was found that the release of cytochrome *c* was triggered by the depletion of GSH or was impaired by the GSH treatment [18]. GSH is widespread distributed in living organisms and is usually present in cells in millimolar concentrations [19,20]. Besides its role in maintaining the redox states, GSH has other important functions: GSH is a free radical scavenger [21]; GSH is a nucleophile that detoxifies xeno- and endobiotic electrophiles [22]; and under oxidative conditions, GSH is oxidized to GSSG that can form disulfide linkages with protein cysteinyl thiols [23]. Formation of mixed disulfide linkages between GSH and cysteines in proteins (protein glutathionylation) is reversible and is an important mechanism to regulate protein functions and to protect proteins from irreversible oxidation [24–26]. A number of proteins including transporters, receptors, protein kinase and phosphatase, proteases, chaperones, and cytoskeletal proteins have been found to be glutathionylated during oxidative stress. Significant increases in glutathionylated proteins have been found in human diseases such as hyperlipidemia and chronic renal failure [27].

These studies greatly enhance our understanding of the roles of cytochrome *c* and GSH in oxidative stress, apoptosis, and iron deposition in hemoglobinpathic red blood cells. Is there a direct interaction between cytochrome *c* and GSH? The two cysteine residues in cytochrome *c* bound to heme via thioether bonds are not available to form disulfide linkages with glutathione. No reports have been found on in vivo glutathionylation of apocytochrome *c*. However, direct in vitro interactions between cytochrome *c* and GSH have been reported [28–30]. The reduction rate of cytochrome *c* by GSH was dependent on the conformation of cytochrome *c* and the reaction was catalyzed by trisulfides and cysteine trisulfides [28]. Although no reaction products were identified, it was proposed that GSH interacted with the edge of the heme moiety to form a GSH–cytochrome *c* intermediate. Recently, Burkitt and colleagues [29] found that a peroxidase compound I-like intermediate was formed from reactions of cytochrome *c* with $H_2O_2$ and this intermediate oxidized GSH to its radical form. A detailed study of heme degradation in the presence of GSH has provided important information for understanding the reaction mechanisms of heme with GSH [30]. It was proposed that GSH reduced $Fe^{3+}$ of heme to $Fe^{2+}$. The $Fe^{2+}$ of heme was re-oxidized by $O_2$ to $Fe^{3+}$ and generated $O_2^-$ species that initiated the heme degradation. $O_2$ is required in this reaction and the reaction is pH dependent.

These studies indicate that the reaction of cytochrome *c* with glutathione is a complex process. A crucial question that still needs to be answered is what the reaction products of cytochrome *c* with GSH are. In the present work, mass spectrometry was used to characterize the reaction of cytochrome *c* with GSH under physiologically relevant experimental conditions. The reaction of cytochrome *c* with GSH produces a complex set of products including oxidized and reduced apocytochrome *c*, glutathionylated apocytochrome *c*, and GSH-modified cytochrome *c*.

## Materials and methods

Horse heart cytochrome *c*, glutathione, Hepes, and trifluoroacetic acid were purchased from Sigma–Aldrich and used without further purification. HPLC grade acetonitrile and water were purchased from Burdick and Jackson. The dithiothreitol (DTT) and Tris(2-carboxyethyl)phosphine (TCEP) were from Calbiochem. Urea was from Roche. All other reagents were of reagent grade quality.

*Reactions of cytochrome c with GSH.* The reaction was studied by mixing the proper quantity of solutions of cytochrome *c* with GSH at physiologically relevant concentrations. In most cases, the concentration of cytochrome *c* was 20 μM and concentrations of GSH ranged from 100 μM to 20 mM. The reaction mixtures were incubated at 37 °C under air or $N_2$ for a desired time. Some of the reaction products were further treated with 100 mM DDT or TCEP to determine the effects of reducing reagents on the reaction products. All reaction products were analyzed using MALDI-TOF or LC–MS.

*Protein analysis by mass spectrometry.* The reaction products of cytochrome *c* with GSH were analyzed by MALDI-TOF mass spectrometry. Briefly, samples were mixed 1:1 (vol/vol) with a solution of α-cyno-4-hydroxycinnamic acid in 50% aqueous acetonitrile/0.1% aqueous trifluoroacetic acid (1:1, vol/vol) and 1 μL was spotted onto a stainless steel MALDI target plate and allowed to dry before analysis by MALDI-TOF MS. Protein molecular ions were analyzed in linear, positive ion mode using a Voyager DE STR mass spectrometer (Applied Biosystems). The resulting spectra were analyzed by DataExplorer (Applied Biosystems, Inc.). The instrument was calibrated using an external apomyoglobin standard and the resulting spectra were Gaussian-smoothed and baseline-corrected. Intact cytochrome *c* and its reaction products were also analyzed using LC–MS. The reaction products were separated by HPLC on a 5 cm × 75 micron i.d. reversed-phase C4 column (Micro-Tech Scientific) using a 60 min linear gradient of 10–70% acetonitrile in 0.1% formic acid at a flow rate of 200 nL/min.

The samples of cytochrome *c* alone and its reaction products were digested with Sequence Grade Modified Trypsin (Promega), Sequence Grade Glu-C (Roche), and chymotrypsin (Boehringer–Mannhein), respectively, in ammonium bicarbonate buffer at 37 °C overnight. All the digestion products were analyzed using a Voyager DE STR MALDI-TOF mass spectrometer and nano-LC–MS/MS. For LC–MS/MS analysis, each digestion product was separated by gradient elution with a Dionex capillary/nano-HPLC system and analyzed by an Applied Biosystems QSTAR XL mass spectrometer using information-dependent, automated data acquisition. The peptide mixtures were separated on a 15 cm × 75 micron i.d. reversed-phase C18 column (Micro-Tech Scientific) using a 40 min linear gradient of 5–75% acetonitrile in 0.1% formic acid and at a flow rate of 200 nL/min.

## Results

### *Analysis of reaction products of cytochrome c with GSH*

The horse heart cytochrome *c* has 104 residues including 2 cysteine residues that are covalently linked to heme. The molecular weight of cytochrome *c* is 12,361 for singly protonated species as shown in Fig. 1A. The monoisotopic mass of GSH is 307.07. The reaction products from incubation of cytochrome *c* (10 μM) with GSH (10 mM) in water at 37 °C for 4 h were analyzed by MALDI-TOF and the mass spectrum is displayed in Fig. 1B. Multiple reaction products were observed. The mass of peak 1 is 11,745

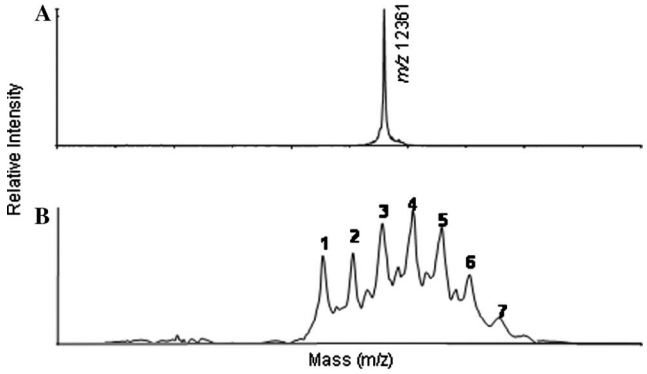


Fig. 1. MALDI mass spectra obtained for the native cytochrome *c* (A) and for reaction products of cytochrome *c* (10 μM) with GSH (10 mM) in water at 37 °C for 4 h (B).

(±3) Da corresponding to apocytochrome *c*. The mass difference between the neighboring peaks is about 306 Da. It is also noted that the peak width of these products is significantly wider than that of cytochrome *c* (Fig. 1A). This suggests that each peak is comprised of several ions that are not resolved in MALDI-TOF analysis. To better resolve these products, an in-line combination of HPLC separation and ESI mass spectrometry was used to separate the reaction products with a gradient elution on a $C_4$ reversed-phase column. The reaction products were eluted earlier compared to the unreacted cytochrome *c*. The deconvoluted mass spectrum for ions eluting at the earlier time (~35% of acetonitrile) is displayed in Fig. 2A and that for ions eluting at 50% of acetonitrile in Fig. 2B. The peak pattern in Fig. 2A is similar to that of the MALDI-TOF mass spectrum in Fig. 1B with multiple peak groups separated by about 306 Da. The higher resolving power of LC–MS allows us to determine the masses of ions at the mass measurement error of ±1 Da. The difference between the consecutive peaks in each group is either 16 or 17 Da. The number of the peaks in each group increases with the mass. The *m*/*z* of singly protonated cytochrome *c* (12,361) is close to the mass of doubly glutathionylated apocytochrome *c* (*m*/*z* 12,356) and both species are irresolvable in the MALDI-TOF mass spectrum (Fig. 1B). However, these two species eluted from the C4 column at different time as observed in the ESI mass spectra (Figs. 2A and B).

As shown in Figs. 1B and 2A, some reaction products consist of multiple glutathione groups. To determine if GSH is covalently bound to apocytochrome *c* or cytochrome *c*, the reaction products were diluted 100-fold in 7 M urea or a 0.1% (v/v) aqueous TFA solution and analyzed with MALDI-TOF mass spectrometry. No changes were observed in the mass spectra indicating the bonding is covalent in nature. The apocytochrome *c* in the reduced form has two free cysteine residues that can form disulfide linkages with GSH. The peaks corresponding to mono- and di-glutathionylated apocytochrome *c* were observed in Figs. 2A and 1B. To confirm that the reaction products contain the disulfide linkages, the reaction products were treated with 100 mM DTT for 1 h at 55 °C. The DTT treatment led to the change of the product distribution as shown in Fig. 3A. Compared to the spectrum from the untreated sample (Fig. 3B), the peak intensity of apocytochrome *c* is significantly enhanced as the peaks of mono- and di-glutathionylated apocytochrome *c* are diminished. This indicates that the products represented by the second and third peak groups in Fig. 3B consist of disulfide linked GSH. The peak groups at masses larger than that of cytochrome *c* also shift to lower masses after DTT treatment. Increasing the concentration of DTT or extending the

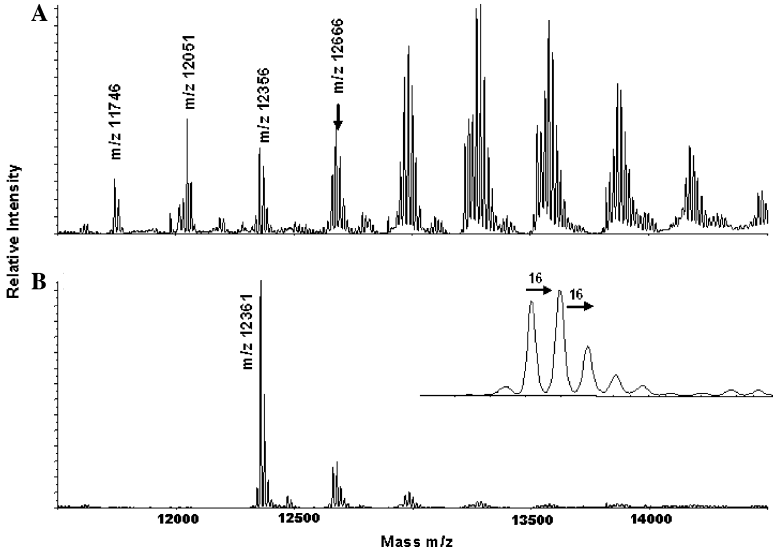


Fig. 2. The deconvoluted LC–MS mass spectra obtained for the reaction products of cytochrome *c* with GSH eluted from the reversed-phase $C_4$ column at (A) 29 min and (B) at 39 min. The reaction products including apocytochrome *c* and its glutathionylation products elute earlier than cytochrome *c* does. The insert shows the mass difference for neighboring peaks in each group is 16 or 17 Da.

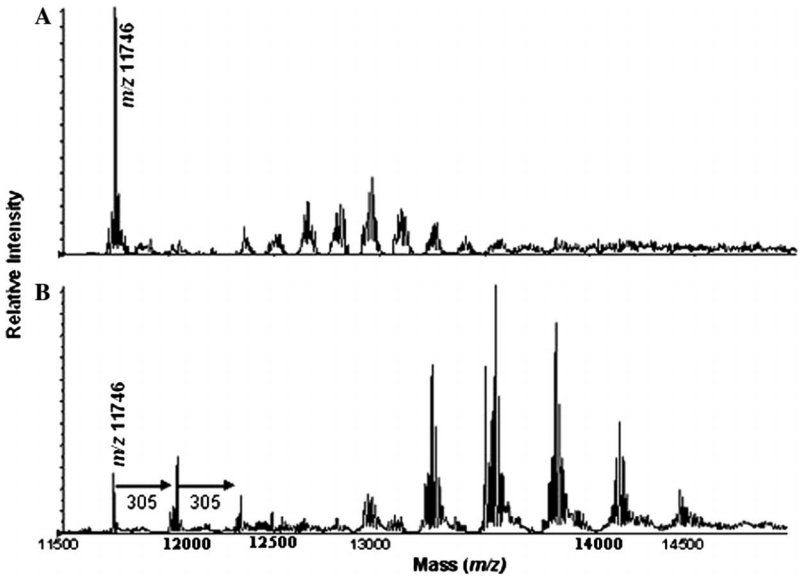


Fig. 3. The deconvoluted ESI mass spectrum obtained for the reaction products of cytochrome *c* with GSH (A) treated with 100 mM DTT for 1 h at 55 °C and (B) without DTT treatment.

incubation time does not lead to further changes in the mass spectra. Similar mass spectra were generated for reaction products treated with TCEP. The fact that treatment of the reaction products with DTT or TCEP is not capable of converting all products to apocytochrome *c* suggests that products are comprised of disulfide- and other covalent bond-linked GSH.

The time course of the reaction was monitored and the mass spectra of reaction products generated at different times show that the adduct of one GSH to cytochrome *c* appears after 1 h of incubation at 37 °C and apocytochrome *c* is formed after 2 h of incubation. Increasing the ratio of GSH to cytochrome *c* speeds up the reaction. These studies demonstrate that the reaction of cytochrome *c* with GSH produce multiple products including apocytochrome *c*, glutathionylated apocytochrome *c*, and GSH-modified cytochrome *c*. To examine the effects of the folding states of cytochrome *c* on the reactions with GSH, cytochrome *c* (10 μM) was dissolved in 7 M urea, and then mixed with GSH (10 mM) at 37 °C for 4 h, and the products were analyzed. Similar products were observed indicating that the unfold cytochrome *c* efficiently reacts with GSH.

However, incubation of a similar reaction mixture for 2 h under $N_2$ does not produce any reaction products. Further increasing the concentration of cytochrome *c* and GSH, temperature or reaction time did not lead to observable products in the mass spectra. This demonstrates that the reaction of cytochrome *c* with GSH is $O_2$ dependent. $H_2O_2$ has been found to play a role for GSH-induced heme degradation [30]. To test if $H_2O_2$ is involved in this reaction, catalase (0.1 μM) was added to cytochrome *c* solution prior to mixing with GSH. The reaction was abolished in the presence of catalase. The reaction was also pH-dependent. The reaction of cytochrome *c* with GSH in 0.1% aqueous TFA solution (pH 2) is slower than that in water under the identical experimental conditions. The reaction is abolished when cytochrome *c* is incubated with GSH in aqueous solution containing 50 mM ammonia bicarbonate (pH 8).

### *Identification of the GSH-modified peptides*

The above analysis of the reaction products demonstrates that the reaction of cytochrome *c* with GSH is $O_2$- and pH-dependent and generates multiple products. Identification of the reaction products is a challenging task involving separation and isolation of each individual reaction product. Therefore, no attempt was made to separate all the reaction products in the present work. Instead, reaction products from incubation of cytochrome *c* and GSH for 8 h were directly digested with three enzymes: trypsin, Glu-C, and chymotrypsin, respectively. The resultant peptide mixtures were analyzed using MALDI-TOF and LC–MS/MS.

All predicted tryptic peptides of cytochrome *c* with mass larger than 500 were observed in the MALDI-TOF mass spectrum including acetylated N-terminal peptide. The free heme was also observed in the MALDI-TOF mass spectrum generated for the un-modified cytochrome *c* sample, but was absent in the mass spectrum generated for the digestion of reaction products of cytochrome *c* with GSH. No peaks corresponding to GSH-modified hemes were identified. This suggests that GSH catalyzes the degradation of heme in consistent with the previous report [30]. The heme-bound peptide (residues 14–22) ion was the most abundant peak in the MALDI-TOF mass spectrum generated for the digestion of cytochrome *c* (Fig. 4A). The major fragment from collision induced dissociation of this peptide ion is the heme ion. However, the intensity of this peptide ion is significantly decreased in the

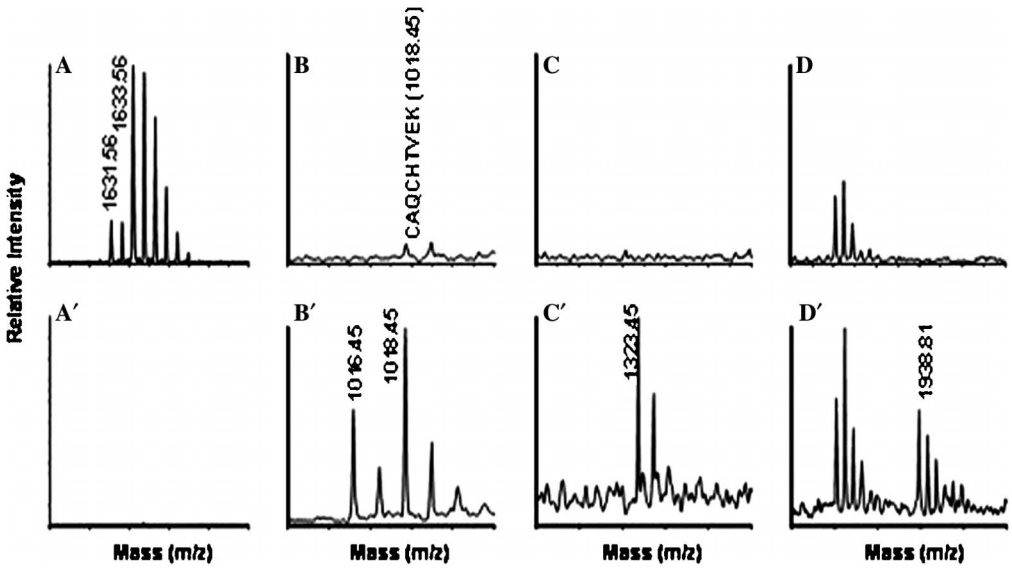


Fig. 4. (A–D) MALDI-Mass Spectra of the selected tryptic peptides from unreacted cytochrome *c*. (A′–D′) MALDI-Mass Spectra of the selected tryptic peptides from the reaction products of cytochrome *c* with GSH.

mass spectrum acquired for the digestion of the reaction products, indicating that heme was removed from the protein during the reaction. On the other hand, the intensity of the peptide (residues 14–22) at *m*/*z* 1018.45 was significantly enhanced in the mass spectrum for the reaction products (Figs. 4B and B′). Furthermore, an ion at *m*/*z* 1016.45 was also observed in Fig. 4B′ indicating that two cysteine residues formed a disulfide linkage in this peptide as confirmed by tandem mass spectrometry.

The mono-glutathionylated peptide (residues 14–22) was also observed (Fig. 4C′). The MS/MS spectrum of this ion showed that GSH either bound to $Cys^{14}$ or to $Cys^{17}$. Interestingly, an ion corresponding to glutathionylated heme-bound peptide (residues 14–22) was also observed in Fig. 4D′. Fragmentation of this ion by collision induced dissociation resulted in two major fragments at *m*/*z* 1018.45 and 617.28 corresponding to the peptide ion (residues 14–22) and protonated heme ion, respectively. This result suggests the cleavage of the thioether bond between heme and Cysteine residue and disulfide formation between Cysteine and glutathione occur synergistically and is consistent with the result from the time-course studies in which the adduct containing one GSH is the early reaction product. The doubly glutathionylated peptide (residues 14–22) was not observed in the mass spectra generated for tryptic peptides of the reaction products. However, the doubly glutathionylated peptide (residues 14–22) was observed in the MALDI-TOF mass spectrum generated for the Glu-C digestion products and its identity was confirmed by MS/MS.

In the mass spectra generated for trypsin and Glu-C digestion of the reaction products, several pairs of peaks that were separated by 305 were observed. One of the pairs from analysis of the Glu-C digestion of the untreated and GSH-treated cytochrome *c* is displayed in Fig. 5. The mono-isotopic mass for the peak at 895.05 ($MH_5^{5+}$) is 4468.2 matching a predicted Glu-C fragment (residues 22–62) shown in Figs. 5A and B. The partial sequence of this peptide by MS/MS confirms that it contains YTDANK (residues 48–53). In Fig. 5B, an ion with mono-isotopic mass of 4773.3 was also observed indicating that one GS is bonded to this peptide. This peptide (residue 22–61) does not have a cysteine residue. Therefore, its GSH adduct was formed by a non-disulfide linkage. This is consistent with the result discussed earlier, in which DTT treatment was not able to covert all GSH-containing products to apocytochrome *c* (Fig. 3). Due to its low intensity, we were not able to determine the glutathionylation site.

Using mass mapping and tandem mass spectrometry, many oxidized and hydroxylated peptides were identified from the trypsin and Glu-C digestion of the reaction products. In addition to methionine, histidine, and tryptophan residues were also oxidized in this reaction. Three residues, tyrosine, lysine, and proline were found to be hydroxylated. The confirmed oxidation and hydroxylation sites are displayed in Fig. 6.

## Discussion

In this study, the reaction products of cytochrome *c* with glutathione were characterized by mass spectrometry. The reaction is rapid in water at neutral pH and 37 °C, and generates multiple reaction products including apocytochrome *c* and glutathionylated apocytochrome *c*. Some products were comprised of multiple glutathione molecules. The reaction involves oxidation and hydroxylation as multiply oxidized and hydroxylated products were observed in the mass spectra as shown in Fig. 2. We are not able to separate each reaction product by reversed-phase liquid

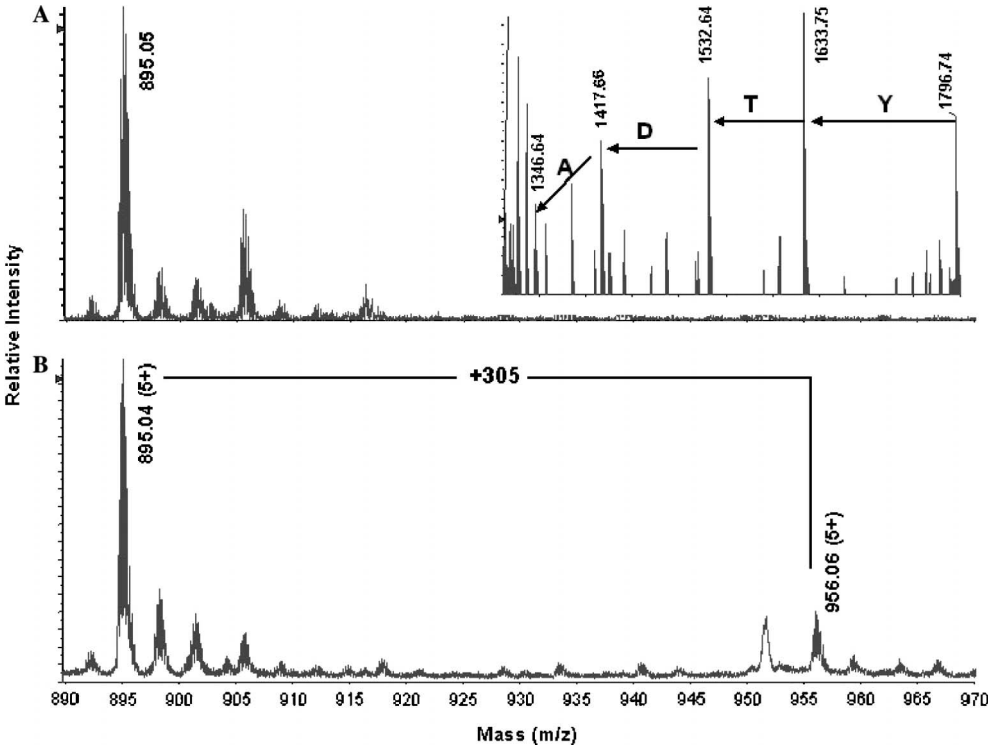


Fig. 5. (A) ESI mass spectra of peptides from Glu-C digestion of Cytochrome *c*; (B) ESI mass spectra of peptides from Glu-C digestion of the reaction products with GSH. The insert shows the MS/MS spectrum in the selected mass range and the partial sequence of the precursor ions.

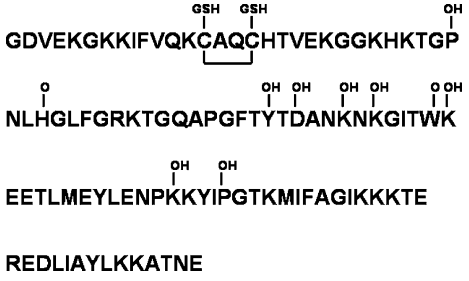


Fig. 6. The major products observed for the reaction of cytochrome *c* with GSH in water at 37 °C for 4 h.

chromatography. But the reaction products of cytochrome *c* elute out earlier from the $C_4$ column than unreacted cytochrome *c* does. The combination of LC separation and mass spectrometric analysis enables us to distinguish the cytochrome *c* (12,361 Da) and di-glutathionylated apocytochrome *c* (12,356 Da). Two forms of apocytochrome *c* (reduced and oxidized) were identified by a combination of trypsin digestion and mass mapping. Apocytochrome *c* in the reduced state forms adducts with glutathione through disulfide linkages. Both mono- and di-glutathionylated apocytochrome *c* were identified by MS and MS/MS analysis. The mass spectra generated from the DTT-treated reaction products suggest that adducts of apocytochrome c/cytochrome *c* with glutathione are formed through disulfide as well as other types of covalent linkages.

Rapid formation of such diverse products from a simple mixture of cytochrome *c* and glutathione must involve complex reaction mechanisms. No attempts were made in this work to characterize the reaction mechanisms. However, studying this reaction under different experimental conditions provides important information for deciphering the reaction process. No mass spectrometrically detectable products were formed under $N_2$ or in the presence of catalase, demonstrating that the reaction is $O_2$-dependent and involves $H_2O_2$. This suggests that heme as the redox reactive center initiates the reaction process. Indeed, the reaction of heme with GSH is rapid and $O_2$-dependent as studied by Atamna and Ginsburg [30]. They found that GSH was able to degrade heme in solution with a pH optimum of 7, resulting in the release of iron from heme. The proposed reaction mechanism for heme degradation includes the redox cycling of $Fe^{3+}$ and $Fe^{2+}$ and production of $H_2O_2$. Since the reaction of cytochrome *c* with glutathione bears similarity to the degradation of heme by GSH, we expect that the reaction proceeds with a similar mechanism. The reduction of cytochrome *c* to ferrocytochrome *c* initiates the reaction process. The re-oxidation of ferrocytochrome *c* to ferricytochrome *c* results in formation of $O_2$ anions and $H_2O_2$ as shown in Eqs. (1)–(4).

$$\text{Cyto-Fe}^{3+} + \text{GSH} \Longleftrightarrow \text{Cyto-Fe}^{2+} + \text{GSSG} \quad (1)$$

$$\text{Cyto-Fe}^{2+} + \text{O}_2 \Longleftrightarrow \text{Cyto-Fe}^{3+} + \text{O}_2^- \quad (2)$$

$$\text{O}_2^- + \text{H}^+ \Longleftrightarrow \text{HO}_2\cdot \quad (3)$$

$$2\text{HO}_2\cdot + \text{O}_2 \Longleftrightarrow \text{H}_2\text{O}_2 + \text{O}_2 \quad (4)$$

This proposed reaction process is pH-dependent and is consistent with our experimental results. The reaction is slower at low pH values because the heme of cytochrome *c* preferentially binds to non-protonated GSH. Under basic conditions, the proton concentration is low and hinders $H_2O_2$ and $O_2$ generation as represented in Eqs. (3) and (4).

Formation of apocytochrome *c* requires the cleavage of thioether linkages between heme and apocytochrome *c*. It is still not clear which reaction intermediate triggers the cleavage of the thioether bond. Based on the report that the thioether linkages were selectively cleaved by Raney nickel [7], we suggest that $Fe^{2+}$ released from heme may play a similar role in the cleavage of thioether bonds between heme and apocytochrome *c*. The sulfhydryl groups in apocytochrome *c* are reactive under the oxidative conditions that form the mixed disulfide linkages. In this work, $Cys^{14}$ or $Cys^{17}$ residues not only form disulfide bonds with one or two GSH, but they also form an intra-disulfide bond as shown in Fig. 6.

Using LC–MS/MS, multiple oxidized and hydroxylated peptides were identified. Oxidation of methionine was found from the unreacted cytochrome *c*. The reaction of cytochrome *c* and glutathione not only significantly enhances methionine oxidation, but it also results in oxidation of histidine and tryptophan. Three residues, tyrosine, lysine, and proline were found to be hydroxylated. The extensive oxidation and hydroxylation products indicate that excessive reactive oxygen species were generated in the reaction. In summary, the present study demonstrates that in vitro reactions of cytochrome *c* with GSH produce a complex set of products under the physiologically relevant experimental conditions.

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