# Inactivation of creatine kinase by S-glutathionylation of the active-site cysteine residue

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Protein S-thiolation, the formation of mixed disulphides of cysteine residues in proteins with low-molecular-mass thiols, occurs under conditions associated with oxidative stress and can lead to modification of protein function. In the present study, we examined the site of S-thiolation of the enzyme creatine kinase (CK), an important source of ATP in myocytes. Inactivation of this enzyme is thought to play a critical role in cardiac injury during oxidative stress, such as during reperfusion injury. Reaction of rabbit CK M isoenzyme with GSSG, used to model protein S-thiolation, was found to result in enzyme inactivation that could be reversed by GSH or dithiothreitol. Measurement of GSH that is released during the thiolation reaction indicated that the maximum extent of CK thiolation was approx. 1 mol of GSH/mol of protein, suggesting thiolation on one reactive cysteine residue. Accordingly, matrix-assisted laser-desorption ionization MS confirmed that the molecular mass of CK was increased, consistent with addition of one GSH molecule/molecule of CK. Using trypsin digestion, HPLC and MS analysis, the active-site cysteine residue ($Cys^{283}$) was identified as the site of thiolation. Reversal of thiolation was shown to be rapid when GSH is abundant, rendering dethiolation of CK thermodynamically favoured within the cell. We conclude that S-glutathionylation of CK could be one mechanism to explain temporary reversible loss in activity of CK during ischaemic injury. The maintainance of GSH levels represents an important mechanism for regeneration of active CK from S-glutathionylated CK.

Key words: glutathione, MALDI mass spectrometry, oxidative stress, S-thiolation.

## INTRODUCTION

Exposure of cells to acute oxidative stress triggers a sequence of events characterized by depletion of antioxidant defences and oxidative modification of intracellular biomolecular constituents including proteins, lipids and nucleic acids, eventually leading to cell dysfunction and death. Proteins that contain thiol groups are particularly susceptible to oxidation, and may represent important targets in redox signalling. During conditions of moderate oxidative stress, intracellular protein thiol groups can be modified by the reversible reaction of S-thiolation, whereas prolonged or increased oxidative stress leads to irreversible thiol modifications such as formation of sulphonic acids [1]. S-thiolation of proteins refers to the formation of mixed disulphides between protein thiols and low-molecular-mass thiols such as cysteine and glutathione (GSH). When the low-molecular-mass thiol GSH forms an adduct with protein thiols, S-thiolation is specifically defined as S-glutathionylation.

S-thiolation of proteins is observed during the respiratory burst of neutrophils [2] and in cells exposed to various chemical oxidants including diamide and t-butyl hydroperoxide [3]. Several important metabolic enzymes including phosphofructokinase [4], glycogen synthase [5], fructose-1,6-bisphosphatase [6], 3-hydroxy-3-methylglutaryl-CoA reductase [7], glyceraldehyde-3-phosphate dehydrogenase [8] and creatine kinase (CK) [9] contain thiol groups critical for their function; thus their activities are potentially influenced by S-thiolation. It is believed that thiolation of enzymes during oxidative stress protects them from irreversible modifications of their thiol groups, often at the expense of temporary loss in their activity [1]. S-thiolation of proteins such as protein kinase C [10], guanylate cyclase [11] or glucocorticoid receptors [12] has been shown to affect their activity. The binding of transcription factors such as c-Jun to DNA appears to be redox regulated by mechanisms that include protein S-thiolation [13,14]. It has been reported recently that exposure of cells to oxidants results in S-glutathionylation of ubiquitin-activating enzymes, with concomitant decrease in activity of the ubiquitinylation pathway [15]. Also, protein S-thiolation induced by hydroperoxides has been shown to mediate the inhibition of protein synthesis in cells [16]. Consequently, this type of protein modification may affect many cellular metabolic and regulatory pathways.

During conditions of oxidative stress in myocytes, the enzyme CK appears to be inactivated via oxidation of the active-site thiol [9,17]. CK is central to energy distribution in cardiac myocytes and catalyses phosphotransfer between creatine phosphate and ATP via the following reaction:

$$\text{Creatine phosphate} + \text{MgADP} \leftrightarrow \text{creatine} + \text{MgATP} \quad (1)$$

Reperfusion injury of the myocardium, a condition in which oxidative stress is believed to play a role, results in a decrease in the flux through the CK reaction as observed by $^{31}$P-NMR [18,19]. Reversible S-glutathionylation of CK may represent one potential mechanism by which the activity of both mitochondrial and cytosolic isoforms of CK are decreased during reperfusion injury. The current studies were designed to demonstrate that inactivation of CK may occur, at least in part, via S-glutathionylation at its active-site thiol.

The precise mechanisms *in vivo* leading to protein S-glutathionylation are not clear. There is neither conclusive evidence for enzymic catalysis nor a generalized non-enzymic

Abbreviations used: CK, creatine kinase; NEM, *N*-ethylmaleimide; MALDI–TOF, matrix-assisted laser-desorption ionization–time-of-flight.

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mechanism for protein S-glutathionylation. One proposed mechanism is the thiol/disulphide mechanism [20], which occurs in response to changes in the GSH/GSSG ratio, and involves reaction of GSSG with protein thiols to form GSH–protein adducts. Since such a mechanism would require considerable levels of GSSG, it would only be relevant in subcellular environments associated with pronounced changes in GSH/GSSG ratios [20,21]. Other mechanisms might include oxidation of protein thiol groups to sulphenic acids [22], which can then react with GSH resulting in S-thiolation [23]. Also, reactive nitrogen intermediates derived from nitric oxide ($NO^{\bullet}$) can subsequently react with protein thiols to form S-nitrosothiols, which then react with GSH leading to protein-S-glutathionylation [14,24–26]. The reversal of S-nitrosation by GSH may thus serve as an important component of interactive redox signalling pathways involving $NO^{\bullet}$-derived species and thiols. Such protein modifications are increasingly being reported to cause cell regulation and are likely to influence CK function *in vivo*.

The aim of the present study was to investigate the inactivation of CK in relation to protein S-thiolation. Previous studies of protein thiolation have used semi-quantitative SDS/PAGE and autoradiographic approaches to measure thiolation status of proteins [27]. In the present study, we used state-of-the-art analytical techniques, including HPLC and matrix-assisted laser-desorption ionization–time-of-flight (MALDI–TOF) MS, to obtain accurate quantitative measures of CK thiolation in relation to enzyme inactivation, and to identify the site of thiolation within CK. GSSG was chosen as the thiolating agent, since this would avoid potential modification of amino acids associated with other oxidant systems. Additionally, thermodynamic measurements were performed to estimate the potential contribution of GSSG to S-glutathionylation of CK *in vivo*.

## EXPERIMENTAL

### Materials

Unless otherwise indicated, reagents were obtained from Sigma (St. Louis, MO, U.S.A.). Glucose-6-phosphate dehydrogenase, from *Leuconostoc mesenteroides*, was purchased from Boehringer Mannheim.

### Incubations of CK with GSH and/or GSSG

Rabbit skeletal muscle CK (5 $\mu$M) was incubated with various concentrations of GSSG or GSH (0–5 mM) or with a mixture containing both GSH and GSSG (total concentrations of substrates maintained at 5 mM) in 10 mM phosphate buffer, pH 7.5, for various time intervals at 37 °C. The intracellular concentration of GSH in many cells varies from 2 to 10 mM [28], hence an average value of 5 mM was used. The activity of CK was measured directly following these incubations using the method described by Collison and Thomas [9]. Briefly, the assay involves measuring NADH formed by the active CK enzyme, by monitoring for absorbance at 340 nm.

### Measurement of the extent of glutathionylation of CK

Thiolation of CK by GSSG was quantified by measuring the release of GSH, which is directly proportional to the extent of glutathionylation of protein:

$$\text{Protein-SH} + \text{GSSG} \rightarrow \text{protein-SSG} + \text{GSH} \quad (2)$$

GSH was assayed by HPLC with fluorescence detection after monobromobimane derivatization [29]. The sample (100 $\mu$l) was incubated with monobromobimane (4 mM, 100 $\mu$l) contained in 50 mM *N*-ethylmorpholine (pH 8.0) for 15 min in the dark, followed by addition of 10 $\mu$l of 100 % trichloroacetic acid. The derivatized sample was injected on a RP-18 RadialPak column (Waters, Milford, MA, U.S.A.) and eluted with 8 % acetonitrile in 0.25 % acetic acid at 1 ml/min for 10 min followed by a column wash with 75 % acetonitrile in water for 5 min to remove any underivatized and/or hydrolysed monobromobimane. The column was re-equilibrated with the initial mobile phase for 5 min. Derivatized thiols were analysed by fluorescence detection (excitation, 394 nm; emission, 480 nm) and quantified using external standards of similarly derivatized GSH, with a detection limit of 1 pmol. The contaminant amounts of GSH present in GSSG were estimated by the above method and subtracted from each of the incubations.

As an alternative means to quantify the extent of protein thiolation, the molecular masses of the intact CK and thiolated CK were determined by MALDI–TOF MS (Biflex III, Brueker, Billerica, MA, U.S.A.).

### Determination of the site of S-glutathionylation of CK

Thiolated CK (5 $\mu$M CK incubated with 5 mM GSSG for 30 min) was subjected to ultrafiltration using Microcon filters (molecular-mass cut-off = 3000 Da) to remove GSSG. The protein was then treated with a 5-fold molar excess of *N*-ethylmaleimide (NEM) (30 min at 37 °C) to block unreacted thiol groups. The protein was then denatured by addition of 70 $\mu$l of 8 M urea in 0.1 M sodium phosphate buffer (pH 7.5) and incubated at 55 °C for 15 min. Following overnight digestion with trypsin (15 $\mu$l of 1 $\mu$g/$\mu$l trypsin), the tryptic fragments were separated using reversed-phase HPLC on a Vydac 218TP52 column (250 mm × 2.1 mm, 300 Å pore size) using a Varian 9010 solvent-delivery system (flow rate 0.3 ml/min) and monitored at 210 and 280 nm using a Hewlett Packard photodiode array (HP 1050) detector. The initial mobile-phase composition was held at 100 % water containing 0.05 % trifluoroacetic acid (TFA) for 2 min, followed by a gradient elution (100 % water with 0.05 % TFA to 90 % acetonitrile with 0.05 % TFA) for 100 min. Fractions were collected over 2 min intervals, dried and reconstituted in a 1:1 acetonitrile/water mixture containing 0.1 % formic acid. The molecular masses of components of the fractions were determined using MALDI–TOF MS on a Biflex III mass spectrometer (Brueker). This instrument has a detection limit of approx. 2 pmol and, as about 3 nmol of digested CK protein was analysed by MALDI–TOF MS, the fractions collected from the column contained sufficient amounts of peptide for accurate analysis.

## RESULTS

### Kinetics and thermodynamics of S-glutathionylation of CK

CK (5 $\mu$M) was incubated with GSSG (1.5 mM) and the activity of CK measured over time (0–80 min) as described in the Experimental section. As illustrated in Figure 1, there was rapid loss in activity of CK, the majority of enzyme inactivation occurring within 20 min. In separate experiments, CK was also incubated with various concentrations of GSSG and/or GSH (0–5 mM) for 30 min. As shown in Figure 2, the activity of CK decreased in the presence of GSSG in a concentration-dependent manner. Nearly 50 % enzyme inactivation occurred in the presence of 0.5 mM GSSG. The loss in activity most probably occurred due to thiolation of CK by GSSG, since the activity of CK was unaffected in the presence of GSH.

To determine if thiolation of CK by GSSG is affected by the presence of GSH, CK was incubated with varying concentrations

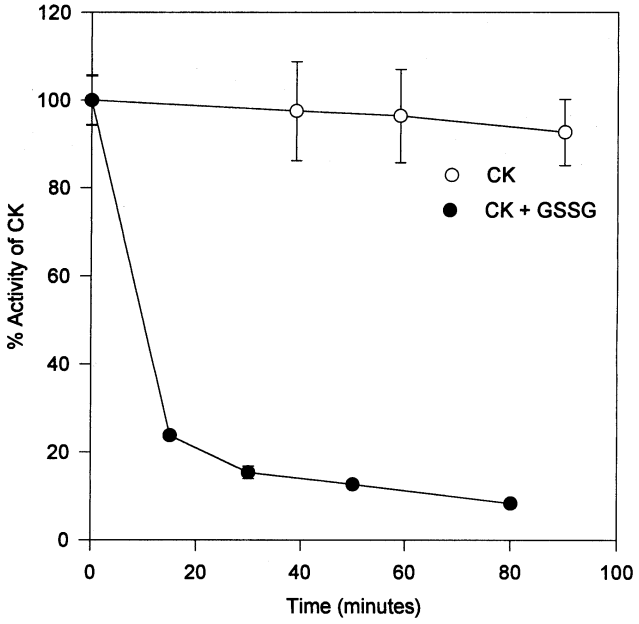


**Figure 1 Kinetics of CK inactivation by GSSG**

CK was incubated in the absence (○) or presence (●) of 1.5 mM GSSG for varying times in 10 mM phosphate buffer, pH 7.5, at 37 °C and changes in the activity of CK were measured as described in the Experimental section. Measurements were performed in triplicate. Error bars represent S.D.

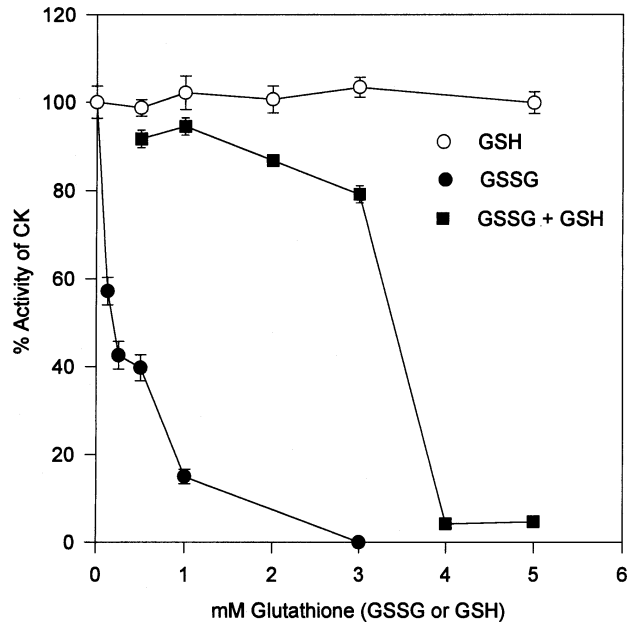


**Figure 2 Inactivation of CK by GSH and/or GSSG**

CK (5 $\mu$M) was incubated for 30 min with various concentrations of GSSG (●) or GSH (○) (0–5 mM) or mixtures of GSH and GSSG (■) (such that [GSH] + [GSSG] = 5 mM). For the sake of simplicity only GSSG concentrations present in mixtures of GSH and GSSG (■) are plotted on the graph. Measurements were performed in triplicate. Error bars represent S.D.

of both GSH and GSSG. In the presence of excess GSH, the activity of CK was preserved. As illustrated in Figure 2, the activity of CK is reduced by 50% in the presence of 0.5 mM GSSG, but this inhibition was only 10% in the presence of 0.5 mM GSSG and 4.5 mM GSH. Thus the thiolation of CK by GSSG is either prevented or reversed in the presence of GSH. To determine the ability of GSH to reverse thiolation of CK, thiolated CK (5 $\mu$M CK incubated with 3 mM GSSG for 30 min) was incubated subsequently with GSH (15 mM; 1:5, GSSG/GSH). The activity of CK was completely restored within 2 min (results not shown), indicating that thiolation of CK is reversed rapidly by excess GSH. A similar rapid restoration in CK enzyme activity was also observed on incubation of thiolated CK with dithiothreitol (results not shown).

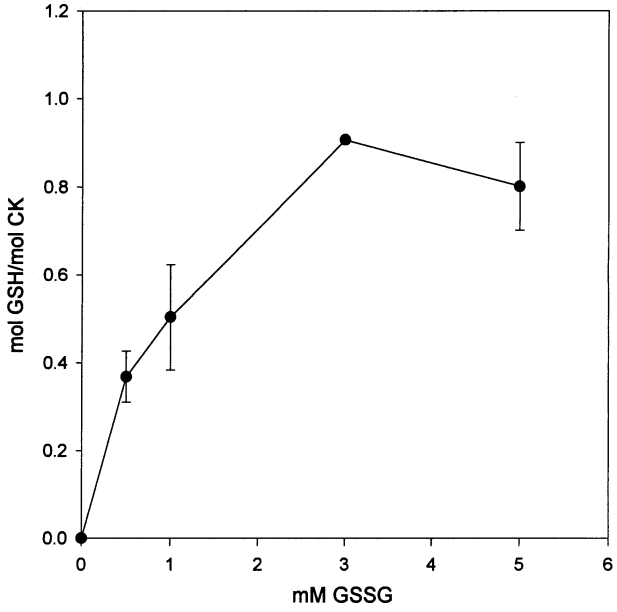


**Figure 3 Determination of the extent of thiolation of CK by GSSG**

CK was incubated with various concentrations of GSSG for 30 min, and the release of GSH during the thiolation reactions was measured by HPLC. Measurements were performed in triplicate. Error bars represent S.D.

Since protein thiolation is reversible (see eqn. 3), the equilibrium constant can be determined by measuring GSH that is generated upon thiolation by GSSG:

$$\text{CK-SG} + \text{GSH} \leftrightarrow \text{CK} + \text{GSSG} \quad (3)$$

The concentrations of CK and GSSG at equilibrium were calculated using the GSH released and the initial concentrations of CK and GSSG. The $K_{eq} = ([\text{GSSG}][\text{CK}])/([\text{CK-SG}][\text{GSH}])$ was determined for various incubations containing varying amounts of GSSG and the average calculated as approx. 320. The half-cell potential for CK glutathionylation calculated from this equilibrium constant was $-0.099 \pm 0.01$ V based upon a reduction potential of $-0.248$ V for the GSH/GSSG half-reaction [30].

## Extent of S-glutathionylation of CK

To determine the number of thiol groups in CK that were modified by GSSG, reaction mixtures were assayed immediately for GSH release using reversed-phase HPLC. The maximum thiolation of CK was found to be nearly 1 mol of GSH/mol of protein (Figure 3), suggesting thiolation of one of the four cysteine residues present in CK. Moreover, the extent of thiolation of CK closely followed the decrease in enzyme activity

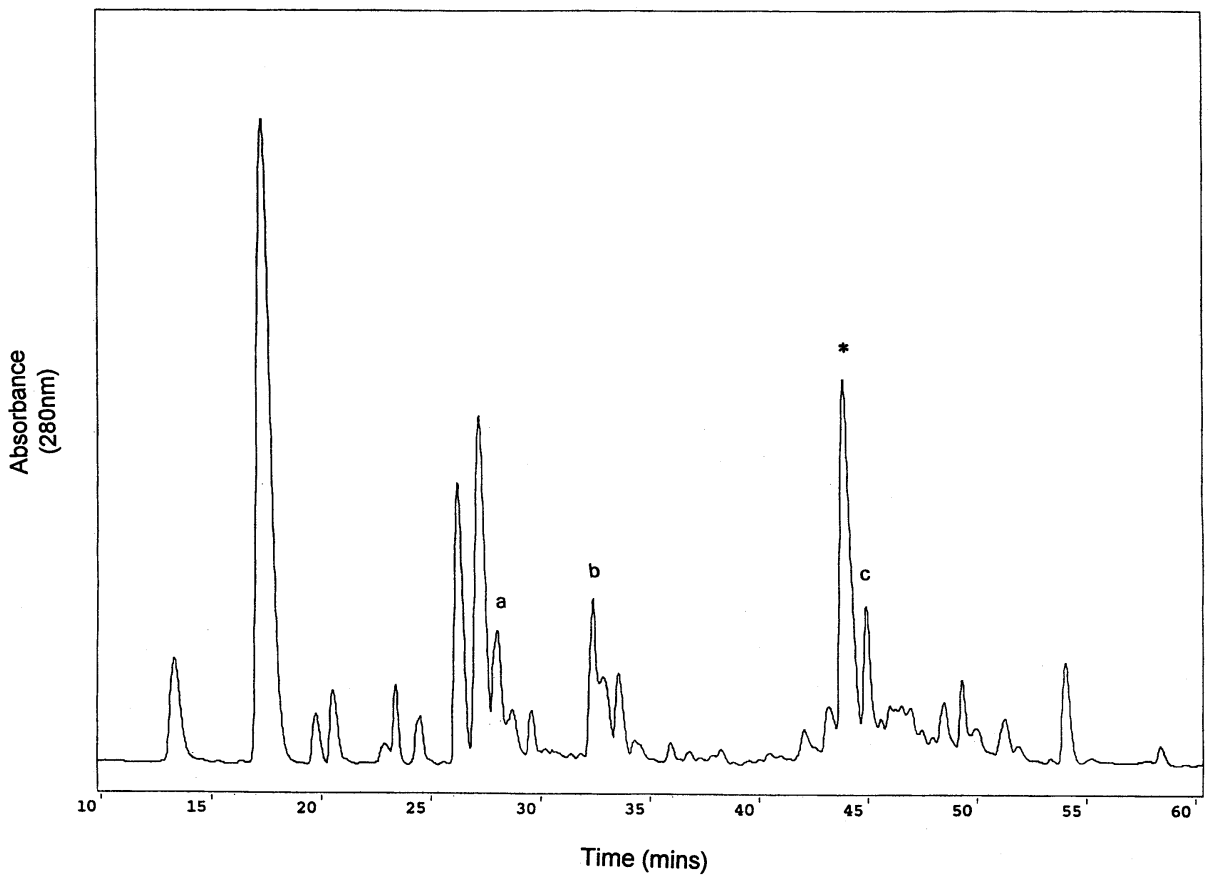


**Figure 4 HPLC separation of tryptic peptides of thiolated CK**

Thiolated CK was digested with trypsin and the resulting tryptic fragments were separated by reversed-phase HPLC and detected by UV (280 nm). The asterisk indicates the elution time of the peptide containing the active site ($Cys^{283}$). Peaks labelled a, b and c refer to the elution positions of the other three cysteine-containing peptides ($Cys^{146}$, $Cys^{254}$ and $Cys^{74}$ respectively) present in CK.

(Figure 2), consistent with the thiolation of the active-site thiol group in CK.

To determine the increase in molecular mass of CK due to thiolation, CK incubated with 5 mM GSSG was directly analysed by MALDI–TOF MS. The MALDI–TOF spectrum of thiolated CK compared with control CK showed an increase in mass of 233 Da (results not shown). Addition of one GSH residue would have resulted in a difference in 307 Da. However, since the intact protein was used for MALDI–TOF analysis, it was difficult to obtain accurate molecular-mass differences because of heterogeneity in CK and the limited resolution of the instrument. Nevertheless, the overall results suggest that a single molecule of GSH is bound to each molecule of the protein, consistent with the results shown in Figure 3.

### Site of S-glutathionylation of CK

To determine the site of thiolation of CK, both unmodified and thiolated proteins were subjected to trypsin digestion and the molecular masses of the tryptic peptides were determined after alkylation of unmodified cysteine residues with NEM and separation by HPLC. Figure 4 shows the HPLC separation of tryptic fragments of S-gluthionylated CK. The tryptic digest of unmodified CK yielded an identical HPLC profile (results not shown). The molecular masses of the peptides collected during HPLC analysis were determined by MALDI–TOF MS. The peptide eluting at 44 min had an average isotopic mass of 2998.2 Da in the case of untreated CK (Figure 5a), compared with 3178.5 Da for the same peptide from thiolated CK (Figure 5b). Subtraction of the molecular mass of either NEM (125.1 Da; untreated CK) or GSH (307.3 Da; thiolated CK) yielded a molecular mass of 2873.2 Da in both cases. The expected molecular mass of the tryptic fragment $[M+H]$ containing the active-site cysteine residue ($Cys^{283}$; AGHPFMWNEHLGYV-LTCPSNLGTGLR) is 2871.3 Da [31]. Thus the peptide eluting at 44 min was identified as the active-site peptide. The discrepancy of about 2 Da between the observed and the theoretical molecular masses of the peptide was probably due to deamidation of the two asparagines in the peptide, which results in a molecular mass increase of 1 Da for each deamidation [32]. No NEM-modified peptide was observed in the MALDI–TOF spectrum of the collected active-site peptide from thiolated CK (Figure 5b), suggesting that thiolation of the active-site peptide was quantitative. The molecular masses of the other three cysteine-containing peptides in the rabbit skeletal muscle M chain are 4374.8 ($Cys^{74}$), 793.4 ($Cys^{254}$) and 1129.5 ($Cys^{146}$) Da [31]. As these cysteine-containing tryptic fragments had unique masses that were distinct from each of the remaining tryptic fragments of CK [31], they could be easily identified by MALDI–TOF MS analysis. The 1129.5 Da peptide eluted at 28 min and was

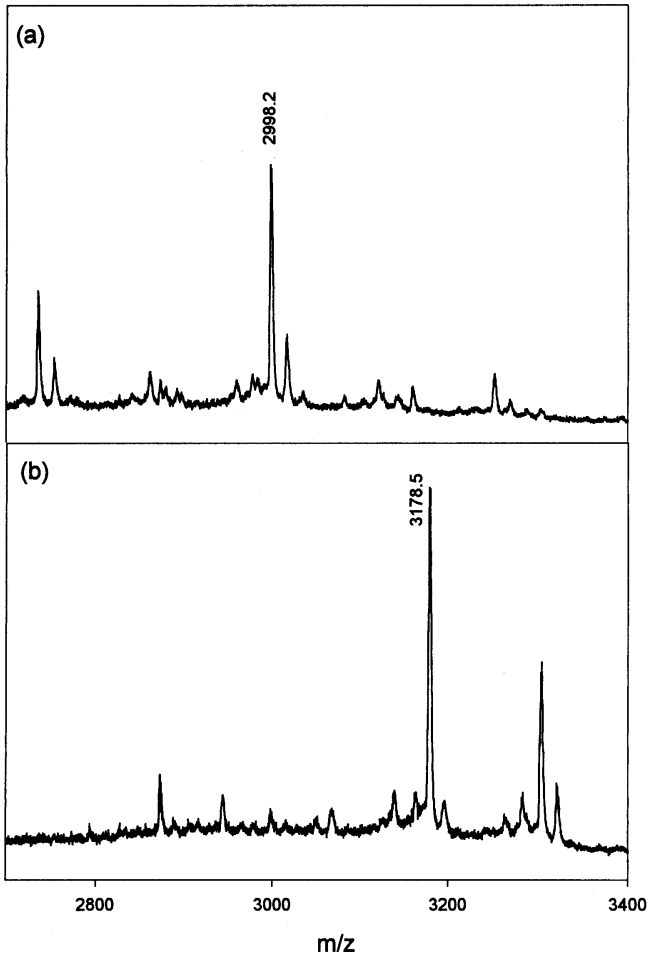


**Figure 5 MALDI–TOF spectra of active-site peptide of unmodified and thiolated CK**

(**a**) MALDI–TOF mass spectrum of the tryptic peptide from unmodified CK, containing the active-site cysteine residue derivatized with NEM (125.1 Da). The molecular mass of the underivatized peptide can be calculated as 2873.2 Da (2998.2 − 125.1 = 2873.1). (**b**) MALDI–TOF mass spectrum of the corresponding peptide of S-glutathiolated CK. The molecular mass of the tryptic fragment was determined by subtracting 305.3 (the molecular mass of GSH-2), yielding a value of 2873.3 Da. The expected mass of the active-site cysteine containing tryptic fragment (AGHPFMWNEHLGYVLTCPSNLGTGLR) from literature values is 2871.3 Da [31].

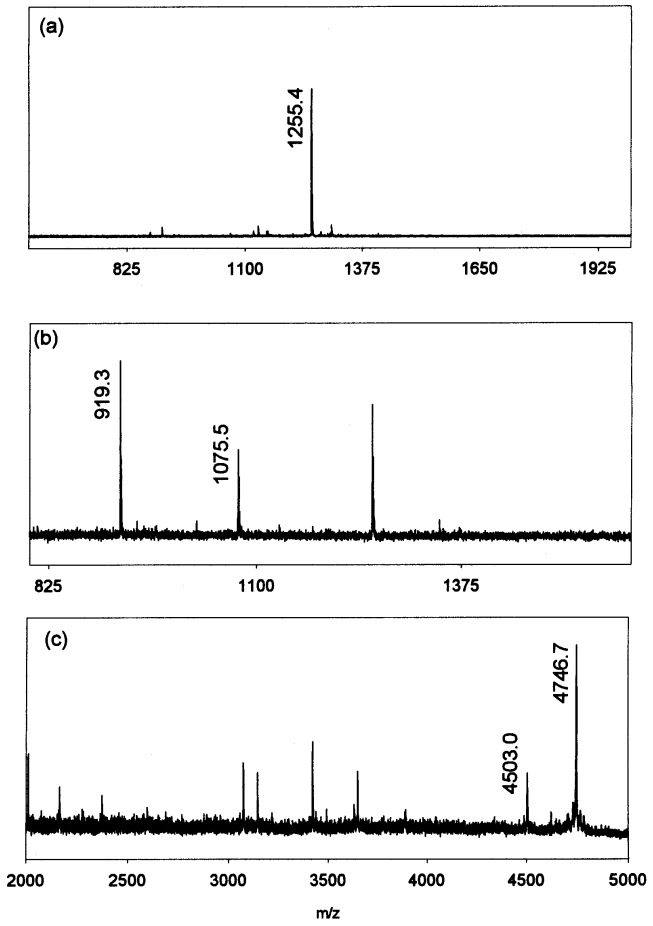


**Figure 6 MALDI–TOF spectra of cysteine-containing peptides of thiolated CK**

(**a**) MALDI–TOF spectrum of peptide a in Figure 4. Subtraction of NEM from the peptide mass results in a mass of 1130.3 Da. This corresponds closely to the mass of the $Cys^{146}$-containing peptide (GYTLPPHCSR), which has a mass of 1129.5 Da. No peak corresponding to the peptide-GSH adduct was detected. (**b**) MALDI–TOF spectrum of peptide b (Figure 4), which has a molecular mass of [919.3 − 125.1 (NEM)] = 794.2 Da. This corresponds to the molecular mass of a $Cys^{254}$-containing peptide (FCVGLQK) that has a mass of 793.4 Da. No GSH-modified peptide (increase of 305 Da) was detected. (**c**) MALDI–TOF spectrum of peptide c (Figure 4), with a mass of approx. 4503 Da; subtraction of NEM from the peptide mass results in 4378 Da. This corresponds closely to the mass of a $Cys^{74}$-containing peptide (ETPSGFTLDDVIQTGV-DNPGHPFIMTVGCVAGDEESYTVFK), which has a mass of 4374.8 Da. The corresponding GSH modification of the peptide was not observed.

observed to be modified by NEM in both control CK and in CK-GSSG ([$M$ + H] of 1255.4 Da; Figure 6a). The other cysteine-containing peptides with molecular masses of 793.99 and 4374.8 Da eluted at 32 and 45 min respectively. Again, only one peak consistent with NEM modification was found (Figures 6b and 6c) in both control and thiolated CK. Thus except for the active-site peptide, the remaining cysteine-containing peptides were not modified by GSSG under our incubation conditions, as only NEM-modified cysteine-containing peptides were detected. Hence, our results indicate that the active-site cysteine residue ($Cys^{283}$) in CK is the sole target of thiolation by GSSG.

## DISCUSSION

Previous studies have shown that the majority of protein S-thiolation *in vivo* involves primarily the formation of protein thiol adducts with GSH, rather than with other low-molecular-mass thiols such as cysteine [8]. Therefore this study was limited to protein S-glutathionylation. Incubation of CK with GSSG resulted in inactivation of the enzyme, which could be reversed in the presence of excess GSH or dithiothreitol, consistent with thiolation of the active-site Cys residue. Using HPLC techniques and MALDI–TOF MS analysis, it was concluded that only one cysteine residue in CK was thiolated. Accordingly, MS analysis of tryptic digests showed thiolation of only the active-site cysteine residue of CK but not the other cysteine residues present in the protein. The reactivity of protein thiol groups is dependent on their p$K_a$, which in turn is affected by microenvironments around the residues. The active-site cysteine may have a lower p$K_a$ compared with the remaining cysteine groups on the protein, thus making it more reactive to GSSG. Previous investigators have shown two reactive cysteine groups within the enzyme that are modified by the thiol-binding agent, dithio-2-nitrobenzoic

acid [33,34]. However, dithio-2-nitrobenzoic acid is more hydrophobic than GSSG and may have greater access to cysteine residues that are more buried in hydrophobic pockets of CK, thus leading to modification of residues other than the active-site cysteine. We used rabbit M chain CK to study thiolation, which has approx. 90 % homology to the B chain of rabbit CK [31] and to human B and M isoenzymes [35]. Thus it is feasible that the kinetics and site of thiolation of human isoenzymes may be similar to those of the rabbit M chain.

Based on the redox half-cell potential for CK S-glutathionylation determined in this study, it would require high, non-physiological concentrations of GSSG to cause protein thiolation by this mechanism [2]. However, we cannot exclude the occurrence of pronounced changes in GSH/GSSG ratios in subcellular compartments or in the extracellular space [36].

S-glutathionylated CK can be dethiolated in the presence of excess GSH, as indicated from the equilibrium constant derived in this study. The dethiolation of proteins is known to be catalysed by enzymes such as thioredoxin, glutaredoxin and protein disulphide-isomerase [30]. The half-cell potential of the reduction of S-glutathionylated CK was determined as $-0.099 \pm 0.01$ V, which suggests that the protein can be dethiolated by thioredoxin (the reduction potential of thioredoxin is $-0.260$). However, thioredoxin does not appear to reduce thiolated CK effectively compared with GSH, suggesting that the dethiolating ability of enzymes such as thioredoxin is not directly related to their reduction potential [37]. The results of our findings suggest that recovery of CK activity after, for example, ischaemic injury, occurs rapidly on restoration of GSH/GSSG ratios in the cell, even without catalytic assistance from glutaredoxin or thioredoxin. Further comparative studies of these reactions under circumstances *in vivo* are warranted.

A speculative implication of our studies is that S-glutathionylation of proteins may have a protective role when the cell experiences oxidative and/or nitrosative stress. During ischaemic injury of cardiac or skeletal muscle, the S-glutathionylation of CK may protect the enzyme from irreversible modifications of its active-site thiol residues. Ischaemic injury is often characterized by a prolonged decrease in phosphotransfer through the CK reaction (eqn. 1). Since the CK can be dethiolated rapidly by GSH, our findings suggest that a prolonged decrease in CK activity would require irreversible modification of CK, although the possibility exists that some compartmental sequestered stores of GSH do not rapidly recover after ischaemic injury. The mitochondria are a major source of reactive oxygen species, thus mitochondrial CK may be a more direct target of oxidation compared with cytosolic CK [38], leading to disruption in the phosphotransfer between creatine phosphate and ATP. Future experiments should investigate the extent of S-glutathionylation of CK and regeneration of active CK in the mitochondria versus the cytosol to further understand the biochemical mechanisms of reperfusion injury in ischaemic muscle tissues.

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## REFERENCES

1 Thomas, J. A. and Sies, H. (1992) Protein S-thiolation and dethiolation. In The Post-Translational Modification of Proteins, Roles in Molecular and Cellular Biology (Tuboi, S., Taniguchi, N. and Katnuma, N., eds.), pp. 35–51, CRC Press, Japan

2 Chai, Y. C., Ashraf, S. S., Rokutan, K., Johnston, R. B. and Thomas, J. A. (1994) S-thiolation of individual human neutrophil proteins including actin by stimulation of the respiratory burst: evidence against a role for glutathione disulfide. Arch. Biochem. Biophys. **310**, 273–281

3 Collison, M. W., Beidler, D., Grimm, L. M. and Thomas, J. A. (1986) A comparison of protein S-thiolation (protein mixed-disulfide formation) in heart cells treated with t-butyl hydroperoxide or diamide. Biochim. Biophys. Acta **885**, 58–67

4 Gilbert, H. F. (1982) Biological disulfides: the third messenger? Modulation of phosphofructokinase activity by thiol/disulfide exchange. J. Biol. Chem. **257**, 12086–12091

5 Ernest, M. J. and Kim, K. H. (1974) Regulation of rat liver glycogen synthetase D. Role of glucose 6-phosphate and enzyme sulfhydryl groups in activity and glycogen binding. J. Biol. Chem. **249**, 5011–5018

6 Nakashima, K., Horecker, B. L. and Pontremoli, S. (1970) Regulatory sulfhydryl groups and activation by homocystine in liver fructose diphosphatase. Arch. Biochem. Biophys. **141**, 579–587

7 Cappel, R. E. and Gilbert, H. F. (1989) The effects of mevinolin on the thiol/disulfide exchange between 3-hydroxy-3-methyglutaryl-coenzyme A reductase and glutathione. J. Biol. Chem. **264**, 9180–9187

8 Ravichandran, V., Seres, T., Moriguchi, T., Thomas, J. A. and Johnston, R. B. (1994) S-thiolation of glyceraldehyde-3-phosphate dehydrogenase induced by the phagocytosis-associated respiratory burst in blood monocytes. J. Biol. Chem. **269**, 25010–25015

9 Collison, M. W. and Thomas, J. A. (1987) S-thiolation of cytoplasmic cardiac creatine kinase in heart cells treated with diamide. Biochim. Biophys. Acta **928**, 121–129

10 Ward, N. E., Pierce, D. S., Chung, S. E., Gravitt, K. R. and O'Brian, C. A. (1998) Irreversible inactivation of protein kinase C by glutathione. J. Biol. Chem. **273**, 12558–12566

11 Brandwein, H. J., Lewicki, J. A. and Murad, F. (1981) Reversible inactivation of guanylate cyclase by mixed disulfide formation. J. Biol. Chem. **256**, 2958–2962

12 Silva, C. M. and Cidlowski, J. A. (1989) Direct evidence for intra- and intermolecular disulfide bond formation in the human glucocorticoid receptor. Inhibition of DNA binding and identification of a new receptor-associated protein. J. Biol. Chem. **264**, 6638–6647

13 Klatt, P., Molina, E. S., Lacoba, M. C., Padilla, C. A., Martinez-glaisteo, E., Barcena, J. A. and Lamas, S. (1999) Redox regulation of c-Jun DNA binding by reversible S-glutathiolation. FASEB J. **13**, 1481–1490

14 Klatt, P., Molina, E. S. and Lamas, S. (1999) Nitric oxide inhibits c-Jun DNA binding by specifically targeted S-glutathionylation. J. Biol. Chem. **22**, 15857–15864

15 Jahngen-Hodge, J., Obin, M. S., Gong, X., Shang, F., Novell, T. R., Gong, J., Abasi, H., Blumberg, J. and Taylor, A. (1997) Regulation of ubiquitin-conjugating enzymes by glutathione following oxidative stress. J. Biol. Chem. **272**, 28218–28226

16 Latour, I., Tos, E. D., Denef, J-F. and Calderon, P. D. (1999) Protein S-thiolation can mediate the inhibition of protein synthesis induced by tert-butyl hydroperoxide in isolated rat hepatocytes. Toxicol. Appl. Pharmacol. **160**, 1–9

17 Mekhfi, H., Veksler, V., Mateo, P., Maupoil, V., Rochette, L. and Ventura-Clapier, R. (1996) Creatine kinase is the main target of reactive oxygen species in cardiac myofibrils. Circ. Res. **78**, 1016–1027

18 Bittl, J. A., Balschi, J. A. and Ingwall, J. S. (1987) Contractile failure and high-energy phosphate turnover during hypoxia: $^{31}$P-NMR surface coil studies in living rat. Circ. Res. **60**, 871–878

19 Neubauer, S., Hammon, B. L., Perry, S. B., Bittl, J. B. and Ingwall, J. S. (1988) Velocity of the creatine kinase reaction decreases in postischemic myocardium: a $^{31}$P-NMR magnetization transfer study of the isolated ferret heart. Circ. Res. **63**, 1–15

20 Gilbert, H. F. (1995) Thiol/disulfide exchange equilibria and disulfide bond stability. Methods Enzymol. **251**, 8–28

21 Thomas, J. A., Poland, B. and Honzatko, R. (1995) Protein sulfhydryls and their role in the antioxidant function of protein S-thiolation. Arch. Biochem. Biophys. **319**, 1–9

22 Lee, S.-G., Kwon, K.-S., Kim, S.-R. and Rhee, S. G. (1998) Reversible inactivation of protein-tyrosine phosphatase 1B in A431 cells stimulated with epidermal growth factor. J. Biol. Chem. **272**, 15366–15372

23 Barette, W. C., DeGnore, J. P., Konig, S., Fales, H. M., Keng, Y.-F., Zhang, Z.-h., Yim, M. B. and Chock, P. B. (1999) Regulation of PTP 1B via glutathionylation of the active site cysteine 215. Biochemistry **38**, 6699–6705

24 Padgett, C. M. and Whorton, A. R. (1998) Cellular responses to nitric oxide: role of protein S-thiolation/dethiolation. Arch. Biochem. Biophys. **358**, 232–242

25 Ji, Y., Akerboom, T. P. M., Sies, H. and Thomas, J. A. (1999) S-nitrosylation and S-glutathiolation of protein sulfhydryls by S-nitroso glutathione. Arch. Biochem. Biophys. **362**, 67–78

26 Mohr, S., Hallak, H., Boitte, A. and Lapetina, E. G. (1999) Nitric oxide-induced S-glutathionylation and inactivation of glyceraldehyde-3-phosphate dehydrogenase. J. Biol. Chem. **274**, 9427–9430

27 Thomas, J. A and Biedler, D. (1986) A thin-gel isoelectric focusing method for quantitation of protein S-thiolation. Anal. Biochem. **157**, 32–38

28 Kosower, N. S. and Kosower, E. M. (1998) The glutathione status of cells. Int. Rev. Cytol. **54**, 109–159

29 Newton, G. L., Dorain, R. and Fahey, R. C. (1981) Analysis of biological thiols: derivatization with monobromobimane and separation by reverse-phase high-performance liquid chromatography. Anal. Biochem. **114**, 383–387
30 Jung, C. H. and Thomas, J. A. (1996) S-glutathiolated hepatocyte proteins and insulin disulfides as substrates for reduction by glutaredoxin, thioredoxin, protein disulfide isomerase, and glutathione. Arch. Biochem. Biophys. **335**, 61–72
31 Pickering, L., Pang, H., Biemann, K., Munro, H. and Schimmel, P. (1985) Two tissue-specific isozymes of creatine kinase have closely matched amino acid sequences. Proc. Natl. Acad. Sci. U.S.A. **82**, 2310–2314
32 Wood, T. D., Chen, L. H., Kelleher, N. L., Little, D. P., Kenyon, G. L. and McLafferty, F. W. (1995) Direct sequence data from heterogeneous creatine kinase (43 kDa) by high-resolution tandem mass spectrometry. Biochemistry **34**, 16251–16253
33 Zhou, H.-M. and Tsou, C.-L. (1987) The presence of reactive SH groups in the enzymatically active dicyano derivative of creatine kinase. Biochim. Biophys. Acta **911**, 136–143
34 Yang, Y. and Zhou, H.-M. (1998) Reactivation kinetics of 5,5′-dithiobis-(2-nitrobenzoic acid)-modified creatine kinase reactivated by dithiothreitol. Biochim. Biophys. Acta **1388**, 190–198
35 Perryman, M. B., Kerner, S. A., Bohlmeyer, T. J. and Roberts, R. (1986) Isolation and sequence analysis of a full-length cDNA for human M creatine kinase. Biochem. Biophys. Res. Commun. **140**, 981–989
36 Hochachka, P. W. (1999) The metabolic implications of intracellular circulation. Proc. Natl. Acad. Sci. U.S.A. **96**, 12233–12239
37 Park, E. M. and Thomas, J. A. (1989) Reduction of protein mixed disulfides (dethiolation) by *Escherichia coli* thioredoxin: a study with glycogen phosphorylase b and creatine kinase. Arch. Biochem. Biophys. **272**, 25–31
38 Stachowiak, O., Dolder, M., Walliman, T. and Richter, C. (1998) Mitochondrial creatine kinase is a prime target of peroxynitrite-induced modification and inactivation. J. Biol. Chem. **273**, 16694–16699

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