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A new method for the detection and characterization of α-lipoic acid mixed disulphides

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Abstract

α-Lipoic acid (LA) acts as a direct regulator of intracellular redox status through the formation of mixed disulphides. However, as this is reversible, the evidence for the mixed disulphides has not been obtained. This study established a method for the detection and characterization of mixed disulphides by mass spectrometry (MS) and was the first to provide direct evidence for their formation. When cysteine methyl ester was incubated with LA in the presence of iodeacetamide (IAA), a mixed disulphide with mono carbamidomethylation was observed. MS/MS analysis indicated that the LA forms a mixed disulphide with the cysteinyl sulphydryls, while the other sulphydryl group is the carbamidomethylated. The same results were obtained from the incubation of sulfphydryl peptides such as glutathione with LA in the presence of IAA. These results may provide further biological evidence that LA is a potential modifier of intracellular sulphydryls through mixed disulfide formation.

Keywords: *α-Lipoic acid, glutathione, mixed disulfide, sulphydryls, mass spectrometry*

Abbreviations: *CAM, carbamidomethylated; CaMKII, calmodulin-dependent protein kinase II 281–289; CysOMe,* L-*Cysteine methyl ester; DHLA, dihydrolipoic acid; GSH, reduced glutathione; GSSG, oxidized glutathione; HPLC, high performance liquid chromatography; IAA, iodeacetamide; LA, α-Lipoic acid; MS, mass spectrometry; MS/MS, tandem mass spectrometry; MALDI-TOF, matrix-assisted laser desorption ionization-time-of-flight; PBS, phosphate-buffered saline; TFA, trifluoroacetic acid.*

Introduction

The thiol redox status of the intracellular compartment is a critical determinant of protein structure, regulation of enzyme activity and control of transcription factor activity and binding [1]. Thiol antioxidants act through a variety of mechanisms, including as components of the general redox process (thiol/disulphide), as radical quenchers, metal chelators, substrates for specific redox reactions and as specific reductants of individual protein disulphide bonds [2]. Because of the free thiol group in the cysteine residue and its abundance within cells, reduced glutathione (GSH) is a major endogenous antioxidant within cells. Elevated levels of oxidized glutathione (GSSG) can affect the function of multiple proteins via glutathionylation and cause cell death through decreasing reduced GSH levels and increasing accumulation of GSSG [3,4]. Thus, the normal thiol/disulphide redox potential maintains the functions of many cellular proteins and enzymes, including many of those involved in cell death and survival cascades.

α-Lipoic acid (LA), a dithiol compound, is a necessary protein-bound cofactor for mitochondrial enzymes, such as *α*-ketoacid dehydrogenases, and thus serves a critical role in mitochondrial energy metabolism. In its oxidized form, LA is synthesized in the body as well as absorbed from the diet, which is then taken up by cells and tissues and reduced to

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dihydrolipoic acid (DHLA). Orally-derived LA has unique biochemical properties separate from its normal metabolic function. Although both LA and DHLA have been reported to act as a pro-oxidant under certain circumstance, they are generally thought to be strong antioxidants [5]. The antioxidant efficiency of LA has been attributed to the unique properties of the LA/DHLA system, such as its reactive oxygen species scavenging ability and its significant effect on tissue concentrations of the reduced forms of other antioxidants [6]. Moreover, LA appears to affect gene expression and a number of signal transduction pathways due to its direct chemical reactivity with redox-sensitive thiol groups [7,8]. It may regulate redox status through alteration of the redox balance via the formation of mixed disulphides. However, as this is reversible, the evidence for mixed disulphide formation has been sparse (Figure 1A) [9].

In the present study, we report a new method for the detection and characterization of a mixed disulphide composed of LA with sulphydryls using mass spectrometry (MS).

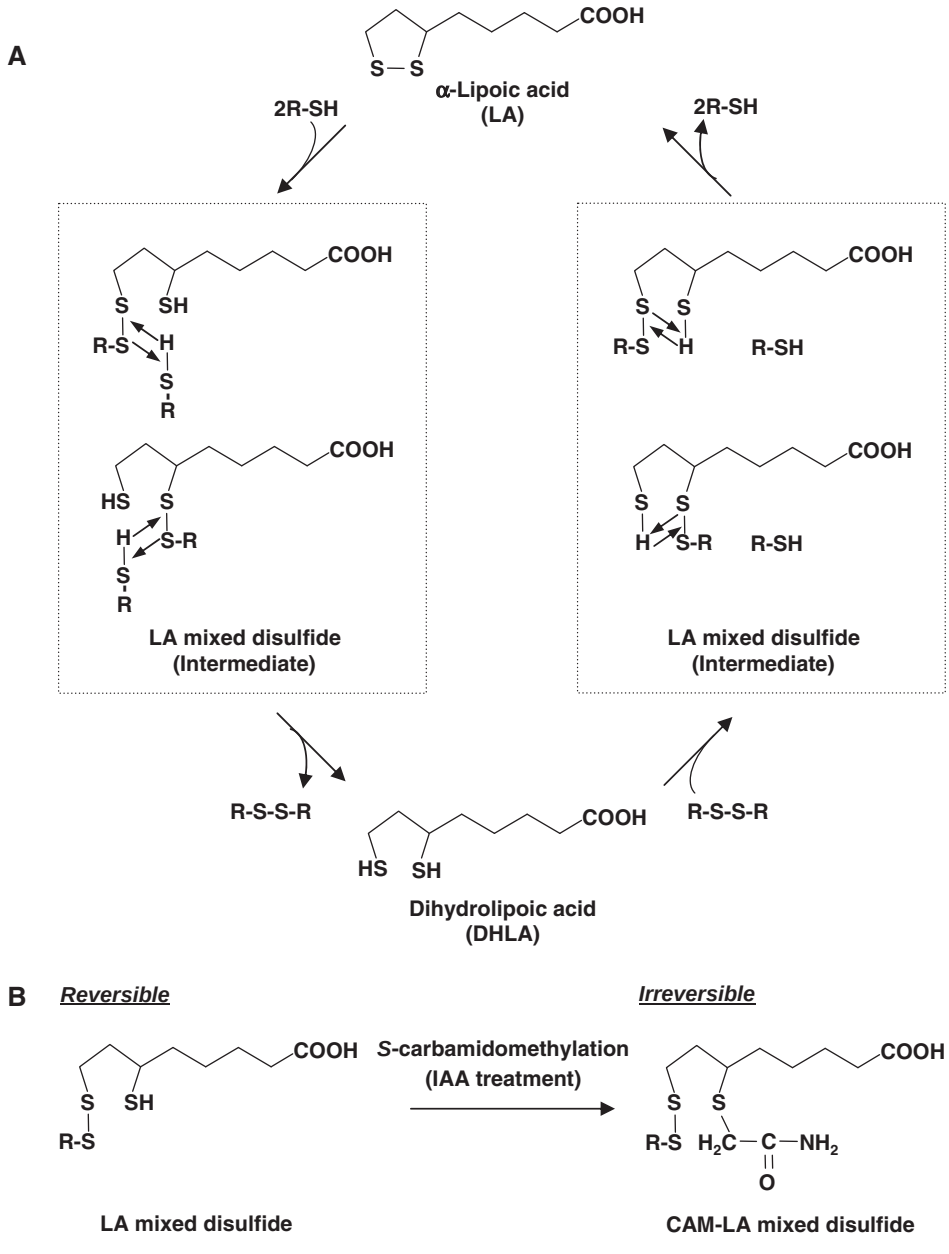

Figure 1. Mixed disulphide formation of *α*-lipoic acid. (A) Mechanism for reversible sulphydryl modification of *α*-lipoic acid. *α*-Lipoic acid (LA/DHLA) reacts with sulphydryls (R-SH/R-S-S-R) for the formation of LA mixed disulphide through thiol/disulphide exchange reaction. LA mixed disulphide is unstable because of inter- or intra-molecular thiol/disulphide exchange reactions. (B) Stabilization of LA mixed disulphide by *S*-carbamidomethylation. A free thiol group on LA is carbamidomethylated (CAM) by IAA during the formation of intermediate sulphydryls. The CAM-LA mixed disulphide is more stable than LA mixed disulphide containing a free thiol group because the ability of thiol/disulphide exchange is blocked.

Materials and methods

Materials

L-Cysteine methyl ester (CysOMe), GSH and LA were purchased from Sigma (St. Louis, MO). Calmodulin-dependent protein kinase II 281−289 (CaMKII, Seq.: MHRQETVDC) and all other reagents were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

High performance liquid chromatography-tandem MS (HPLC-MS/MS)

CysOMe (2.5 mM) or GSH (2.5 mM) were incubated with 2.5 mM LA at 37°C for 1 h in phosphate-buffered saline (PBS) containing 10% ethanol with or without 50 mM iodeacetamide (IAA). Each sample was analysed by high performance liquid chromatography-tandem MS (HPLC-MS/MS). Products were separated on an 1100 series HPLC system (Agilent Technologies, Santa Clara, CA) using a ZORBAX StableBond C18 column (5 μm, 150 × 0.5 mm i.d., Agilent Technologies). Samples were eluted with a linear gradient of 0.1% formic acid in water (solvent A) and 0.1% formic acid/acetonitrile (solvent B) (time = 0 min, 1% B; 0–4 min, 1% B; 4–8 min, 30% B; 8–15 min, 75% B; 15–20 min, 100% B; 20–23 min, 100% B; 23–25 min, 1% B). The flow rate was 15 μL/min and the column temperature was controlled at 37°C. The MS (MS/MS) analyses were performed on an LCQ ion trap mass system (Thermo Fisher Scientific, Inc., San Jose, CA) equipped with an electrospray ion source. Collision-induced dissociation experiments in the positive or negative ion mode were performed by setting the relative collision energy at 30% and using helium as the collision gas.

Matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS)

CaMKII (0.1 mg/mL) was incubated with 100 μM LA at 37°C for 1 h in PBS with or without 10 mM IAA and then mixed with trifluoroacetic acid (TFA). To improve the ionization efficiency of MS, samples were purified with Zip Tip μ-C18 (Millipore, Bedford, MA) before MALDI-TOF MS analysis. Peptides were mixed with 2.5 mg/ml α-cyano-4-hydroxycinnamic acid containing 50% acetonitrile and 0.1% TFA and dried on stainless steel targets at room temperature and pressure. The analyses were performed using an UltraFLEX MALDI-TOF MS (Bruker Daltonics, Ltd., Bremen, Germany). All analyses were carried out in the positive ion mode and the instrument was calibrated immediately prior to each series of studies.

Results and discussion

Development of a method for the detection and characterization of mixed disulphides by mass spectrometry

To indicate the evidence for mixed disulphide formation of LA, we established a method for the detection and characterization of mixed disulphides by MS. In this method, a free thiol group on LA was carbamidomethylated (CAM) by IAA during the formation of intermediate sulphydryls. The CAM-mixed disulphide is more stable than mixed disulphide containing a free thiol group because the ability of thiol/disulphide exchange is blocked (Figure 1B). Figure 2A presents the total ion chromatogram (TIC) for each sample. When CysOMe was incubated with LA in the presence of IAA, ~ 12% of LA was lost after a 1-h incubation, suggesting that LA reduced by CysOMe through the formation of mixed disulphide (data not shown). However, the LA mixed disulphide was less detected by HPLC with UV detection. On the other hand, a new-negative ion peak at *m/z* 397.5 (namely P-1; t_R 10.7 min), which corresponded to the formation of mixed disulphide with mono carbamidomethylation (+57 Da), was observed (Figure 2A, right panel). No P-1 peak was seen when incubating in the absence of IAA (Figure 2A, left panel), however. Furthermore, the P-1 peak was not detected following a reaction between *S*-oxidized CySOMe and LA (data not shown). These results strongly suggest that one of the two thiols in LA forms a mixed disulphide with the cysteinyl thiol in CysOMe, while the other thiol is carbamidomethylated with IAA. Moreover, several fragment ions were observed in MS/MS analysis of the $[M - H]^-$ at *m/z* 397.5 (Figure 2B). The fragment ions (366.1 338.9, 309.1, 263.8, 230.8, 185.0 and 171.1) revealed that P-1 is S1- or S2-CAM-LA with CysOMe at the other sulphydryl on the dithiolane moiety (Figure 2C). These results indicate that LA forms a mixed disulphide with the cysteinyl sulphydryl, while the S2 sulphydryl group is carbamidomethylated with IAA.

Detection and characterization of LA mixed disulfides with GSH

Reed et al. [10] reported that lipoic acid *in vivo* might exist as mixed disulphide with GSH [10]. To indicate the evidence for the LA mixed disulphide with GSH, we evaluated the formation of the mixed disulphide in the present method. When GSH was incubated with LA in the presence of IAA, a new-positive ion peak at *m/z* 571.1 (namely P-2; t_R 11.6 min), which corresponded to the formation of mixed disulphide with mono carbamidomethylation (+57 Da), was observed (Figures 3A, right panel and B, left panel). However,

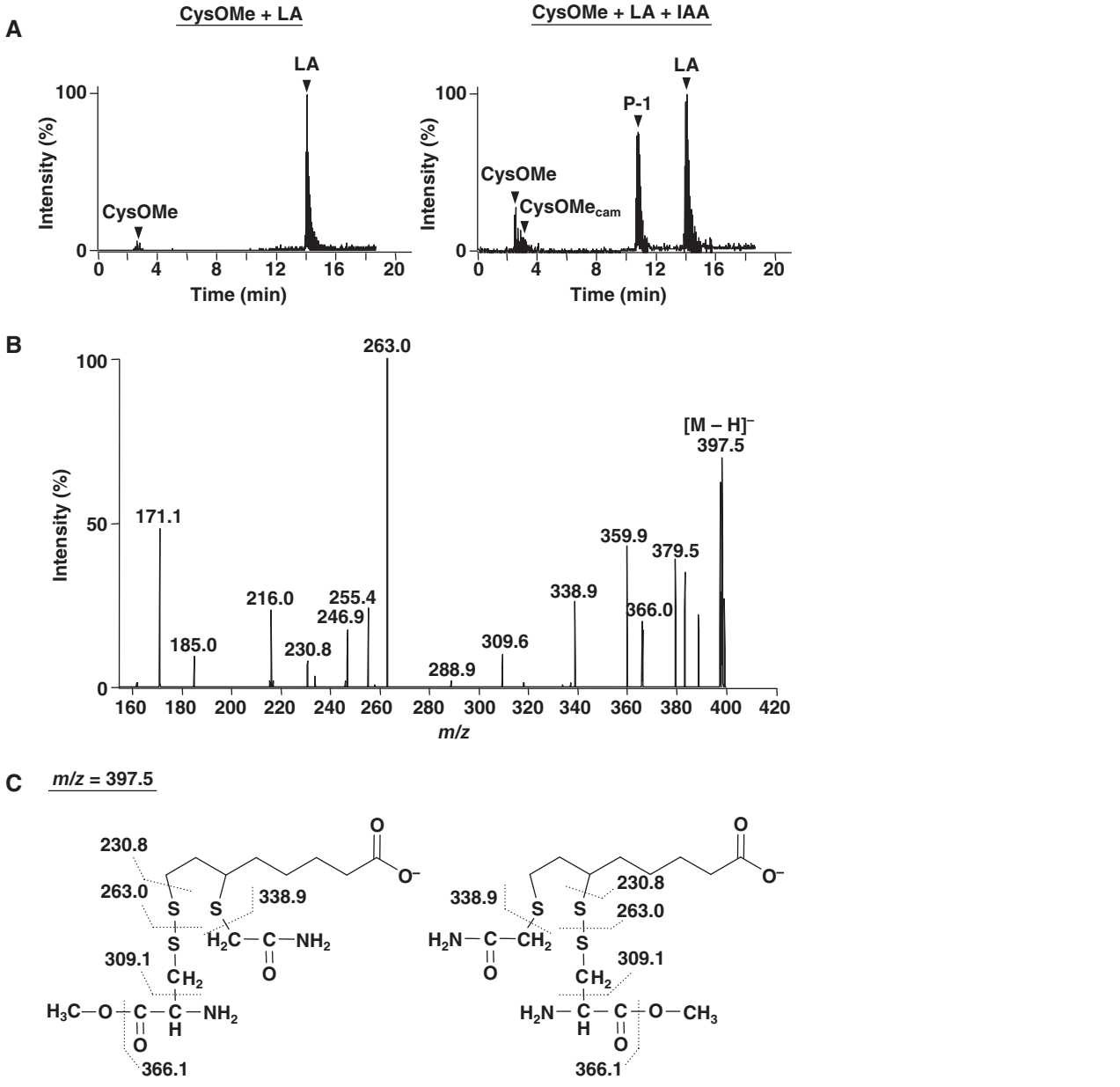

Figure 2. Detection and characterization of LA mixed disulphides with CysOMe. (A) Total ion chromatogram of LA-treated CysOMe. CysOMe (2.5 mM) was incubated with 2.5 mM LA at 37°C for 1 h in PBS with (right) or without (left) IAA. (B) MS/MS spectrum of the $[M - H]^-$ ion at *m/z* 397.5 from P-1. (B) Characterization of P-1 by assignment of the fragmentation pattern. Unassigned fragment ions may be neutral loss ion. The data do not support an assignment of which of the LA thiols (S1 and S2) is modified by CysOMe forming the mixed disulphide and which one is carbamidomethylated.

no P-2 peak was seen when incubating in the absence of IAA (Figure 4A, left panel). Furthermore, the P-2 peak was not detected following a reaction between GSSG and LA (data not shown). These results suggest that LA form CAM-mixed disulphide with the GSH. Moreover, several fragment ions were observed in MS/MS analysis of the $[M + H]^+$ at *m/z* 571.1 (Figure 3B, right panel). The fragment ions (496.0 441.9, 339.0, 295.9, 264.0 and 231.2) revealed that P-2 is S1- or S2-CAM-LA with GSH at the other sulphydryl on the dithiolane moiety (Figure 3C). These results show the direct evidence that LA forms a mixed disulphide with GSH.

S-Thiolation of protein sulfhydryls by LA

LA is thought to modulate intracellular function via effect on the redox status of thiol-containing proteins through its reactive sulphydryls. To gain further details about the formation of the mixed disulphide by LA, we used the model peptide CaMKII (MHRQET-VDC). CaMKII is a convenient model protein that contains one sulphydryl group per sub-unit and is known to be highly sensitive to modification by electrophiles *in vitro* [11]. As shown in Figure 4A, MALDI-TOF MS analysis of the native peptide revealed a peak at *m/z* 1118.5, which is in agreement

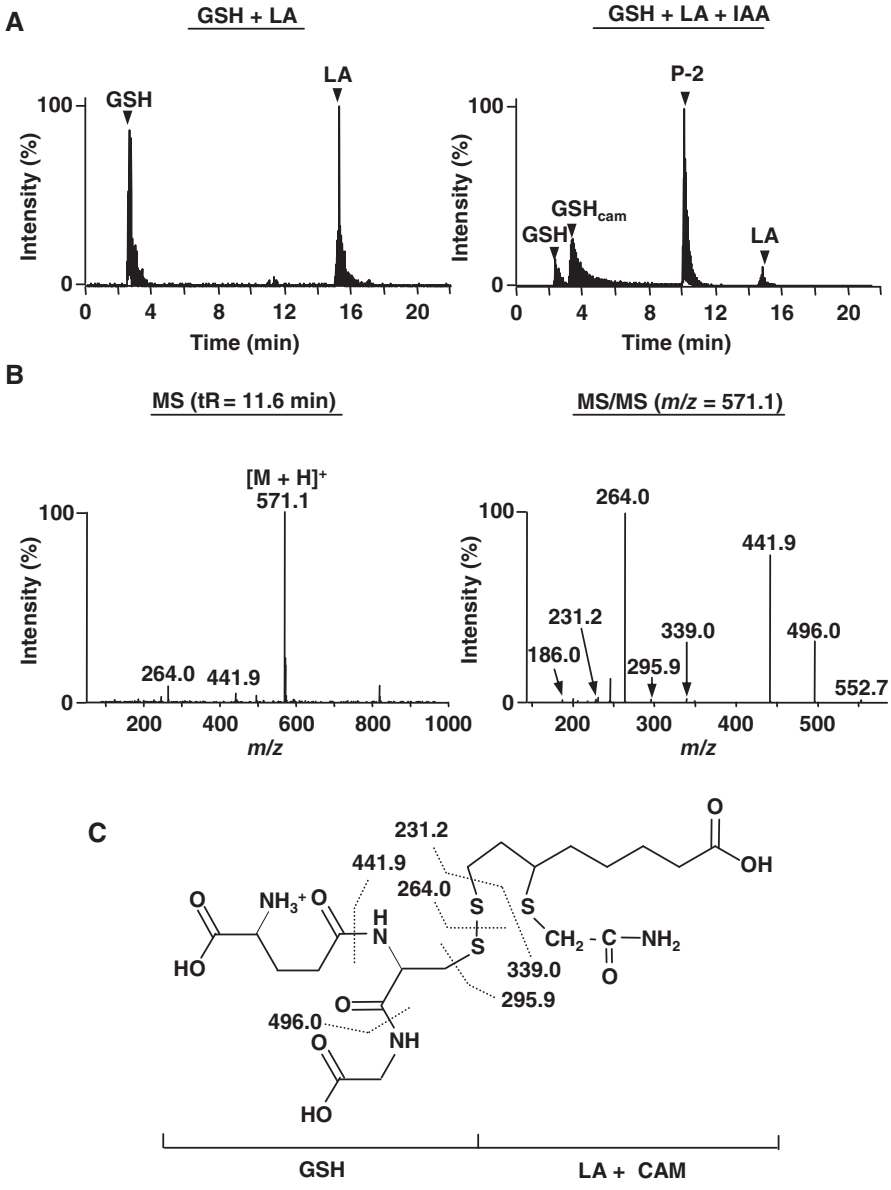

Figure 3. Detection and characterization of LA mixed disulphides with GSH. (A) Total ion chromatogram of LA-treated GSH. GSH (2.5 mM) was incubated with 2.5 mM LA at 37°C for 1 h in PBS with (right) or without (left) IAA. (B) MS (left) and MS/MS (right) spectra of the $[M + H]^+$ ion at *m/z* 571.1 from P-2. (C) Characterization of P-2 by assignment of the fragmentation pattern. Unassigned fragment ions may be neutral loss ion. The data do not support an assignment of which of the LA thiols (S1 and S2) is modified by GSH forming the mixed disulphide and which one is carbamidomethylated.

with the theoretical molecular mass from the sequence MHRQETVDC. When LA (0.1 mM) was incubated with CaMKII, LA formed an adduct with the peptide at *m/z* 1324.5. By incubation of CaMKII with LA in the presence of IAA, a mono-CAM native peptide at *m/z* 1175.5 was formed. In addition, formation of the LA adduct at *m/z* 1381.7 (namely P-3) was strongly promoted via mono-carbamidomethylation. In MS/MS analysis of the $[M + H]^+$ at *m/z* 1381.7, singly charged *N*-terminal product ions (a2, a7, b2, b3, b5, b7 and b8), NH_3 loss fragment ions (b3−17 ~ b6−17) and an H_2O adduct fragment ion (b8+18) were observed and *C*-terminal product ions (y7* and y8*) were observed with a 263 Da increase (LA addition; 206-Da, CAM; 57-Da, namely CAMLA) (Figure 4B). MS/MS analysis of P-3 showed that incubation of the LA adduct with IAA resulted in a 263 Da increase on the cysteine residue in the peptide. In addition, a peak corresponding to CAM lipoic acid molecular mass at *m/z* 263.8 was detected independently. These results show that one of the two thiols in LA forms a mixed disulphide with the cysteinyl thiol in the peptide, while the other thiol is carbamidomethylated with IAA.

S-Thiolation is an oxidative, reversible post-translational modification of cysteine residues of

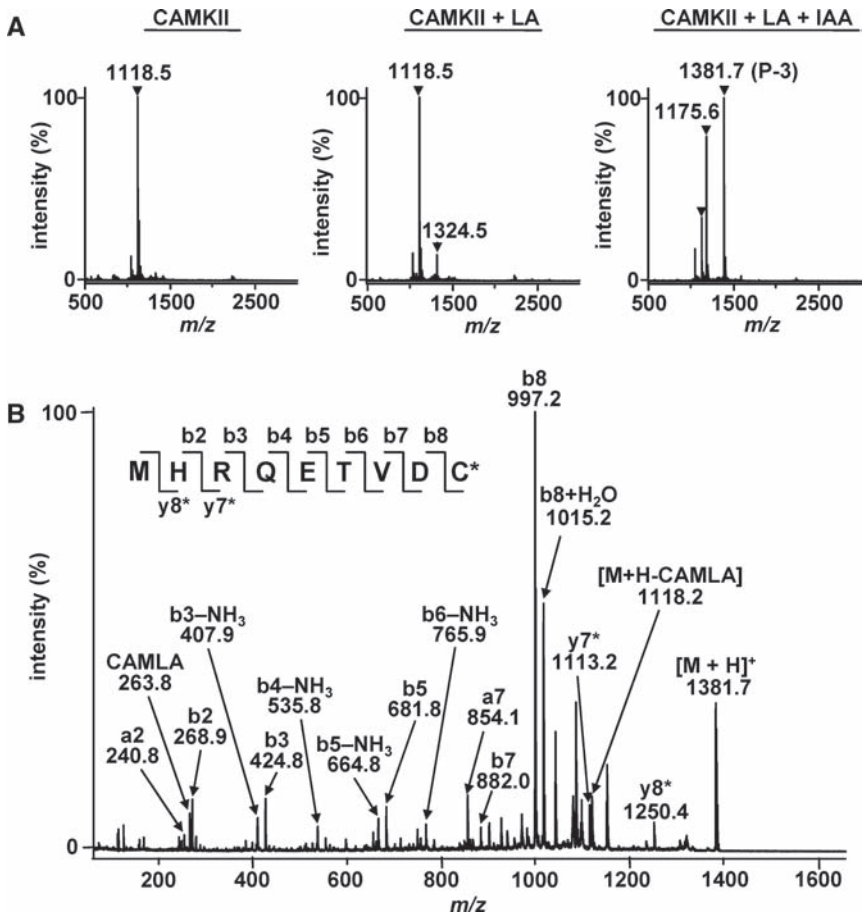

Figure 4. Detection and characterization of LA mixed disulphides with CaMKII. (A) MS analyses of LA-treated CaMKII. CaMKII (0.1 mg/mL) was incubated in the absence (left) or presence of 100 μM LA at 37°C for 1 h in PBS with (middle) or without (right) 10 mM IAA. (B) MS/MS spectra of the $[M + H]^+$ ion at *m/z* 1381.7 from P-3. The data do not support an assignment of which of the LA thiols (S1 and S2) is modified by CaMKII forming the mixed disulphide and which one is carbamidomethylated. Asterisks denote modification by both LA and IAA.

proteins and can be viewed as a protective mechanism that guards against the terminal or irreversible oxidation of these residues. Protein *S*-thiolation can be directly coupled to cellular redox status and has no absolute requirement for specialized regulatory enzymes, although thiol transferase enzymes can catalyse these reactions [12]. It has long been recognized that low-molecular-weight thiols, such as GSH, can interact in a reversible manner with the cysteine sulphydryl groups in many cellular proteins [13,14]. In particular, protein *S*-thiolation/dethiolation is a dynamic process that occurs in cells under physiological conditions as well as following exposure to an oxidative stress [15–17]. In this study, we show the direct evidence that LA forms a mixed disulphide with not only GSH but also the sulphydryl peptide (Figures 3 and 4). DHLA also form a mixed disulphide with oxidized thiol compounds such as GSSG, but can complete the reduction via intramolecular thiol exchange. In our experimental condition, CAM-GSH was detected in the incubation of DHLA with GSSG with IAA, but LA-GSH mixed disulphide was not. These results show that DHLA is a powerful reductant for intracellular disulphide linkage through mixed disulphide formation. These results and observation suggest that lipoic acid may be a potential modifier of redox-dependent cellular events through both oxidation and reduction of intracellular sulphydryls such as glutathione and protein cysteine residues.

In conclusion, we successfully performed mass spectrometric detection of mixed disulphide formation between LA and sulphydryls following treatment with IAA. *S*-Thiolation/dethiolation and formation of disulphide bonds in proteins are critical post-translational modifications, having the dual role of modulating protein functions and protecting from irreversible modification [18–21]. These modifications also comprise a dynamic reversible process that occurs in cells under physiological conditions. Searching for the target proteins of the mixed disulphide formation is the first step to understanding the molecular and biochemical mechanisms of the functional effects of LA. In conclusion, we successfully performed mass spectrometric detection of mixed disulphide formation between LA and sulphydryls following treatment with IAA. *S*-Thiolation/dethiolation and formation

of disulphide bonds in proteins are critical post-translational modifications, having the dual role of modulating protein functions and protecting from irreversible modification [18–21]. These modifications also comprise a dynamic reversible process that occurs in cells under physiological conditions. Searching for the target proteins of the mixed disulphide formation is the first step to understanding the molecular and biochemical mechanisms of the functional effects of LA. Our results may provide a foundation for future studies on the mechanism of action of LA.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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