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TIPS

Mass Spectrometry Detection and Reduction of Disulfide Adducts Between Reducing Agents and Recombinant Proteins With Highly Reactive Cysteines

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Recombinant proteins with highly reactive thiol groups can form disulfide adducts with reducing agents commonly used in protein purification, such as β-mercaptoethanol and dithiothreitol. These adducts can interfere with protein-protein or protein-ligand interactions. This report describes the reduction of persistent disulfide adducts between the reducing agents glutathione or β-mercaptoethanol and the recombinant protein Cyto-MelCAM, which were detected using matrix-assisted laser desorption and ionization (MALDI) mass spectrometry. These adducts were effectively reduced using the trialkylphosphine reducing agent tris(2-carboxyethyl)phosphine hydrochloride. (J Biomol Tech 1999;10: 17–20)

KEY WORDS: matrix-assisted laser desorption and ionization mass spectrometry, disulfide adduct, recombinant protein, reducing agent, tris(2-carboxyethyl)phosphine hydrochloride (TCEP-HCl).

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The cytoplasm is a reducing environment because of the presence of various reducing compounds, including glutathione. Protein purification strategies often include the use of reducing agents such as β-mercaptoethanol or dithiothreitol (DTT) to prevent oxidation and possible inactivation of cytoplasmic proteins when the cell is disrupted. Oxidation of critical thiol groups can lead to the formation of artifactual intramolecular or intermolecular disulfide bonds that may interfere with protein-protein or protein-ligand interactions. Matrix-assisted laser desorption and ionization mass spectrometry (MALDI-MS) is an effective and rapid screening tool for detecting adducts formed between reducing reagents and unusually reactive cysteines during purification of some recombinant proteins.

This report describes detection and reduction of disulfide adducts of the cytoplasmic domain of MelCAM, a cell adhesion molecule expressed in malignant melanoma cells.[1,2] The approximately 7-kd Cyto-MelCAM protein was expressed in *Escherichia coli* as a GST fusion protein and purified using affinity chromatography with a reduced glutathione–Sepharose column. The peptide was cleaved from the GST moiety with thrombin and purified for sedimentation equilibrium experiments designed to determine its oligomeric state. However, Cyto-MelCAM contains a single cysteine residue that proved to be highly reactive, readily forming intermolecular disulfide bonds and disulfide adducts with glutathione and β-mercaptoethanol under conditions in which disulfide adducts would not normally form. These disulfide adducts were detected using MALDI–time of flight (MALDI-TOF) mass spectrometry and reduced using the highly effective reducing agent tris(2-carboxyethyl)phosphine hydrochloride (TCEP-HCl; Pierce, Rockford, IL, USA).

METHODS

Expression and Purification of Cyto-MelCAM

Cyto-MelCAM was expressed as a GST fusion protein in *E. coli*. Construction of the expression plasmid and the expression and purification of the CytoMelCAM-

GST fusion protein was performed as previously described.[3,4] Cyto-MelCAM was cleaved from the GST molecule using bovine thrombin (Sigma) at 37°C for 3 hours (1 U thrombin/1 mg fusion protein) and purified by rechromatography on a reduced glutathione–Sepharose 4B column (Pharmacia). The eluted Cyto-MelCAM was further purified by HPLC gel filtration on a preparative Superdex 75 column (16 × 600 mm, Pharmacia) in a buffer comprising 10 mM sodium phosphate, 500 mM NaCl, 1 mM EDTA, 0.15 mM phenylmethylsulfonyl fluoride, and 0.05% sodium azide at pH 7.3.

Mass Spectrometry

MALDI-TOF mass spectrometry was carried out on a Voyager RP mass spectrometer (PerSeptive Biosystems, Framingham, MA, USA). Peptide solutions (1 μL) were mixed 1:1 with a saturated solution of sinapinic acid in 33% acetonitrile (ACN) and 0.1% trifluoroacetic acid and allowed to air dry on the sample target before analysis. Cytochrome *c* and protein A were used as external standards.

RESULTS

A MALDI-MS spectrum of Cyto-MelCAM, purified as described in Methods, is shown in Figure 1*A*. The masses of the major peaks correspond to the expected sequence molecular mass of Cyto-MelCAM monomer (MW_{seq} = 7,146) and a Cyto-MelCAM dimer (MW_{seq} = 14,290). Another significant peak was detected, with an observed mass 310 daltons

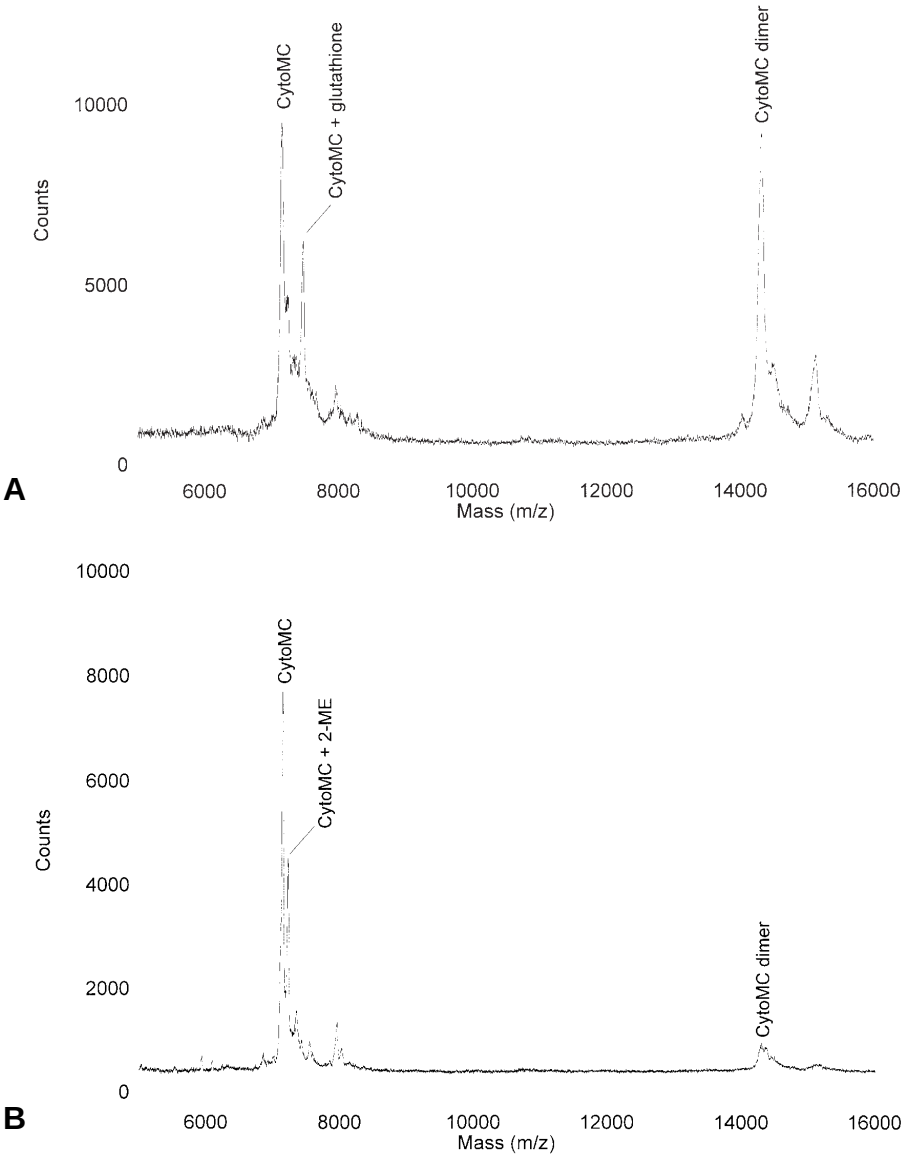

FIGURE 1

(A) MALDI-TOF mass spectrometry of Cyto-MelCAM immediately after purification. A disulfide-linked dimer and glutathione adduct of Cyto-MelCAM (CytoMC) are detected. **(B)** The same sample after incubation with 100 mm β-mercaptoethanol for 1 hour at 37°C, followed by dialysis against a buffer containing 10 mM sodium phosphate, 150 mM NaCl, and 1 mM β-mercaptoethanol at pH 7.3. A β-mercaptoethanol (2-ME) adduct of Cyto-MelCAM is formed.

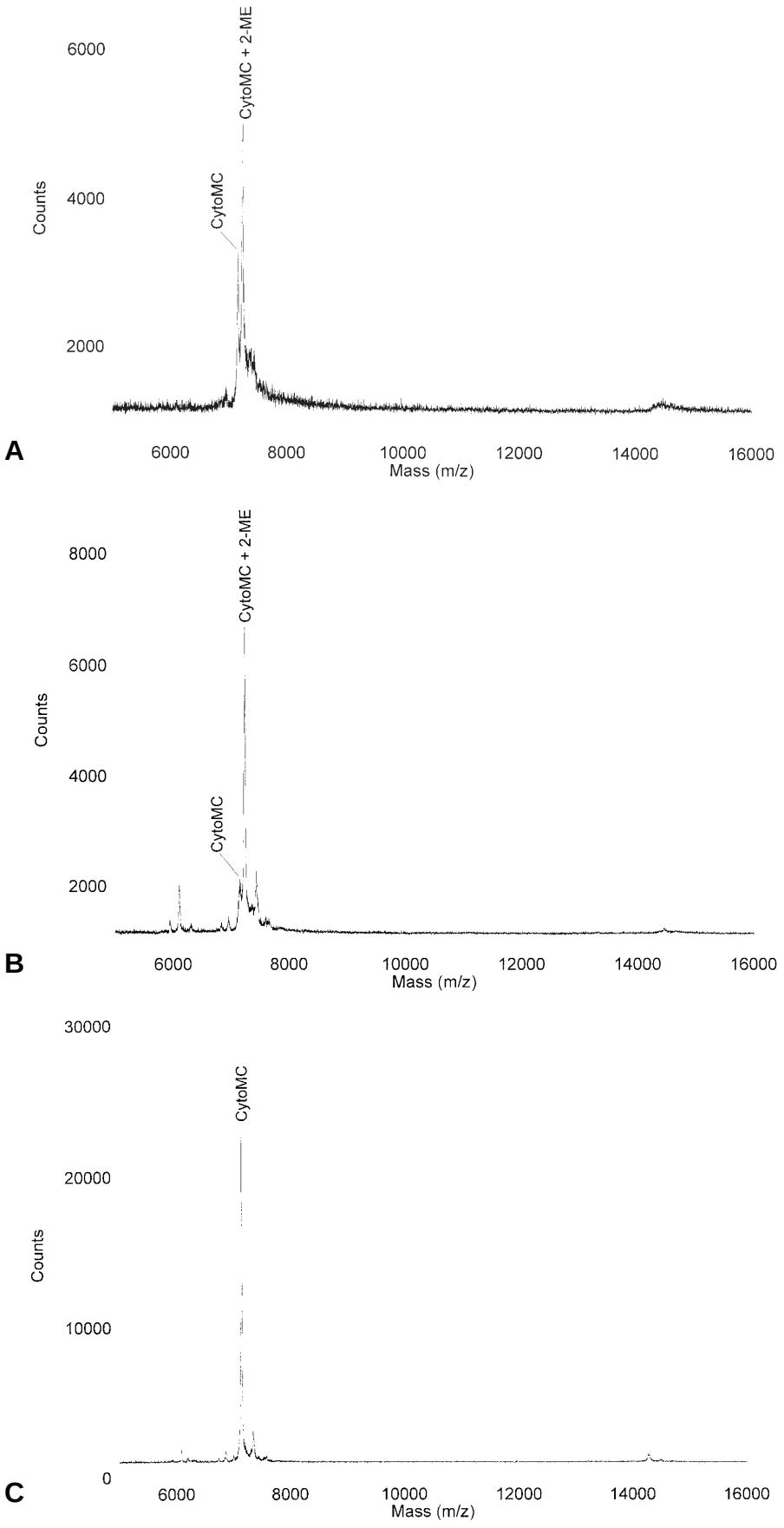

FIGURE 2

(A) MALDI-TOF mass spectrometry of Cyto-MelCAM after gel filtration with 1 mM β-mercaptoethanol in the buffer. No Cyto-MelCAM dimer is formed; however, a large proportion of Cyto-MelCAM is present as the β-mercaptoethanol adduct. **(B)** The same sample as in **A** after incubation in 100 mM β-mercaptoethanol for 1 hour at 37°C, followed by dialysis against a buffer containing 10 mM sodium phosphate and 150 mM NaCl at pH 7.3, which had been degassed with argon. The proportion of Cyto-MelCAM/β-mercaptoethanol adduct is increased. **(C)** The same sample as in **A** after dialysis against a buffer containing 20 mM Tris, 130 mM NaCl, and 1.4 mM TCEP-HCl at pH 7.4 for 16 hours at 4°C. The TCEP-HCl completely reduced the Cyto-MelCAM/β-mercaptoethanol adduct.

greater than that of the monomer, indicative of a potential disulfide adduct between Cyto-MelCAM and glutathione (expected mass increase = 305.1 daltons). This adduct formed during elution of the Cyto-MelCAM fusion protein from the glutathione-Sepharose column with a buffer containing 10 mM glutathione. The formation of disulfide adducts between proteins (ie, hen egg white lysozyme) and reducing agents (ie, β-mercaptoethanol and DTT) has previously been observed to occur during electrophoresis.[5]

To determine whether the Cyto-MelCAM dimer was disulfide linked and to reduce the glutathione adduct, a sample of the protein was incubated with 100 mM β-mercaptoethanol for 1 hour at 37°C under argon, followed by dialysis against a buffer containing 1 mM β-mercaptoethanol. As Figure 1*B* shows, this reducing treatment almost completely eliminated the dimer peak, confirming that the Cyto-MelCAM dimer was disulfide linked. The glutathione disulfide adduct was also reduced. However, another peak appeared with an observed mass 74 daltons larger than Cyto-MelCAM, consistent with a disulfide adduct between the peptide and β-mercaptoethanol (expected mass increase = 76.0 daltons).

Figure 2*A* shows the MALDI-MS spectrum of another preparation of Cyto-MelCAM that was purified with 1 mM β-mercaptoethanol in the gel filtration buffer to prevent the formation of disulfides during the purification. The presence of β-mercaptoethanol prevented the formation of disulfide-linked dimers of Cyto-MelCAM; however, the β-mercaptoethanol adduct of Cyto-MelCAM was again present. This sample was then incubated with 100 mM β-mercaptoethanol for 1 hour at 37°C, followed by dialysis against a buffer that contained no reducing agents but had been thoroughly degassed and was blanketed with argon. As shown in Figure 2*B*, this treatment only increased the proportion of the β-mercaptoethanol adduct; the reduced form of Cyto-MelCAM was barely detectable. These results indicated that the reactivity of the thiol group on Cyto-MelCAM was comparable to that of β-mercaptoethanol and that a stronger reducing agent would be required to completely reduce the protein.

The reducing agent TCEP-HCl was evaluated. It is resistant to air oxidation, highly stable in most aqueous solutions, substantially more effective than DTT, and cannot form covalent interactions with cysteines.[6] The sample shown in Figure 2*B* was dialyzed against a buffer containing 1.4 mM TCEP-HCl for 16 hours at 4°C. The MALDI-MS spectrum of the dialyzed sample is shown in Figure 2*C*. Treatment of the sample with TCEP-HCl resulted in complete reduction of the Cyto-MelCAM/β-mercaptoethanol adduct.

CONCLUSIONS

The tendency for proteins with free thiol groups to form artifactual disulfide-linked dimers during and after purification is well known and can often be detected by comparing apparent size using reducing and nonreducing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Although all free thiol groups can form disulfides under oxidizing conditions, highly reactive cysteines occasionally are encountered that readily form disulfide adducts with commonly used reducing agents. This report demonstrates the reduction of these adducts with the use of TCEP-HCl, a reducing agent that is more effective than DTT and cannot form covalent interactions with the protein.[6] The presence and reduction of disulfide adducts can be readily monitored using MALDI-MS.

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