The Disulfide Structure of Mouse Lysosome-associated Membrane Protein 1*

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The disulfide structure of mouse lysosome-associated membrane protein 1 has been determined by reverse-phase isolation and sequence analysis of the cysteine-containing tryptic fragments of the reduced and nonreduced deglycosylated protein. Half-cystines were distinguished (a) by their localization within tryptic or chymotryptic peptides that formed reverse-phase peaks unique to the reduced digests and (b) by their \(^2\)-carboxymethylation only after reduction of the protein. The disulfide arrangement of the cysteines was assigned after isolation of disulfide-linked peptide pairs. Each pair chromatographed as a peak present in the nonreduced (but not the corresponding reduced) tryptic digest. NH₂-terminal sequencing as well as reduction, alkylation, and rechromatography of the tryptic fragments led to the following assignment of disulfide bonds: Cys\(^{11}\) and Cys\(^{296}\), Cys\(^{125}\) and Cys\(^{161}\), Cys\(^{188}\) and Cys\(^{321}\), and Cys\(^{333}\) and Cys\(^{340}\). This structure creates four 36–38-residue loops that are symmetrically placed within the two halves of the protein's intraluminal domain. The loops formed by the Cys\(^{11}\)–Cys\(^{296}\) and Cys\(^{125}\)–Cys\(^{161}\) bridges are homologous, and the Cys\(^{188}\)–Cys\(^{321}\) and Cys\(^{333}\)–Cys\(^{340}\) loops form a second set of homologous domains. The conservation of cysteine residues among lysosome-associated membrane proteins 1 and 2 suggests that this disulfide arrangement is common to both members of this family of lysosomal membrane glycoproteins.

mLAMP-1 \(^1\) (1, 2) is a member of a highly conserved family of lysosomal membrane glycoproteins that also includes human LAMP-1 (3, 4), chicken LEP100 (5), and rat lgp120 (6) (reviewed in Ref. 7). These proteins are major components of the lysosomal membrane, and several of them have also been found on the plasma membrane of some embryonic, transformed, and metastatic cell types (3, 8, 9). In addition to a presumed role in the lysosome, these glycoproteins may function as regulated receptors for the extracellular matrix and play a role in the malignant transformation of cells. Specifically, mLAMP-1 is the predominant carrier of complex N-linked oligosaccharides with an unusual \(\beta\)1–6-trimannosyl core branching pattern that has been correlated with an increased metastatic potential in mouse lymphoma cells (10).

In addition, mLAMP-1 molecules bind to an Arg-Gly-Asp (RGD) peptide column; and the deglycosylated form of the molecule binds to collagen, laminin, and fibronectin (11). Another potential function for LAMP-1 has been suggested by Febraro et al. (12), who recently found that surface expression of human LAMP-1, although low on resting platelets, is markedly increased on thrombin-activated platelets and may function in platelet adhesion.

The primary structure of mLAMP-1, deduced from a cDNA clone (2), consists of a 382-residue (42-kDa) protein with a large (346-residue) extracytoplasmic NH₂-terminal domain followed by a 24-residue hydrophobic transmembrane region and a short (12-residue) COOH-terminal cytoplasmic tail. The extracytoplasmic domain of mLAMP-1 is composed of two \(\sim\)160-residue homology units that are separated by a Pro/Ser-rich region. Each of these homologous domains contains 4 uniformly spaced cysteine residues, with two intercysteine intervals of 36–38 residues and one of 68 or 76 residues. The LAMP-1 proteins from each of the animal species contain these \(\sim\) regularly spaced cysteines as well as many of the same flanking residues. Moreover, a second class of lysosomal membrane glycoproteins, the LAMP-2 family, which have about 30% sequence homology to the LAMP-1 molecules, also contains these \(\sim\) regularly spaced cysteines and several common surrounding residues (6, 13). The regular spacing of these residues and the presence of some sequences characteristic of immunoglobulins also suggested the possible occurrence of a pair of disulfide-bridged immunoglobulin-like domains (2, 13).

In this study, the disulfide structure of mLAMP-1 has been elucidated. The data provide evidence that the first and second, third and fourth, fifth and sixth, and seventh and eighth cysteines are disulfide-linked in this protein.

EXPERIMENTAL PROCEDURES AND RESULTS

The data presented in this study collectively indicate that the 8 Cys residues in mLAMP-1 form four intrachain disulfide bonds. Each bond links a pair of adjacent Cys residues such that Cys\(^{11}\) and Cys\(^{296}\), Cys\(^{125}\) and Cys\(^{161}\), Cys\(^{188}\) and Cys\(^{321}\), and Cys\(^{333}\) and Cys\(^{340}\) are bridged within the molecule. These bridges create four 36–38-residue loops in the protein (Fig. 6) such that the two extracytoplasmic homology units in the

DISCUSSION

The data presented in this study collectively indicate that the \(\sim\)8 Cys residues in mLAMP-1 form four intrachain disulfide bonds. Each bond links a pair of adjacent Cys residues such that Cys\(^{11}\) and Cys\(^{296}\), Cys\(^{125}\) and Cys\(^{161}\), Cys\(^{188}\) and Cys\(^{321}\), and Cys\(^{333}\) and Cys\(^{340}\) are bridged within the molecule. These bridges create four 36–38-residue loops in the protein (Fig. 6) such that the two extracytoplasmic homology units in the
protein each contain two disulfide-bonded domains. There is no homology between the disulfide-bonded domains within the same homology unit. However, the disulfide-linked domains between the two units are homologous. Hence, the loop formed by Cys11 and Cys46 in the first unit is homologous to the Cys235-Cys303 loop in the second unit (25% identical and 48% similar residues), and the domain formed by Cys125 and Cys46 in the first unit is homologous to the Cys235-Cys303 loop in the second unit (21% identical and 48% similar residues) (Fig. 7).6

The mLAMP-1 molecule contains 20 Asn-X-Ser/Thr glycosylation sites in its extracytoplasmic domain, most of which appear to be modified with complex N-linked sugar moieties (28). The loops formed by the disulfide bonds between Cys11 and Cys125, Cys235 and Cys245, and Cys303 and Cys310 each contain several potential N-linked oligosaccharide sites, whereas the fourth loop, formed by Cys80 and Cys106, has none. This study revealed that one of those sites, Asn224 on the Cys125-Cys235 loop, was not glycosylated since there was an 87-100% stepwise yield of the nonglycosylated phenylthiohydantoin-Asn during sequencing of the Cys235-containing peptide (Tables I and III). If the residue had been glycosylated, whereas the fourth loop, formed by Cys303 and CYSTS', has hydantoin-Asn during sequencing of the Cys235-containing the terminal GlcNAc would have remained attached to the Cy~"~-Cys 235 loop, was not glycosylated since there was Asn after deglycosylation with trifluoromethanesulfonic acid and Asn'35, Asn105, Asn'105, Asnzz8, and Asnz7' were found to be actual sites for attachment of N-linked oligosaccharides since a phenylthiohydantoin-Asn was sequenced at that residue (Tables I and III).

The loops created by the disulfide bonds would not necessarily cause the protein to fold into a highly globular structure since the bridging of neighboring Cys residues creates relatively small loops. Hence, no major change in mobility on sodium dodecyl sulfate-polyacrylamide electrophoresis gel was observed between the reduced and nonreduced species. However, the disulfide bonds apparently stabilize the protein structure, as evidenced by the inability of several proteases to cleave the nonreduced protein under strongly denaturing conditions. The carbohydrate also protected the molecule from proteolytic digestion. The disulfide bonds and abundant oligosaccharides may thus protect the protein against protease digestion in the hostile lysosomal environment (29). The Chou-Fasman (30) program of secondary structures predicts that each of the loops formed by the disulfide bonds contains β-sheets. The disulfide bonds may therefore stabilize the tertiary sheet structure.

The importance of the Cys residues to the molecule is further suggested by the complete conservation of the cysteines between the LAMP-1 and LAMP-2 groups of proteins. Since the gross structures of these proteins are very similar, they may all have similar disulfide bonding arrangements. Presumably, the role of the disulfide bond is of great enough importance to the structure and/or function of these molecules that these bonds have been conserved throughout evolution of the various animal species (human, mouse, rat, and chicken) as well as between the two different groups of molecules (LAMP-1 and LAMP-2).

The regular spacing of the Cys residues together with the presence of a characteristic immunoglobulin-related sequence common to the V-like domains, Tyr-X-Cys (31), had earlier suggested that the mLAMP-1 molecule might fall into the immunoglobulin superfamily of genes (2, 13). The data generated during this study, however, suggest that mLAMP-1 is not a member of this superfamily. Specifically, the loops formed in the immunoglobulins are composed of ~70 residues, whereas the mLAMP-1 loops are of 36-38 residues. This alone would not exclude LAMP-1 molecules from membership in the immunoglobulin superfamily because another member of the immunoglobulin family, the Fc receptor, has relatively small loops (18). However, the Tyr X Cys sequence found at Cys125 and Cys235 in mLAMP-1 do not fall at the carboxy-terminal end of a disulfide loop in mLAMP-1 as they do in the immunoglobulins. This observation together with the fact that there are no other sequences characteristic of the immunoglobulins in the mLAMP-1 sequence lead us to conclude that mLAMP-1 is not a member of the immunoglobulin superfamily of genes.

During a broad protein search, no proteins outside of the LAMP-1 or LAMP-2 family of proteins were found to be homologous to mLAMP-1 or to contain domains homologous to the disulfide-linked loops of this protein.7 We therefore

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6 Percentage identical residues was assigned by the SEQHP program (GenMenu), and the percentage conserved residues was calculated by the ALIGN program (GenMenu).

7 Protein was searched against the GenBank198 protein sequence data bank using the FASTA program from BIONET (32).
conclude that the LAMP-1 and LAMP-2 lysosomal membrane glycoproteins constitute a distinct class of proteins that are structurally related and contain distinctive disulfide-bonded domains.

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EXPERIMENTAL PROCEDURES

Immunoaffinity Purification of mLAMP-1. mLAMP-1 was purified by immunoaffinity chromatography using a monoclonal antibody from a mouse ascites fluid made in vivo against the 44-kDa fragment of LAMP-1 (mLAMP-1). This fragment proved to be a rich source of mLAMP-1, and resulted in a good yield of purified protein per gram of tissue (4-4.5 mg/g tissue). It was obtained from the lysosomal source, from Swiss Webster mice (4-4.5 mg/g tissue). The purified purification method was modified from the Itoh procedure. Tissue membranes were washed with 0.5% Triton X-100 (pH 7.3) containing 0.1% deoxycholate. The purified protein was eluted with 0.1 M glycine-HCl buffer (pH 2.6) containing 0.05% SDS and 0.1% glycine. The purified protein was stored at -80°C in 0.1 M glycine buffer for up to 3 months.

Results for specificity and activity of trypsin- Tryptic digestion of mLAMP-1 was performed with a specific activity of 0.8 U/mg. The purified protein was activated by addition of 0.36 mmol/mg of buffer to the sample to maximize the activity of the purified protein. The purified protein was stored at -80°C in 0.1 M glycine-HCl buffer for up to 3 months.

Disulfide Structure of mLAMP-1. The mLAMP-1 glycoprotein was also digested with trypsin (United States Biochemical Corp.) using 0.15 U/mg as a standard digestion. The digested samples were analyzed by SDS-PAGE and Western blotting.

To verify the disulfide structure of mLAMP-1, the purified protein was digested with 10% triton X-100, and the disulfide bonds were analyzed by SDS-PAGE and Western blotting. The purified protein was activated by addition of 0.36 mmol/mg of buffer to the sample to maximize the activity of the purified protein. The purified protein was stored at -80°C in 0.1 M glycine-HCl buffer for up to 3 months.

The purified protein was digested with 10% triton X-100, and the disulfide bonds were analyzed by SDS-PAGE and Western blotting. The purified protein was activated by addition of 0.36 mmol/mg of buffer to the sample to maximize the activity of the purified protein. The purified protein was stored at -80°C in 0.1 M glycine-HCl buffer for up to 3 months.
**RESULTS**

The effect of dilution of the structure of the bovine serum albumin (BSA) and the effects of reducing and alkylating mLAMP-1 (12) on the molar ratio of BSA and mLAMP-1 were measured by spectrophotometric methods. The molar ratio of BSA to mLAMP-1 was determined by measuring the absorbance at 280 nm after addition of sodium dodecyl sulfate (SDS) to the sample. The molar ratio of BSA to mLAMP-1 increased linearly with the concentration of BSA, while the molar ratio of mLAMP-1 to BSA remained constant (Fig. 1). The results indicated that mLAMP-1 bound to BSA in a 1:1 molar ratio.

The molar ratio of mLAMP-1 to BSA was determined by spectrophotometric methods. The molar ratio of mLAMP-1 to BSA increased linearly with the concentration of mLAMP-1, while the molar ratio of BSA to mLAMP-1 remained constant (Fig. 2). The results indicated that mLAMP-1 bound to BSA in a 1:1 molar ratio.

**TABLE I**

The specific activity and % recovery of mLAMP-1 from the solution are shown in Table I. The specific activity of mLAMP-1 increased with the concentration of mLAMP-1, while the % recovery of mLAMP-1 remained constant.

**TABLE II**

The specific activity of mLAMP-1 increased with the concentration of mLAMP-1, while the % recovery of mLAMP-1 remained constant.

**DISCUSSION**

The results presented in this study indicate that mLAMP-1 binds to BSA in a 1:1 molar ratio. The specific activity of mLAMP-1 increased with the concentration of mLAMP-1, while the % recovery of mLAMP-1 remained constant. These findings suggest that mLAMP-1 may have potential as a biocatalyst in industrial applications.
Disulfide Structure of mLAMP-1

Fig. 3 - Isoelectric-Focusing Acid Labeling of Reduced and Non-reduced Trypsin-Digested Deglycosylated mLAMP-1. Reduced and non-reduced deglycosylated mLAMP-1 (50 μg each) were alkylated with iodoacetamide or iodoacetate before trypsin digestion. Samples were chromatographed on a hydrogen-oxide column using the standard program. (A) Appearance of some of the peaks are non-reduced tryptic peptides. (B) Relative intensities of the peaks. The first 35% of the chromatogram contains unreacted peaks and free N-iodoacetic acid and have been omitted for simplicity. On-Cys-containing peptide peaks are labeled by Cys residue number.

Fig. 4 - Chromatography of Non-reduced Tryptic Peptides from Deglycosylated mLAMP-1. mLAMP-1 (40 μg) was deglycosylated, alkylated and trypsin digested. The digest was chromatographed on the FPLC C18 column using the standard program. The resulting chromatogram was compared with a partial profile of reduced tryptic peptides (not shown) and peaks unique to the non-reduced profile were tabulated (A - E).

TABLE II
N-terminal sequence analysis of tryptic peptides unique to the non-reduced digest.

Peaks A - E (Fig. 4) were N-terminally sequenced. The sequence of a unique peak isolated from a non-reduced chromatographic digest is also shown. No indication was possible of cysteine residues which do not sequence.

Fig. 5 - Reduction, Alkalization and Carboxypeptidase Treatment of Disulfide-Bonded mLAMP-1. Reduction and alkalization of mLAMP-1 resulted in a peak containing a Cys-Cys or Cys-Cys-Cys peptide fragment (Fig. 5). These fragments were then identified by carboxypeptidase A and D treatment. The peaks were isolated by the retention time of the reduced and alkylated mLAMP-1 (retention time of the reduced mLAMP-1 is longer than that of the alkylated mLAMP-1). On-Cys-containing peaks were identified by retention time and are labeled by Cys residue number. The Cys-Cys-350 peptide was distinguished from the ligation sequence of the disulfide-bonded fragment with Cys-Cys residues 2 and 3.
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