Formation of a Major Histocompatibility Complex Class I Tapasin Disulfide Indicates a Change in Spatial Organization of the Peptide-loading Complex during Assembly*

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The assembly and peptide loading of major histocompatibility complex Class I molecules within the endoplasmic reticulum are essential for antigen presentation at the cell surface and are facilitated by the peptide-loading complex. The formation of a mixed disulfide between the heavy chain of Class I and components of the loading complex (ERp57, protein disulfide isomerase, and tapasin) suggests that these molecules are involved in the redox regulation of components during assembly and peptide loading. We demonstrate here that a disulfide formed between heavy chain and tapasin can occur between cysteine residues located in the cytosolic regions of these proteins following translation of heavy chain in an in vitro translation system. The formation of this disulfide occurs after assembly into the loading complex and is coincident with the stabilization of the α2 disulfide bond within the peptide binding groove. A ternary complex between heavy chain, ERp57, and tapasin was observed and shown to be stabilized by a disulfide between both tapasin-heavy chain and tapasin-ERp57. No disulfides were observed between ERp57 and heavy chain within the loading complex. The results provide a detailed evaluation of the various transient disulfides formed within the peptide-loading complex during biosynthesis. In addition, the absence of the disulfide between tapasin and heavy chain in TAP-deficient cells indicates that a change in the spatial organization of tapasin and heavy chain occurs following assembly into the loading complex.

For major histocompatibility complex (MHC)³ Class I molecules to be presented at the cell surface they must first be assembled within the endoplasmic reticulum, a process requiring the association of three components, namely the heavy chain, β2microglobulin, and a peptide generated in the cytosol. The assembly is coordinated by a specialized group of proteins that collectively form the peptide-loading complex (PepLC), the role of which is both to assemble the Class I molecule and to prevent the premature exit of unassembled heavy chain from the endoplasmic reticulum (1). The main constituents of the PepLC are the two Transporter associated with Antigen Processing (TAP) subunits, tapasin, ERp57, and calreticulin (2–4). In addition, protein disulfide isomerase has recently been suggested to be part of this loading complex (5). The exact role of each of these components is unclear; however, recent advances have led to the idea that tapasin, ERp57, and protein disulfide isomerase are involved in peptide loading by regulating the redox state of a disulfide bond within the peptide binding groove of the heavy chain (5, 6).

During the biosynthesis of MHC Class I molecules, the heavy chain is first translocated across the endoplasmic reticulum membrane prior to assembly with β2microglobulin. There are two intra-chain disulfide bonds within the heavy chain, one of which, in the α3 domain, forms immediately following translocation (7, 8). The second disulfide bond, in the α2 domain, forms concomitant with assembly of the heavy chain-β2microglobulin heterodimer and prior to association with the peptide-loading complex (9, 10). The α2 disulfide bond is located at the base of the peptide binding groove and is thought to be unstable and susceptible to reduction prior to peptide binding (11). It has recently been shown that the presence of tapasin and ERp57 within the loading complex stabilizes the α2 disulfide bond, possibly by tapasin inhibiting the reductase activity of ERp57 (6). In support of this idea, it has been demonstrated that tapasin and ERp57 form a stable interchain disulfide bond between Cys-95 of tapasin and the first active site of ERp57 (12, 13). When the tapasin-ERp57 disulfide bond is prevented from forming by mutation of tapasin Cys-95, the resulting PepLC is unable to stabilize the α2 disulfide bond, giving rise to two redox forms of heavy chain (12). In addition to forming a disulfide bond with ERp57, tapasin has been shown to provide the physical link between the heavy chain-β2microglobulin heterodimer and the TAP subunits, ensuring that the peptide binding subunit remains close to the transporter of peptides (14, 15). ERp57 is a member of the protein disulfide isomerase family of proteins that are involved in the formation of native disulfide bonds (16). ERp57 binds to the lectins calnexin or calreticulin, and hence its substrates are primarily glycoproteins (17). The role of ERp57 in MHC Class I biosynthesis seems to involve stabilization of the peptide-loading complex, as the consequence of its absence is a reduction in cell surface expression of stable Class I molecules and impaired PepLC assembly (18). ERp57 has also been shown to specifically reduce unassembled

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3 The abbreviations used are: MHC, major histocompatibility complex; ER, endoplasmic reticulum; TAP, transporter associated with antigen processing; PepLC, peptide-loading complex; PAS, protein A-Sepharose; SP cells, semi-permeabilized cells.
heavy chain molecules, indicating that it may be involved in preparation for dislocation to the cytosol for degradation (19). In addition, transient mixed disulfides between ERp57 and heavy chain have been observed, which would be predicted if heavy chain is a substrate for ERp57. However, these mixed disulfides were only present when tapasin was absent from cells or when a tapasin Cys-95 mutant was expressed (6). These results suggest that ERp57 may well be involved in the formation or reduction of disulfide bonds within heavy chain but that the activity of ERp57 is inhibited in the PepLC by the formation of an interchain disulfide bond with tapasin.

Further support for a role of ERp57 and tapasin in regulation of the redox state of heavy chain within the PepLC was provided when a disulfide-bonded ternary complex between heavy chain, ERp57, and tapasin was identified (20). In particular, it was suggested that a mixed disulfide forms between ERp57 and heavy chain within the PepLC. If correct, this would indicate that, within the PepLC, ERp57 in a stable complex with tapasin is able to catalyze the reduction of the α2 disulfide bond. To investigate these observations further we have undertaken a series of experiments to determine the proteins forming mixed disulfides in the PepLC and to identify the specific cysteine residues involved. We found no evidence that ERp57 forms a mixed disulfide with heavy chain within the PepLC. We show that tapasin itself forms a disulfide with heavy chain when heavy chain is translated in an in vitro translation system and that this disulfide can exist in a ternary complex with ERp57. The disulfide between heavy chain and tapasin is mediated by cysteine residues that are within or close to the transmembrane domain. The timing of the formation of the disulfide and the fact that it is co-immunoprecipitated with antibodies to TAP indicate that it forms within the loading complex. In addition, the disulfide between heavy chain and tapasin does not form in cells devoid of TAP subunits. Hence, we have been able to use the fact that a disulfide forms between heavy chain and tapasin in our experimental system as a tool to study the orientation of these molecules during incorporation into the loading complex.

EXPERIMENTAL PROCEDURES

**Cell Lines**—HT1080 cell lines were maintained in Dulbecco’s modified Eagle’s medium supplemented with 4.5 g/liter glucose, 10% v/v fetal bovine serum, and 0.56 mM L-glutamine. HT1080 cell line stably expressing ERp57-V5 Cys60 and Cys-409 was generated as described previously (21). The TAP-negative B-cell line.174 (22) was maintained in Dulbecco’s RPMI medium supplemented with 10% v/v fetal bovine serum and 0.56 mM L-glutamine. All cell lines were treated with γ-irradiation (200 units/ml) for 48 h prior to the preparation of semi-permeabilized (SP) cells.

**Antibodies**—Monoclonal anti-V5 antibody was purchased from Invitrogen. PaSta.1 (mouse monoclonal antibody to tapasin) and R.RING.4C (purified rabbit serum to TAP-1) were a kind gift from Peter Cresswell (Yale University School of Medicine). ERp57 anti-peptide antibody was raised as described previously (23).

**Transcription and Translation in Vitro**—DNA was transcribed, proteins translated, and SP cells prepared as described previously (24). Human leucocyte antigen-B35 cDNA cloned into pcDNA3.1 was a gift from Simon Powis, University of St. Andrews, UK. Plasmid DNA was linearized with HindIII, and mRNA was prepared by transcription with T7 RNA polymerase. RNA transcripts were translated in a rabbit reticulocyte lysate (Flexylsate; Promega) in the presence of SP cells. Translation reactions (25 μl) containing 16.5 μl of reticulocyte lysate, 40 μM methionine-free amino acid mixture, 45 mM KCl, 15 μCi of [35S]methionine (PerkinElmer Life Sciences), 1 μl of transcripted mRNA, and SP cell preparation (~2 × 10^6 cells) were incubated at 30 °C. When carrying out a time course, 1 mM aurin tricarboxylic acid (Sigma) was added after 5 min of translation to prevent reinitiation. All translation reactions were stopped at the required incubation time by the addition of N-ethylmaleimide (Sigma) to a final concentration of 40 mM and incubation at 4 °C for 20 min to prevent any disulfide exchange reactions occurring and to freeze the redox state of the translation product. SP cells were isolated and washed in KH buffer (110 mM KOAc, 20 mM HEPES, 2 mM MgOAc) before preparation for SDS-PAGE or immunoprecipitation.

**Immunoprecipitation**—For immunoprecipitation under denaturing conditions, cell pellets from translation reactions were lysed in IP buffer (1% (v/v) Triton X-100, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, and 0.5 mM phenylmethylsulfonyl fluoride). Supernatant was isolated, and SDS was added to 2% (w/v) and incubated at 100 °C before 100-fold dilution in IP buffer. Samples were pre-cleared with protein A-Sepharose (PAS) (Invitrogen) for 1 h at 4 °C before incubation with fresh PAS beads and specific antibodies at 4 °C for 18 h. PAS beads were washed in 3 × 1 ml of IP buffer before boiling in SDS-PAGE sample buffer (0.25 mM Tris–HCl, pH 6.8, 2% (w/v) SDS, 20% (v/v) glycerol, 0.004% (w/v) bromphenol blue) and separation on a 10% polyacrylamide gel.

For immunoprecipitation under native conditions, cell pellets from translation reactions were lysed in TBS (0.15 M NaCl, 0.01 M Tris–Cl, pH 7.4) containing 10% w/v digitonin (Calbiochem) and 0.5 mM phenylmethylsulfonyl fluoride. Samples were pre-cleared with PAS before incubation with fresh PAS beads and specific antibodies at 4 °C for 18 h. PAS beads were washed in 3 × 1 ml containing 1% (w/v) digitonin and 0.5 mM phenylmethylsulfonyl fluoride before boiling in SDS-PAGE sample buffer and separation on a 10% polyacrylamide gel. When sequential immunoprecipitation was performed, the second round of immunoprecipitation was performed under denaturing conditions.

**Site-directed Mutagenesis**—The cysteine mutants in B35 were created using the QuikChange procedure as outlined by Stratagene (La Jolla, CA). Mismatch oligonucleotides were designed to replace the cysteine codon with one for alanine (sequences available upon request).

**RESULTS**

**MHC Class I Heavy Chain Forms Mixed Disulfides with Components of the PepLC**—We have demonstrated previously that the initial stages in the folding and assembly of MHC Class I molecules can be faithfully reproduced by translation of heavy chain in the presence of SP cells (25) (see Fig. 1A). In such a system, translated, radiolabeled heavy chain is translocated into the endoplasmic reticulum of the SP cells where it associates
with endogenous β2microglobulin. Hence, only the translated heavy chain is radiolabeled and can be detected by autoradiography. In this study we investigated the changes in redox state during synthesis of the tapasin-dependent heavy chain B35 when translated in the presence of human HT1080 cells. The majority of protein present after 60 min migrated as a single band under reducing conditions (Fig. 1B, lane 1). We have demonstrated previously that this corresponds to glycosylated heavy chain with the minor product being unglycosylated protein (25). Newly synthesized heavy chain was treated with N-ethylmaleimide at various time points, following initiation of translation, to freeze the redox state of the translation product. It has been shown previously that when separated by non-reducing SDS-PAGE, heavy chain can be resolved into two distinct redox forms containing either the α3 disulfide or both the α2 and α3 disulfides (6). During our time course of synthesis, we clearly saw two different redox forms, the first of which appeared at early time points whereas the second form predominated after 30 min (Fig. 1B, lanes 2–8, annotated 1 and 2). A time course of immunoprecipitation of heavy chain with antibodies to β2microglobulin demonstrates that only the fully oxidized heavy chain assembles to form a heterotrimer (Fig. 1C). Hence, the SP cell translation system reproduces the previously characterized time course of assembly and disulfide bond formation clearly differentiating the formation of the α2 and α3 disulfide bonds.

We noticed that several minor radiolabeled higher molecular mass proteins were present in our translation products (Fig. 1B). These could be derived from mixed disulfides or stable disulfides formed between the translated heavy chain and endogenous proteins that are trapped following alkylation with N-ethylmaleimide. A mixed disulfide is an intermediate that is formed when one thiol group reacts with a disulfide bond to form a transient covalently linked intermediate. They are very unstable and can only be seen following alkylation with reagents that react with a resolving thiol group. To investigate whether any complexes were formed during translation of heavy chain, we carried out immunoisolation with antibodies to components of the PepLC. When the immunoisolation was carried out under native conditions and separated under non-reducing conditions, two higher molecular mass species were observed (Fig. 1D). The complexes were clearly disulfide-bonded complexes between the translated heavy chain and unlabeled endogenous protein as only radiolabeled heavy chain was observed when these same samples were separated after reduction (Fig. 1E). To identify which component was disulfide-bonded with heavy chain we needed to carry out the immunoisolations under denaturing conditions.
ditions to first disrupt the PepLC. Under these conditions the lower 94-kDa product was immunoisolated with tapasin, whereas the higher 150-kDa molecular mass product was, albeit faintly, immunoisolated with ERp57 and tapasin (Fig. 1D, lanes 6 and 7). These results confirm previously published results that demonstrated a mixed disulfide between heavy chain, ERp57, and tapasin (20) and show for the first time that a disulfide can form between heavy chain and tapasin. It should be stressed that the formation of disulfide bonds in these experiments could well be a result of the prevailing redox conditions within the *in vitro* translation system (see “Discussion”).

It has been shown that a complex between ERp57 and tapasin, stabilized by the formation of a labile disulfide bond, is formed within the PepLC (12, 13). To confirm that the 150-kDa complex seen in our immunoprecipitations was a ternary complex between heavy chain, tapasin, and ERp57, we took advantage of the fact that the ERp57-tapasin interchain disulfide can be stabilized by mutation of the second cysteine residues in the ERp57 active sites (26). We synthesized heavy chain in SP cells isolated from a stable cell line expressing a V5-tagged ERp57 that carries a mutation of the second cysteine residues in each of the A and A’ active sites (ERp57-V5 C60A,C409A) (21). Products of translation were immunoisolated with antibodies to the V5 tag or tapasin. The 150-kDa product was again immunoisolated by both V5 tag and tapasin antibodies (Fig. 2A, lanes 1 and 2) and was linked via a disulfide as only heavy chain was seen upon reduction (Fig. 2B). Note that only heavy chain is radiolabeled during the *in vitro* translation reaction, so no radiolabeled ERp57 or tapasin is seen. Additional mixed disulfides between ERp57 and heavy chain were seen in this cell line (Fig. 2A, lane 1, asterisks), but these were not present in the PepLC as they are absent from the tapasin immunoisolate (lane 2). In addition, when the tapasin immunoisolate was denatured and immunoisolated with ERp57 antibodies, only the 150-kDa product was reisolated (Fig. 2, A and B, lanes 3). This immunoprecipitation was specific as no product was seen when protein disulfide isomerase rather than ERp57 antibodies were used (Fig. 2, A and B, lanes 4). These results confirm that the 150-kDa product is a ternary complex between ERp57, tapasin, and heavy chain.

Identification of the Heavy Chain Cysteine Residues Involved in Disulfide Formation with Tapasin—Having identified a novel disulfide between tapasin and heavy chain, we were interested to determine which cysteine residues within heavy chain were involved. A schematic detailing the positions of the cysteine residues in B35 heavy chain is depicted in Fig. 2C. As tapasin and ERp57 have been postulated to be involved in the formation or reduction of the α2 disulfide, our initial experiments focused on the cysteines involved in formation of this bond, namely Cys-101 and Cys-164. These residues were mutated to alanine either singly or together. The resulting

![FIGURE 2. Heavy chain forms a disulfide with tapasin that is not dependent upon the cysteines forming the α2 domain disulfide bond.](image)
mutant heavy chains were then translated in the presence of SP cells. When separated under non-reducing conditions, all mutants had a slower mobility than the wild-type protein, indicating a lack of formation of the α2 disulfide (Fig. 2D, lanes 1 and 2 for Cys-164, and results not shown). In addition, all the α2 mutants were able to form a disulfide that was immunoisolated with tapasin or TAP under native conditions (Fig. 2D, lanes 3–10). The disulfide formed with the mutants had a slightly slower mobility, indicating a lack of formation of the α2 disulfide within the complex. When the translation products were immunosolated with tapasin antibody under denaturing conditions, the complex was still present with all mutants (Fig. 2D, lanes 11–14). These results clearly demonstrate that the disulfide-linked complex between tapasin and heavy chain is not via the α2 cysteine residues. In addition, the fact that the complex is immunosolated with TAP antibodies demonstrates that the α2 disulfide bond does not need to form prior to incorporation of heavy chain into the PepLC.

A ternary complex between ERp57, tapasin, and heavy chain has previously been identified that was suggested to include a mixed disulfide between ERp57 and heavy chain via the α2 cysteine residues (20). To determine whether the ternary complex we observed in our experimental system also contains a mixed disulfide between ERp57 and the α2 cysteines of heavy chain, we used the approach that maximized the formation of this complex. Hence, we translated either wild-type or the α2 double cysteine mutant in the presence of cells expressing ERp57-V5 C60A,C409A and determined whether the ternary complex could still be immunoisolated with antibodies to tapasin. Removal of the α2 disulfide bond had no effect on the formation of the ternary complex or on its ability to be immunosolated under native conditions with TAP or tapasin (Fig. 3). In addition, the ternary complex formed with both the wild-type and α2 mutant heavy chain was also immunosolated with antibodies to tapasin or V5-tagged ERp57 under denaturing conditions (Fig. 3, lanes 7–10). No complexes were isolated with the TAP antibody under denaturing conditions (lanes 11–12), further confirming these complexes were present within the PepLC. Interestingly, several ERp57/α2 mutant heavy chain mixed disulfides were seen exclusively with the wild-type protein (lanes 3 and 9, asterisks) that were not present within the PepLC, as judged by their absence from the TAP and tapasin immunosolates. These mixed disulfides must therefore be via the heavy chain α2 cysteine residues and ERp57. Such mixed disulfides have been observed previously and are likely to form between heavy chains that are either misfolded or are excluded from the PepLC (6).

Our failure to prevent disulfide formation with the α2 mutant heavy chain led us to consider which other cysteine residues within B35 heavy chain could be responsible for the linkage to tapasin. B35 contains five cysteine residues, four of which form the α2 and α3 disulfide and one (Cys-308) is located within the transmembrane domain or just within the cytosolic tail (Fig. 2C). We created a double mutant removing both the α3 disulfide cysteine residues, but this did not fold correctly as judged by its poor assembly with β2microglobulin and so was unable to interact with tapasin (data not shown). When we mutated the Cys-308 cysteine to alanine and translated the resulting heavy chain in the presence of SP cells, the heavy chain folded correctly as judged by formation of the α2 and α3 disulfide bonds and formation of the heavy chain β2microglobulin heterodimer (Fig. 4, A and C). However, this mutation completely abolished the disulfide with tapasin (Fig. 4A). In addition, when this heavy chain was translated in the ERp57-V5 C60A,C409A cell line, no ternary complex was observed (data not shown). These results demonstrate that the disulfide between tapasin and heavy chain is formed via the Cys-308 cysteine within heavy chain. Tapasin also contains a cysteine

**FIGURE 3.** The ternary complex formed between ERp57, tapasin, and heavy chain is not mediated by the α2 disulfide cysteines. RNA coding for either wild type (wt) B35 heavy chain (lanes 1, 3, 5, 7, 9, 11) or the C101A,C164A (lanes 2, 4, 6, 8, 10, 12) was translated in the presence of SP cells isolated from an HT1080 cell line expressing V5-ERp57 C60A,C409A. Translation products were immunosolated using the indicated antibodies under either native (lanes 1–6) or denaturing (lanes 7–12) conditions and separated by non-reducing SDS-PAGE. Asterisks in lanes 3 and 9 indicate the positions of mixed disulfides between ERp57 and heavy chain.
residue at the C-terminal end of its transmembrane domain that could potentially form the disulfide linkage with heavy chain; it is unlikely that any cysteine within the luminal domain of tapasin could form this disulfide.

**Tapasin Heavy Chain Disulfide Forms during Assembly of the PepLC**—Having established that heavy chain forms a disulfide with tapasin via Cys-308, we were keen to determine the timing of the formation of this complex during the biosynthesis of the MHC Class I molecule. To this end we carried out a time course of assembly and immunopurified the translation products with the tapasin antibody (Fig. 5A). The results clearly demonstrate that at early time points tapasin interacts non-covalently with the newly synthesized heavy chain with little or no disulfide formation (Fig. 5A, lanes 2 and 3). However, after 20 min over half the translation product immunopurified with the tapasin antibody is present as a disulfide-linked complex, and after 30 min all the immunopurified heavy chain was trapped as a disulfide-linked complex with tapasin (lanes 4–7). These results suggest that heavy chain and tapasin first associate as a non-covalent complex that then undergoes a change in conformation, allowing the formation of a disulfide bond.

The timing of the appearance of the disulfide-linked complex coincided with the formation of the α2 disulfide within heavy chain. At this moment during the synthesis of the MHC Class I molecule, the heavy chain has assembled with β2microglobulin and is being incorporated into the PepLC (see Fig. 1A). To determine whether assembly of the PepLC is necessary for the formation of the heavy chain-tapasin disulfide, we studied the interaction of heavy chain and tapasin in a cell line devoid of TAP. It has been shown previously that in .174 cells heavy chain and tapasin still interact but as TAP is absent, no PepLC is formed (2). When heavy chain was translated in the presence of .174 cells, heavy chain formed intra-chain disulfide bonds with a similar time scale to translations in HT1080 cells (Fig. 5B, compare lane 1 to lanes 3–8). Hence, the delay in formation of the α2 disulfide bond is not dependent upon formation of the PepLC. In addition, heavy chain was immunopurified with the tapasin antibody; however, no disulfide-linked complex was observed at any time point (Fig. 5C, lanes 1–7). These results clearly demonstrate that in the absence of TAP, the heavy chain-tapasin complex cannot form a disulfide. Hence, in the presence of TAP, heavy chain associates with tapasin non-covalently at an early stage during biosynthesis, and some time after assembly of the PepLC a change in the relative position of the tapasin and heavy chain occurs, resulting in the potential to form a disulfide.

**DISCUSSION**

The ability to detect mixed disulfide complexes between heavy chain and components of the PepLC has previously provided insights into the redox regulation of the peptide-loading complex (5, 6, 12, 20). It is clear from the previous work that tapasin, ERp57, and possibly protein disulfide isomerase function in some way to regulate the formation or reduction of the α2 disulfide bond within heavy chain and thereby potentially regulate the binding of peptide for ultimate presentation at the cell surface. However, reliance on the formation of a mixed disulfide complex to indicate a functional role of an oxidoreductase in a disulfide exchange process is not without problems. One needs to be sure which cysteines are involved in...
the disulfide and to be confident that the disulfide is not formed merely because of the proximity of the cysteine residues and the prevailing redox conditions. In this report we have demonstrated the ability of tapasin to form a disulfide with heavy chain and shown that this disulfide bond is mediated via cysteines well away from the peptide-loading region.

Although the formation of a disulfide between tapasin and heavy chain has not been reported previously, the formation of a ternary complex between heavy chain, ERp57, and tapasin has been. It was concluded that the complex was stabilized by the formation of mixed disulfides between ERp57/heavy chain and tapasin/ERp57 (20). Here we also show that a ternary complex can be generated between these three components but that the complex is stabilized by a disulfide bond between tapasin and heavy chain. In the previous work it was assumed that the disulfide bond did not form between heavy chain and tapasin, based upon an experiment where the cytosolic tail of the heavy chain (B27) was deleted, thereby removing a free cysteine in this region. However, a conserved cysteine at the cytosolic end of the transmembrane region still exists in the truncated B27 that is equivalent to the Cys-308 in B35. Here we show that mutation of Cys-308 in B35 abolishes the formation of a ternary complex with tapasin and ERp57. Our conclusion is that a disulfide bond can form between Cys-308 in heavy chain and Cys-420 in tapasin when these two molecules are in the correct orientation to bring the cysteines into close enough proximity. Although we have not shown conclusively that the cysteine in tapasin forming the disulfide with heavy chain is Cys-420, it is unlikely to be any of the luminally located cysteines as they are not in a position to interact with the Cys-308 in heavy chain. Any low molecular weight oxidant such as glutathione disulfide, hydrogen peroxide, or even oxygen could facilitate the formation of this disulfide bond. Hence, in our experiments the formation of a disulfide bond between two proteins does not mean they would form a disulfide under normal physiological conditions and is likely to be because of the prevailing redox conditions. Indeed, positioning of cysteine residues within transmembrane domains and the formation of disulfide bonds following addition of oxidizing agents is an approach often used to demonstrate association of transmembrane sequences (27).

Is there any functional significance to the formation of the disulfide bond between tapasin and heavy chain? It is likely that the disulfide bond itself does not play a role in Class I assembly and is only formed under the redox conditions present during cell-free translation or conditions of oxidative stress in whole cells (20). However, the fact that the bond only forms after the PepLC has assembled and does not form in the absence of TAP suggests that a conformational change must occur between heavy chain and tapasin after PepLC assembly. It has been shown previously that the membrane domain of tapasin is essential for association with TAP (14); hence, our results further support a model whereby free tapasin first interacts with heavy chain and then binds subsequently to the TAP complex prior to peptide loading. Peptide loading can occur in the absence of such an interaction, but it is much more efficient when the PepLC is formed (28). In addition, our results show that the α2 disulfide is not required for association with tapasin or for assembly into the PepLC. Hence, the α2 disulfide is not required for assembly with β2microglobulin and, therefore, folding of heavy chain. The lack of a requirement of this disulfide for folding and assembly into the PepLC suggests that its
role is more likely related to stabilization of the peptide binding groove following peptide binding.

The ability to form mixed disulfides between heavy chain and ERp57 was also confirmed in the course of our work. However, as shown previously these were only present between ERp57 and heavy chain molecules that had not assembled into the PepLC. The mixed disulfides were specifically between ectopically expressed V5-tagged ERp57 C60A,C409A and one of the two cysteines that form the α2 domain disulfide bond. The fact that we trapped these mixed disulfides with the ERp57 C60A,C409A mutant would suggest that outside the PepLC the α2 disulfide may form but is reduced by ERp57. This conclusion is based on the fact that the C60A,C409A form of ERp57 is unable to act as an oxidase and can only act as a reductase. Hence, under these conditions mixed disulfides could only form if the α2 disulfide were oxidized by protein disulfide isomerase or endogenous wild-type ERp57 and subsequently reduced by the ectopically expressed mutant ERp57. One potential role for ERp57 therefore could be to act as a reductase maintaining the α2 disulfide in a reduced form prior to the assembly of the PepLC. Such a role for ERp57 as a reductase has been suggested previously (19); our results and others (5) indicate that this role would be limited to heavy chains outside the PepLC.

The role of disulfide bond formation during the peptide-loading process remains to be fully elucidated. It is tempting to speculate that the redox state of the heavy chain in the PepLC complex could regulate peptide loading or even dissociation of the fully assembled MHC Class I complex from the PepLC for subsequent transport from the endoplasmic reticulum to the cell surface. We are not currently in a position to fully understand the mechanism underlying this redox regulation; however, our results go some way toward clarifying the observed disulfide-linked ternary complex between ERp57, tapasin, and heavy chain.

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