Structural characterization of a soluble and partially folded class I major histocompatibility heavy chain/β_2m heterodimer

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Class I major histocompatibility (MHC) heavy chain (HC) must fold and assemble with β_2 microglobulin (β_2m) prior to binding peptides in the endoplasmic reticulum (ER). Each of these events is mediated by association with chaperones and other proteins and is an essential requirement for the maturation and normal cell surface expression of stable class I MHC–peptide complexes. Here we describe the biochemical and structural characterization of a soluble HC (B*0702)/β_2m heterodimer, apparently free of peptide. Results suggest that the peptide binding domains (α1 and α2) of this folding intermediate are unstable and possess many of the properties ascribed to the molten globule state. The partially folded state of the HC/β_2m heterodimer is consistent with the suggestion that it is stabilized by chaperones and other proteins in the ER. This soluble intermediate may be useful for studying protein-assisted folding and peptide binding of class I MHC molecules.

The mechanism by which the immune system responds to viral infections is initiated by intracellular processing of antigens into short peptide fragments that subsequently bind to class I MHC molecules in the lumen of the ER. Class I MHC–peptide complexes are transported through the Golgi apparatus and to the cell surface where they interact with specific protein receptors on cytotoxic T lymphocytes (CTLs).

Maturation of stable peptide-filled class I MHC molecules in the ER is a multi-step process mediated by association with chaperone proteins, including calnexin and calreticulin, the peptide transporter TAP, and the associated protein tapasin[1,2]. Results from immunoprecipitation studies using cell lines with specific defects have suggested that the assembly process is initiated by formation of the HC/β_2m heterodimer and involves the stepwise participation of calnexin and calreticulin[3–5]. The HC/β_2m heterodimer remains associated with calreticulin during its subsequent interaction with TAP through a newly identified protein named tapasin[5,6]. The heterodimeric TAP protein is a member of the ABC superfamily of transmembrane transporters and promotes, in some way, the binding of antigenic peptides to protein-associated HC/β_2m heterodimers[7–9]. Formation of stable peptide-filled class I MHC molecules causes dissociation of all associated proteins and initiates intracellular transport of the class I MHC–peptide complex to the cell surface. The specific roles and modes of action of calnexin, calreticulin, TAP, and tapasin in maturation of class I MHC–peptide complexes are not fully understood. Similarly, the conformational changes in HC at the different stages of assembly are largely uncharacterized.

Here, we describe the assembly and characterization of a soluble, recombinant intermediate of human class I MHC molecules: the apparently peptide-free HC(B7)/β_2m heterodimer. This intermediate possesses many of the properties characteristic of the molten globule state[10–12]: increased tendency to aggregate, substantial secondary structure, lack of a cooperative thermal unfolding transition, increased binding of the hydrophobic dye 1-anilinonaphtalene-8-sulphonic acid (ANS), and increased susceptibility to proteolysis. Furthermore, a temperature-dependent proteolytic cleavage of the B7/β_2m heterodimer with thermolysin has provided structural information by identifying the most destabilized regions of this intermediate in the α1 and α2 domains of the HC. These regions of the peptide binding site are highly susceptible to proteolysis by thermolysin in comparison to the α3 domain, which is stabilized by β_2m. In contrast, peptide-filled class I MHC molecules are resistant to proteolysis under identical conditions. These findings suggest a conformational change in the α1 and α2 domains from a more disordered state to a more rigid three-dimensional structure upon binding of a peptide ligand. This is consistent with findings that the peptide is an essential requirement for proper assembly of stable class I MHC–peptide complexes[13–18]. Results presented here also suggest that the intermediate B7/β_2m heterodimer may be useful in association with soluble, recombinant chaperones, transport proteins, and tapasin to clarify the folding and assembly of class I MHC–peptide complexes.

Assembly of class I MHC HC/β_2m heterodimers

Soluble HC/β_2m heterodimers were assembled from the denaturation of peptide-filled class I MHC molecules. This strategy releases bound peptide ligands and provides a source of denatured HC and β_2m with native disulfide bonds that can subsequently be reassembled, in the absence of synthetic peptides, to form HC/β_2m heterodimers. A similar approach was used successfully to exchange a mixture of endogenous peptides with a single synthetic peptide from the binding site of soluble class I MHC molecules[19], and to assemble mouse H-2K^d/human or mouse β_2m heterodimers[20,21] . No HC/β_2m heterodimers were

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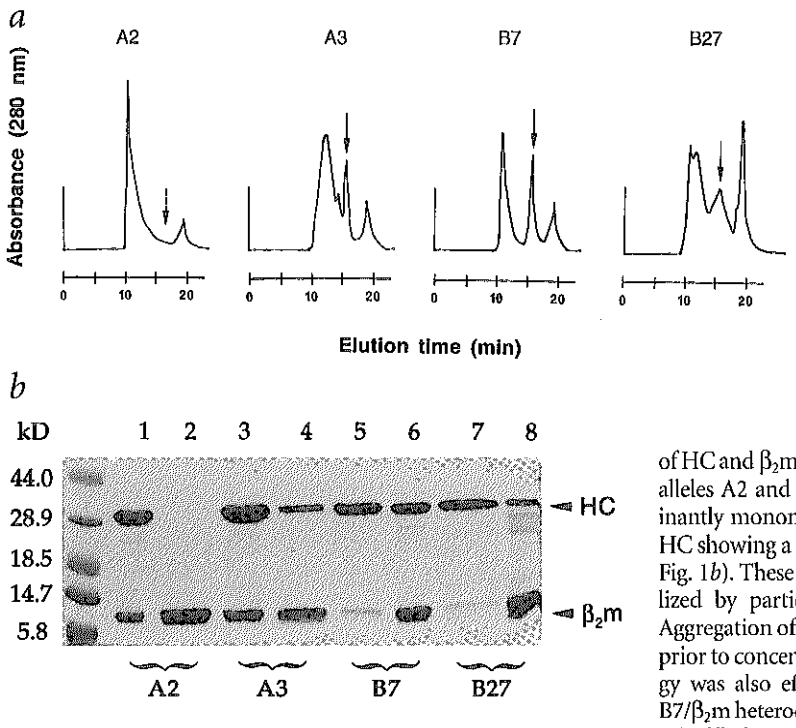


Fig. 1 ***a***, Gel filtration chromatograms obtained from the purification of HC/β_2m heterodimers for alleles A2, A3, B7, and B27. Analysis was done at 4 °C to prevent dissociation of the subunits using a Phenomenex Biosep Sec-S3000 column in 20 mM Tris-HCl pH 7.5 containing 150 mM NaCl (0.7 ml min^{-1}). The HC/β_2m heterodimers, indicated by solid arrows, eluted at 16 min. Adjacent peaks correspond to aggregates (11 min) and excess free β_2m (19 min). ***b***, SDS-PAGE (15%) analysis of fractions collected from (*a*), showing bands for HC and β_2m: lanes 1, 3, 5, and 7, aggregate fractions for alleles A2, A3, B7, and B27 respectively; lane 2, excess free β_2m for allele A2; and lanes 4, 6, and 8, HC/β_2m heterodimer fractions for alleles A3, B7, and B27 respectively (collected with an excess free β_2m). Molecular weight standards are indicated at left.

detected when non-disulfide-bonded denatured HC and β_2m subunits were reassembled in the absence of peptides (this study and ref. 22). Four different human class I MHC alleles (A*0201, A*0301, B*0702, and B*2705) were used to assemble HC/β_2m heterodimers. Since polymorphic residues are predominantly clustered in the binding site, it was expected that, in the absence of a bound peptide ligand, the stability of HC/β_2m heterodimers may vary with class I alleles. Furthermore, the rate and affinity of association between HC and β_2m has previously been reported to show allele and locus-specific differences[23,24]. For these reasons different class I MHC alleles were studied.

Fig. 1*a* shows gel filtration chromatography profiles obtained from the purification of HC/β_2m heterodimers using alleles A2, A3, B7 and B27. The chromatograms for alleles A3, B7 and B27 show a distinct peak (solid arrow) eluting at the expected time for monomeric HC/β_2m heterodimers (16 min). The absence of a peak (dashed arrow) corresponding to HC/β_2m heterodimers in the chromatogram of A2 indicates that separation of the denatured HC and β_2m subunits from peptide ligands was complete, providing supporting evidence that the peak eluting at 16 min in the other chromatograms corresponds to peptide-free HC/β_2m heterodimers. An SDS-PAGE analysis of fractions collected from purification of HC/β_2m heterodimers is shown in Fig. 1*b*. The aggregate fraction (11 min) for alleles A2, A3, B7, and B27 (lanes 1, 3, 5, and 7 respectively, in Fig. 1*b*), contains both HC and β_2m in nearly equimolar ratio for alleles A2 and A3, but contains predominantly HC for alleles B7 and B27. The HC/β_2m heterodimer fraction (16 min) (collected with an excess free β_2m, lane 2) for alleles A3, B7, and B27 (lanes 4, 6, and 8 respectively, in Fig. 1*b*) shows the presence of HC; a separate SDS-PAGE analysis of purified B7–β_2m heterodimer (with no excess free β_2m) showed HC and β_2m in equimolar ratio (data not shown).

These results suggest that the ability of HC to associate with β_2m and form soluble, monomeric HC/β_2m heterodimers differs primarily with locus, but shows also some allelic variations; the reassembly of HC and β_2m subunits yields mostly aggregates of heterodimers for alleles A2 and A3 (Fig. 1*a*; lanes 1 and 3 in Fig. 1*b*), but predominantly monomeric heterodimers for alleles B7 and B27, with only HC showing a tendency to form aggregates (Fig. 1*a*; lanes 5 and 7 in Fig. 1*b*). These results show that HC/β_2m heterodimers can be stabilized by particular residues in the MHC peptide binding site. Aggregation of B7 HC could be reduced by addition of 15 % glycerol prior to concentrating crude B7/β_2m heterodimers. The same strategy was also effective in increasing the recovery yield of purified B7/β_2m heterodimers. Glycerol had no effect on the assembly of peptide-filled B7 molecules. Since the assembly and purification yields of B7/β_2m heterodimers were repeatedly higher than those of other alleles, allele B7 was used for further studies.

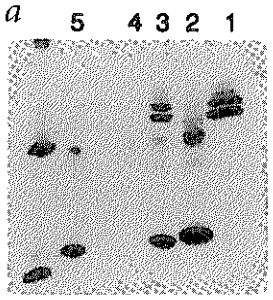
Absence of bound peptides

Purified B7/β_2m heterodimers were analyzed by native isoelectric focusing (IEF) gel electrophoresis and by fast atom bombardment (FAB) mass spectrometry, and the results (Fig. 2) compared with those of the peptide-filled B7 molecules, to provide evidence for the absence of synthetic peptides.

Lane 1 (Fig. 2*a*) shows that the peptide-filled B7 molecules focus as two bands of equal intensity indicating, most probably, the incomplete removal of the N-terminal methionine residue in the HC. In contrast, the B7/β_2m heterodimer (lane 2) shows no bands focusing at the same pHs as those of the peptide-filled B7 molecules. The absence of any intense bands in lane 2, other than that corresponding to free β_2m (lane 5), indicates that these heterodimers are unstable, and most likely dissociate during electrophoresis, causing HC to precipitate in the gel. This behavior indicates that the B7/β_2m heterodimers are devoid of stabilizing peptides.

The B7/β_2m heterodimers were incubated overnight at 37 °C with a large excess of either the B7-restricted APRTVALTA peptide[25] used in the assembly of peptide-filled B7 molecules, or the A3-restricted KLYEKVYTY peptide[26], to determine whether the heterodimers are competent to bind peptides selectively. Results show that the B7/β_2m heterodimers associate with the restricted APRTVALTA peptide to yield considerable amounts of peptide-filled B7 molecules (lane 3), as indicated by the presence of new bands focusing at the same pHs as those in lane 1. These results provide clear evidence that B7/β_2m heterodimers can interact, in some way, with restricted peptides to form molecules that are indistinguishable, by native IEF gel electrophoresis, from peptide-filled B7 molecules. As expected, incubation of the B7/β_2m heterodimers with the A3-restricted peptide (lane 4) failed to form peptide-filled B7 molecules, as evidenced by the lack of bands focusing at the same pHs as those in lane 1.

The FAB mass spectra of peptide-filled class I MHC molecules are typically characterized by intense ion peaks corre-



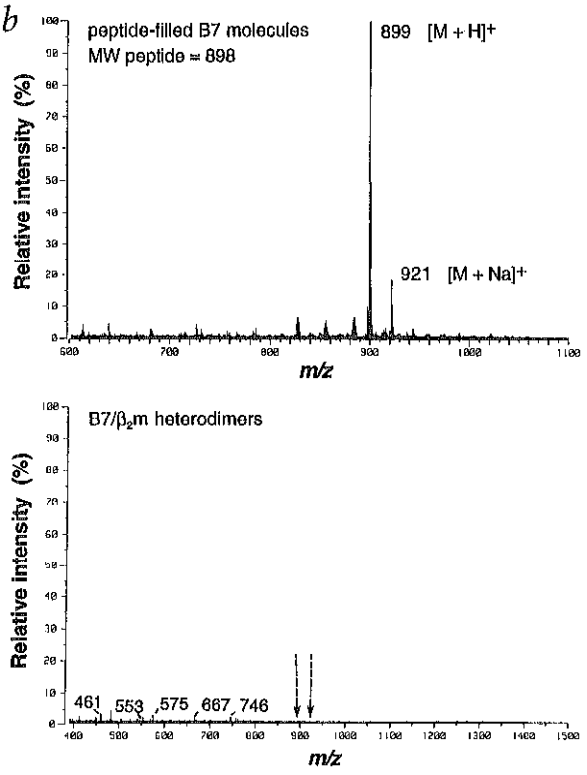


Fig. 2 ***a***, Native IEF gel electrophoresis (pH 4–6.5) carried out at 10 °C: lane 1, peptide-filled B7 molecules, peptide ligand is APRTVALTA[25]; lane 2, B7/β_2m heterodimers; lane 3, HC/β_2m heterodimers incubated overnight at 37 °C with the B7-restricted peptide APRTVALTA; lane 4, HC/β_2m heterodimers incubated overnight at 37 °C with the A3-restricted peptide KLYEKVYTY[26]; and lane 5, free β_2m. Unlabeled lane corresponds to IEF standards. ***b***, FAB mass spectrum of purified peptide-filled B7 molecules showing the expected peptide ion peaks for APRTVALTA ([M+H]$^+$ = 899 and [M+Na]$^+$ = 921). These peaks are absent (dashed arrows) in the FAB mass spectrum of purified B7–β_2m heterodimers.

sponding to the molecular weight of the bound peptide ligand[22,27]. This is illustrated in Fig. 2*b* with the FAB mass spectrum of the peptide-filled B7 molecules showing two intense ion peaks corresponding to the APRTVALTA peptide (898 M_r). To obtain additional evidence that the B7/β_2m heterodimers are devoid of peptide ligands, the FAB mass spectrum of these heterodimers was recorded (Fig. 2*b*). The spectrum shows no major peaks (dashed arrows) at mass-to-charge ratio (*m/z*) 898 and 921. No other major peaks in the range *m/z* 700–1000 were observed suggesting the absence of any short peptides associated with the B7/β_2m heterodimers. A series of small peaks (labeled in Fig. 2*b*) could not be accounted for by fragmentation of the APRTVALTA peptide and was also different with each new stock solution of B7/β_2m heterodimers, suggesting that they are background peaks. These results provide further evidence that the reassembly of denatured B7 and β_2m subunits, after removal of the synthetic peptide, leads to the formation of peptide-free B7/β_2m heterodimers.

Circular dichroism and fluorescence

Circular dichroism (CD) spectroscopy was used to characterize the B7/β_2m heterodimers and the results were compared with those of the peptide-filled B7 molecules. CD spectra were recorded between 250–198 nm (Fig. 3*a*) and thermal denaturation curves were obtained by monitoring the change in CD signal at 218 nm in the range 15–90 °C (Fig. 3*b*).

The CD spectrum of the B7/β_2m heterodimers (curve at 15 °C in Fig. 3*a*) shows a well-defined maximum centered at 218 nm which is characteristic of proteins having a high content of β-sheet[28]. This CD spectrum is distinctively different from that of the unfolded B7/β_2m heterodimers (curve at 70 °C), but remarkably similar to that of the peptide-filled B7 molecules (curve at 15 °C). These results suggest that, at low temperature, the B7/β_2m heterodimers possess elements of secondary structure that are comparable to those of the peptide-filled B7 molecules. The thermal denaturation curve of the B7/β_2m heterodimers (Fig. 3*b*) shows a steady decrease in CD signal as the temperature increases with no distinct unfolding transition other than a subtle change in the range 25–35 °C. The lack of a cooperative thermal transition is generally associated with the absence of a rigid tertiary structure in proteins[10]. The denaturation curve of the peptide-filled B7 molecules shows, in contrast, a single sharp transition centered at 67.8 °C (melting temperature), reflecting the cooperative nature of the unfolding process. Similar profiles with steep transitions were previously reported for the thermal denaturation of peptide-filled class I and class II MHC molecules[20–22,29,30], including some very unstable peptide-filled A2 molecules with steep thermal denaturation transitions as low as 43 °C[22].

The small hydrophobic dye ANS has been used widely as a probe to identify the molten globule state[31,32]. Molten globules typically bind more ANS molecules than native proteins due to the increased mobility of their hydrophobic core structure, which allows diffusion of the probe into the globule. Binding of ANS to hydrophobic regions of proteins causes a dramatic increase of its fluorescence and a blue-shift of its λmax. The fluorescence emission spectra of ANS in the presence of B7/β_2m heterodimers and peptide-filled B7 molecules were recorded and compared (Fig. 3*c*). Results show that the B7/β_2m heterodimers and the peptide-filled B7 molecules bind ANS, as indicated by the increase in emission intensity and by the shift of λmax to lower wavelengths. Both of these changes are more significant for ANS bound to the B7/β_2m heterodimers, as would be expected if B7/β_2m heterodimers had partially folded regions that lack rigid tertiary structure like molten globules. Interpretation of these fluorescence data is, however, complicated by the difficulty of determining the contribution of the peptide *per se* in binding ANS; the emission spectrum of ANS may show different spectral features with each peptide-filled class I MHC molecule.

Proteolysis with various enzymes

Susceptibility of proteins to proteolytic cleavage provides a simple way to determine the presence of rigid three-dimensional structure; exposed or flexible regions of proteins are more sensitive to proteolysis in comparison to more buried or ordered regions. The B7/β_2m heterodimers and peptide-filled B7 molecules were incubated with six different enzymes to compare their stability to proteolysis (Fig. 4).

SDS-PAGE analysis of the proteolytic products from incubation of B7/β_2m heterodimers with trypsin, elastase, V8-protease,

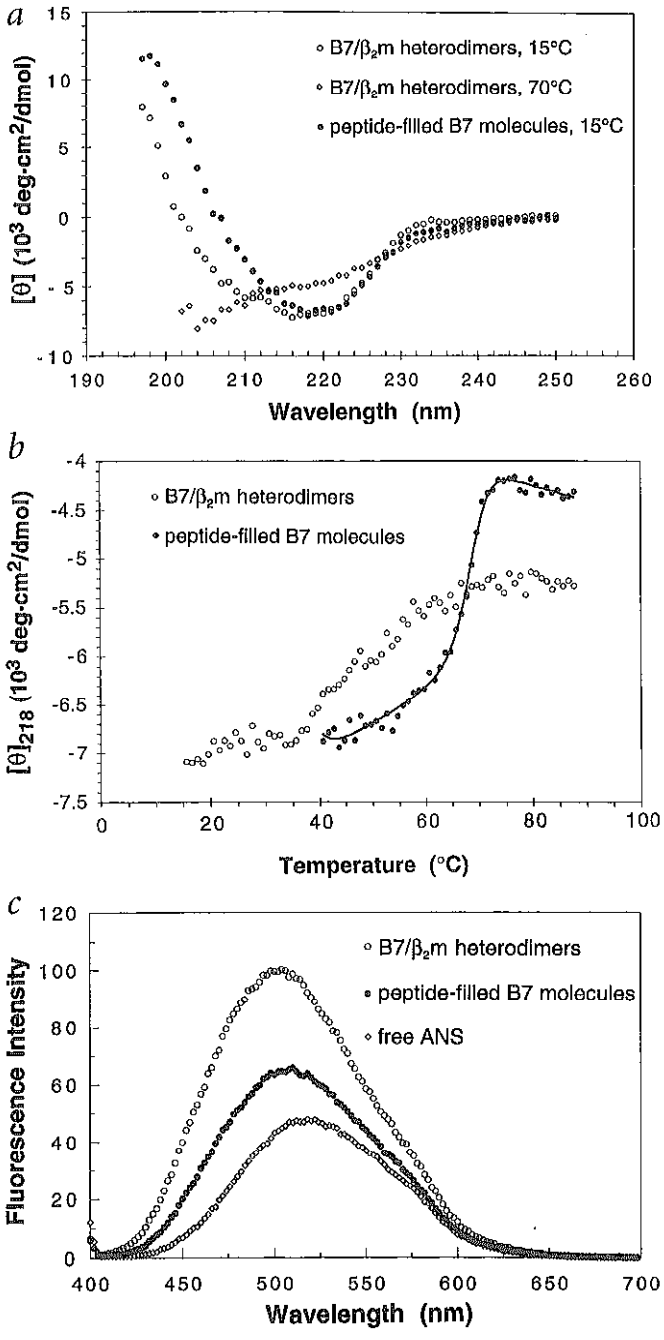


Fig. 3 ***a***, CD spectra (250–198 nm) of B7/β_2m heterodimers at 15 °C (open circles) and at 70 °C (open diamonds), and of peptide-filled B7 molecules at 15 °C (closed circles). Protein concentrations were 0.18 mg ml^{-1} in 20 mM Tris-HCl pH 7.5 containing 15% glycerol and 150 mM NaCl. ***b***, Thermal denaturation curves (15–90 °C) for B7/β_2m heterodimers (open circle) and peptide-filled B7 molecules (closed circles). Curves were obtained in triplicate by monitoring the change in CD signal at 218 nm. A two-state unfolding model was assumed to generate the theoretical line through the data points of peptide-filled B7 molecules[22]. Protein concentrations were 0.18 mg ml^{-1} in 20 mM Tris-HCl pH 7.5 containing 15% glycerol and 150 mM NaCl. ***c***, Fluorescence emission spectra of ANS (200 μM) recorded at 15 °C in the presence of B7/β_2m heterodimers (3.9 μM) (open circle) and peptide-filled B7 molecules (4.5 μM) (closed circle). The spectrum of free ANS (200 μM) is also shown (open diamonds). Buffer was 20 mM Tris-HCl pH 7.5 containing 15% glycerol and 150 mM NaCl.

α-chymotrypsin, proline specific endopeptidase, and thermolysin is shown in Fig. 4*a* (lanes 1–6 respectively). The B7/β_2m heterodimer is sensitive to cleavage with four of the enzymes (lanes 1, 3, 4 and 6) as indicated by the change in the intensity of the HC and β_2m bands in comparison to the control (lane C). These results also show that HC is more susceptible to proteolysis than β_2m; HC is cleaved by trypsin, V8-protease, α-chymotrypsin, and thermolysin (lanes 1, 3, 4 and 6), however, β_2m is cleaved only by trypsin and thermolysin (lanes 1 and 6). The digest of HC with trypsin and V8-protease (lanes 1 and 3) yields stable fragments migrating at ~13,000 M_r on the gel. Incubation of peptide-filled B7 molecules with the same six enzymes leads, in contrast, to dramatically different results (Fig. 4*b*). The peptide-filled B7 molecules are completely resistant to proteolysis with all enzymes, as indicated by the intensity of the HC and β_2m bands in comparison to those of the control (lane C). Considering the broad range of substrate specificity of these enzymes, and the distinct patterns of fragmentation obtained in Fig. 4*a*, these results suggest that the B7/β_2m heterodimers possess a more disordered and destabilized three-dimensional structure than the peptide-filled molecules, but still retain some stable structure that prevents complete degradation by proteolysis.

An SDS-PAGE analysis of aliquots taken from the tryptic mixture of B7/β_2m heterodimers after 1, 3 and 8 h incubation is shown in Fig. 4*c* (lanes 1, 2 and 3). Results show that proteolysis of HC reaches an endpoint after ~1 hour of incubation and leads to the accumulation of two fragments migrating at ~14,000 M_r and ~13,000 M_r. The fragment of ~14,000 M_r is fully digested over a period of 8 hours into the more stable ~13,000 M_r fragment (lane 3). The susceptibility of β_2m to proteolysis in the HC/β_2m heterodimers is apparently linked to the digestion of HC since it occurs only after the digestion of HC has been initiated (lane 2) and, more evidently, because β_2m is resistant to cleavage in the peptide-filled B7 molecules (Fig. 4*b*).

The results obtained from spectroscopy and proteolysis provide clear evidence that the B7/β_2m heterodimer and the peptide-filled B7/β_2m molecule are two different structural states with distinct properties. The increased tendency to form aggregates, the relatively high content of secondary structure, the lack of a cooperative thermal transition, the increased tendency to bind ANS, and the increased susceptibility to proteolysis, are all properties shared by B7/β_2m heterodimers and partially folded states like molten globules. The destabilized three-dimensional structure of this soluble intermediate is consistent with the findings that although β_2m can associate with HC before peptide binding in the assembly process, the peptide ligand is an essential requirement to achieve a stable and biologically functional structure[13–18,33]. Class II MHC molecules that lack bound peptides were also reported to have a more 'floppy' structure[34,35] and form aggregates[36,37].

Temperature-dependent thermolytic digest

To gain further insight into the structure of the partially folded state of the B7/β_2m heterodimers, thermolytic digests were carried out at different temperatures (Fig. 5). Since thermolysin displays enzymatic activity over a broad range of temperature, this enzyme can be used to estimate the thermal stability of proteins[38,39]. In the case of peptide-filled class I MHC molecules, this analysis also revealed differences in the stability of HC and β_2m[22].

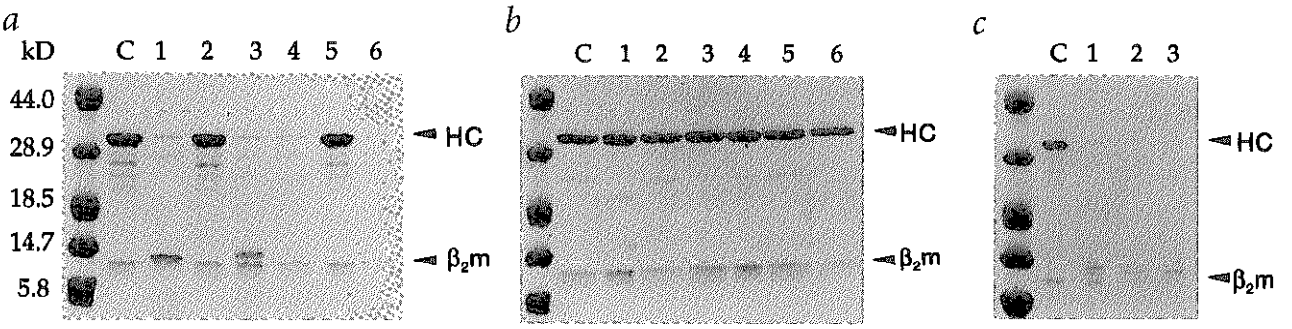


Fig. 4 SDS-PAGE (15%) analysis of products from the digest of ***a***, B7/β_2m heterodimers and ***b***, peptide-filled B7 molecules with trypsin, elastase, V8-protease, α-chymotrypsin, proline specific endopeptidase, and thermolysin (lanes 1–6 respectively) showing HC and β_2m bands. Digests were carried out at 25 °C for 24 h at enzyme:substrate ratio 1:100 (W/W) in 20 mM Tris-HCl pH 7.5 containing 15 % glycerol and 150 mM NaCl. Lane C in (*a*) and (*b*) is protein without added enzymes, loaded at the same concentration as in lanes 1–6. Molecular weight standards are indicated at left in (*a*). **c**, SDS-PAGE (15 %) analysis of products from the digest of B7/β_2m heterodimers with trypsin showing HC and β_2m bands. Aliquots were removed after 1, 3 and 8 h (lanes 1–3 respectively) incubation. Same digest conditions as given above in (*b*).

An SDS-PAGE analysis of products from the thermolytic digest of B7/β_2m heterodimers is shown in Fig. 5*a*. These results show that proteolysis of B7/β_2m heterodimers is initiated by the breakdown of HC in the temperature range 15–37 °C yielding a series of medium size fragments, as indicated by several new bands (bands 1–5) that are absent in the control (lane C). The cleavage of HC leads to a stable fragment, migrating at ~14,000 M_r (band 5), that disappears from the proteolytic mixture above 37 °C. The temperature range for the digestion of HC correlates with the subtle changes between 25–35 °C of the thermal denaturation curve (Fig. 3*b*) of B7/β_2m heterodimers, suggesting that the unfolding of HC is responsible for the appearance of the thermal denaturation curve in this temperature range. The disappearance of β_2m from the proteolytic mixture occurs between 55–60 °C (Fig. 5*a*), which is consistent with previously reported results for both free β_2m and peptide-filled A2 molecules[22].

The results obtained in Fig. 5*a* show clearly that the increased susceptibility of the B7/β_2m heterodimers to proteolysis is due predominantly to the destabilized structure of HC with respect to that of β_2m. The three-dimensional structure of β_2m in B7/β_2m heterodimers is likely to be similar to that determined in peptide-filled class I MHC molecules[40,41]. Previous studies[22] have shown that the temperature-dependent thermolytic digestion of very unstable peptide-filled A2 molecules ($T_m \cong 43$ °C) yielded no medium size fragments of HC, as obtained here with the B7/β_2m heterodimers (Fig. 5*a*), but rather an all-or-none disappearance of the HC band on SDS-PAGE gel (at ~43 °C). These differences in the pattern of HC fragmentation reflect a change in the unfolding process from a cooperative transition in peptide-filled molecules (Fig. 3*b*; see below), even with unstable peptide-filled molecules, to a non-cooperative transition in HC/β_2m heterodimers (Fig. 3*b*).

Table 1 Identification of cleavage sites in fragments of HC produced by the temperature-dependent thermolytic digest of B7/β_2m heterodimers

Band[1]	Cleavage site[2]	Domain in HC[3]
1	Leu 81	α1
2	Leu 95	α2
3	Ile 124	α2
4	Ile 142	α2
5	Leu 160	α2

[1]Bands 1–5 (Fig. 5*a*).
[2]Cleavage sites were determined by N-terminal amino acid sequencing (10 cycles) of bands 1–5 excised from the electroblotted SDS-PAGE gel shown in Fig. 5*a*. Residues are numbered from Gly 1.
[3]See refs 40, 51.

An SDS-PAGE analysis of products from the thermolytic digestion of the peptide-filled B7 molecules is shown in Fig. 5*b*. The results show that these molecules are completely resistant to proteolysis below 60 °C, as indicated by the intensity of the HC and β_2m bands in comparison to the control (lane C). The peptide-filled molecules are digested in the range 60–64 °C as evidenced by the simultaneous disappearance of both the HC and β_2m bands on gel. These results correlate with the profile of the thermal denaturation curve (Fig. 3*b*) showing a single sharp transition, and with the measured melting temperature (T_m = 67.8 °C). The concomitant unfolding, and thus digestion, of HC and β_2m have been observed in the temperature-dependent proteolysis of other peptide-filled class I MHC molecules with thermolysin[22].

Structural characterization of B7/β_2m heterodimers

Insight into the three-dimensional structure of the B7/β_2m heterodimers were obtained by directly identifying the sites of thermolytic cleavage in HC. This was done by electroblotting the SDS-PAGE gel shown in Fig. 5*a* onto a transfer membrane followed by N-terminal amino acid sequencing of the excised bands corresponding to thermolytic fragments of HC (bands 1–5). The data in Table 1 firmly establish that all cleavage sites are within the peptide binding site of HC (α1 and α2 domains) with no evidence of proteolysis in the α3 domain. The locations of the cleavage sites in the structure of the class I MHC molecule are shown in Fig. 6. These results provide direct evidence that, in the absence of a bound peptide ligand, the peptide binding site of the B7/β_2m heterodimers is unstable, adopting a partially folded conformation with properties like those typical of molten globules. Upon binding of a peptide, the conformational changes in the α1 and α2 domains result in a more rigid and stable tertiary structure with increased resistance to proteolysis. These results are consistent with previous studies and models proposing that HC in HC/β_2m heterodimers assumes an 'open' conformation[15,18,42–46].

The most stable fragment produced by the temperature-dependent thermolytic digest of B7/β_2m heterodimers (band 5 in Fig. 5*a*) corresponds to the α3 domain and 22 addi-

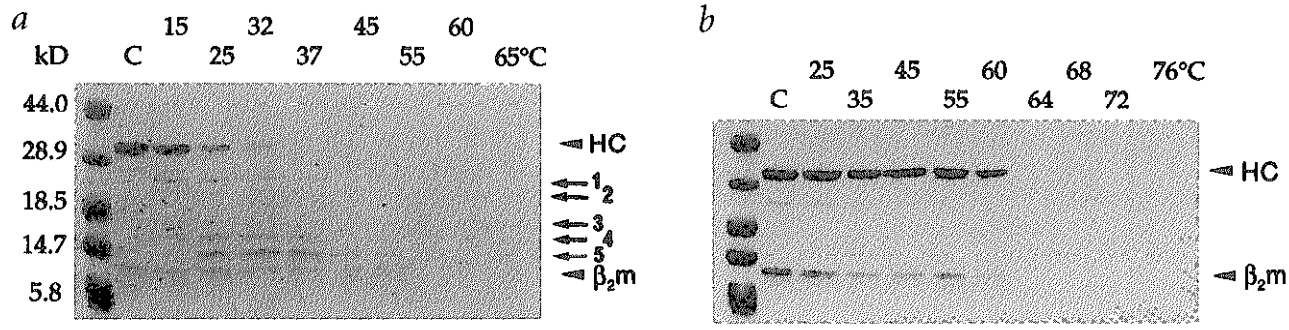


Fig. 5 **a**, SDS-PAGE (15%) analysis of products from the thermolytic digest of B7/β_2m heterodimers showing HC and β_2m bands. Digests were carried out at the indicated temperatures for 15 min at enzyme:substrate ratio 1:300 (W/W) in 20 mM Tris-HCl pH 7.5 containing 15% glycerol and 150 mM NaCl. Bands labeled 1–5 correspond to proteolytic fragments of HC that were analyzed by N-terminal amino acid sequencing. The control lane (lane C) is protein without added enzymes, loaded at the same concentration as in other lanes. Molecular weight standards are indicated at the left. ***b***, SDS-PAGE analysis of products from the thermolytic digest of peptide-filled B7 molecules. Same digest conditions as given above in (*a*).

tional residues from the α2 domain (Table 1). The size of this fragment on the gel is comparable to those of the stable fragments produced in the digests of B7/β_2m heterodimers with trypsin and V8-protease (lanes 1 and 3 in Fig. 4*a*), suggesting that the α3 domain can fold into a stable structure independently from the α1 and α2 domains[47]. Association of the α3 domain with β_2m may contribute to this increased stability[47]. Finally, results from Fig. 5*a* indicating that the α3 domain unfolds above 37 °C, correlates with our previous proteolysis studies showing that the thermolytic digestion of HC in very unstable peptide-filled A2 molecules occurs in the range 34–39 °C[22]. Collectively, these results suggest that the affinity of HC for β_2m is sufficiently high at physiological temperature to allow the assembly and transport of peptide-filled class I molecules with suboptimal peptides.

In summary, results presented here demonstrate that the renaturation of correctly disulfide-bonded B7 HC and β_2m subunits yields a soluble folding intermediate referred to as the B7/β_2m heterodimer. Biochemical and structural studies of the soluble B7/β_2m heterodimer indicate that it has a partially folded structure and displays many properties characteristic of the molten globule state including: an increased tendency to form aggregates; a relatively high content of secondary structure; the lack of a cooperative thermal transition; an increased tendency to bind ANS; and an increased susceptibility to proteolysis. Incubation of the B7/β_2m heterodimers with a B7-restricted peptide leads to the formation of molecules that are indistinguishable, by IEF gel electrophoresis, from the native peptide-filled B7 molecules. Stabilization of molten globule states by ligand binding has been observed in other proteins[48, 49]. N-terminal amino acid sequencing of fragments produced by the thermolytic digest of B7/β_2m heterodimers has provided evidence that the structure of the α1 and α2 domains that form the peptide binding site of HC is partially folded and unstable. The structure of the α3 domain is more rigid and is stabilized by association with β_2m.

The finding that the HC/β_2m heterodimer is in an unstable, partially folded state is consistent with the evidence that this intermediate associates with chaperone proteins in the ER[1,2]. These interactions provide a mechanism to stabilize the HC/β_2m heterodimers and to retain them in the lumen of the ER until peptides are translocated from the cytosol by TAP and assembly of the class I MHC-peptide complex is completed in the presence of tapasin[5,6]. Although crystallization trials are in progress (see also ref. 20), crystals of the B7/β_2m heterodimers may prove difficult to grow considering the physical properties of these molecules. The soluble B7/β_2m heterodimer provides a molecule that may be useful in association with chaperones, transport proteins, and tapasin to study directly the stepwise folding and assembly of peptide-filled class I MHC molecules. Such complexes may also be amenable to crystallization.

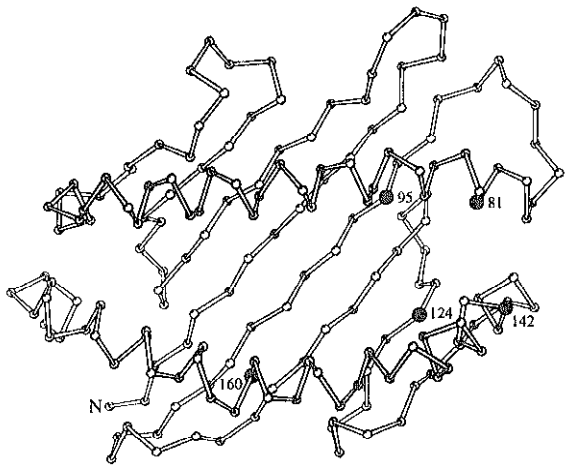


Fig. 6 The location of the thermolytic cleavage sites in the structure of the class I MHC molecule. Coordinates are from the HLA-A2–peptide model, with the peptide left out of the figure. Only the α1 and α2 domains, residues 1–181, are shown.

Methods

Assembly of class I MHC–peptide complexes. Class I MHC-peptide complexes were assembled from *E. coli*-expressed HC (1 μM) and β_2m (2 μM) in the presence of excess synthetic peptides (10 μM) [50]. Refolding mixtures were incubated at 10 °C for two days and purified by gel filtration chromatography. Several class I MHC alleles (A*0201, A*0301, B*0702, and B*2705) were used in the refolding experiments. Purified class I MHC–peptide complexes were characterized by FAB mass spectrometry and by native IEF gel electrophoresis in the pH range 4–6.5.

Assembly of class I MHC HC/β_2m heterodimers. Soluble HC/β_2m heterodimers were assembled from the denaturation of purified peptide-filled class I MHC molecules. Class I MHC–peptide

complexes were incubated at room temperature for 4 h in buffered 6 M guanidine hydrochloride. Denatured HC and β_2m were separated from peptide ligands by gel filtration chromatography in buffered 6 M guanidine hydrochloride. Fractions corresponding to HC and β_2m subunits were combined, diluted to 0.1 mg ml^{-1}, and dialyzed at 15 °C overnight in buffered 8 M urea. Renaturation of subunits was initiated by extensive dialysis at 4 °C in 20 mM Tris-HCl pH 7.5 containing 150 mM NaCl (some initial experiments included the addition of an excess of β_2m at this step). Glycerol (to 15%) was added to assembled HC/β_2m heterodimers prior to concentration (some initial experiments omitted the addition of glycerol). The HC/β_2m heterodimers were purified by gel filtration chromatography at 4 °C, and fractions concentrated in the presence of 15% glycerol. Stock solutions were kept frozen at -20 °C. Purified HC/β_2m heterodimers were characterized by FAB mass spectrometry and by native IEF gel electrophoresis in the pH range 4–6.5.

Circular dichroism measurements. CD experiments were done using an Aviv 62DS spectropolarimeter equipped with a thermoelectric temperature controller. Protein concentrations (0.18 mg ml^{-1}) in 20 mM Tris-HCl pH 7.5 containing 15% glycerol and 150 mM NaCl were determined by amino acid analysis using norleucine as an internal standard. Ellipticities are expressed on a molar residue basis. CD spectra were recorded in the range 250–198 nm at the indicated temperatures using a 1 mm cell. Denaturation curves were obtained in triplicate by monitoring the change in CD signal at 218 nm in the range 15–90 °C[22].

Fluorescence measurements. Fluorescence spectra were recorded at 15°C using a Hitachi F-2000 spectrofluorometer. Binding of ANS to proteins was measured by adding 250 µl of ANS stock solution (400 µM) to 250 ul of protein stock solutions (8.0–9.0 µM) in 20 mM Tris-HCl pH 7.5 containing 15% glycerol and 150 mM NaCl. After 1 h incubation, samples were excited at 390 nm and the ANS emission fluorescence spectra were recorded in the range 400–700 nm. Protein concentrations were determined by amino acid analysis using norleucine as an internal standard.

Proteolysis with various enzymes. Digestions with trypsin, elastase, V8-protease, α-chymotrypsin, proline specific endopeptidase, and thermolysin were carried out at 25 °C for 24 h at enzyme:substrate ratio of 1:100 (W/W) in 20 mM Tris-HCl pH 7.5 containing 15% glycerol and 150 mM NaCl. Kinetic experiments were performed using identical conditions, aliquots were removed from the proteolytic mixture after 1, 3 and 8 h incubation. Proteolysis was terminated by the addition of SDS-PAGE solubilization buffer. Products of digests were analyzed by 15% SDS-PAGE.

Temperature-dependent proteolysis with thermolysin. Digestions with thermolysin were carried out at different temperatures for 15 min at enzyme:substrate ratio of 1:300 (W/W) in 20 mM Tris-HCl pH 7.5 containing 15% glycerol, 150 mM NaCl, and 2 mM $CaCl_2$ (ref. 22). Proteolysis was terminated by the addition of EDTA (to 10 mM) and immediately cooling samples on ice. Products of digests were analyzed by 15% SDS-PAGE.

N-terminal amino acid sequencing. The SDS-PAGE gel from the temperature-dependent thermolytic digest of B7/β_2m heterodimers (Fig. 5a) was electroblotted in a Bio-Rad Trans-Blot system onto a polyvinylidene difluoride membrane (Millipore). The membrane was stained with Ponceau S and bands corresponding to the desired fragments of HC were cut out, washed, and analyzed by N-terminal amino acid sequencing.

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1. Solheim, J.C., Carreno, B.M. & Hansen, T.H. Are transporter associated with antigen processing (TAP) and tapassin class I MHC chaperones? *J. Immunol.* **158**, 541–543 (1997).
2. Williams, D.B. & Watts, T.H. Molecular chaperones in antigen presentation. *Curr. Opin. Immunol.* **7**, 77–84 (1995).
3. Sugita, M. & Brenner, M.B. An unstable β2-microglobulin: Major histocompatibility complex class I heavy chain intermediate dissociates from calnexin and then is stabilized by binding peptide. *J. Exp. Med.* **180**, 2163–2171 (1994).
4. Nöbner, E. & Parham, P. Species-specific differences in chaperone interaction of human and mouse major histocompatibility complex class I molecules. *J. Exp. Med.* **181**, 327–337 (1995).
5. Sadasivan, B., Lehner, P.J., Ortman, B., Spies, T. & Cresswell, P. Roles for calreticulin and a novel glycoprotein, tapassin, in the interaction of MHC class I molecules with TAP. *Immunity* **5**, 103–114 (1996).
6. Ortmann, B. *et al.* A critical role for tapasin in the assembly and function of multimeric MHC class I-TAP complexes. *Science* **277**, 1306–1309 (1997).
7. Ortmann, B., Androlewicz, M. & Cresswell, P. MHC class I/β2microglobulin complexes associate with TAP transporters before peptide binding. *Nature* **368**, 864–867 (1994).
8. Suh, W.-K. *et al.* Interaction of MHC class I molecules with the transporter associated with antigen processing. *Science* **264**, 1322–1326 (1994).
9. Androlewicz, M.J., Ortmann, B., van Endert, P.M., Spies, T. & Cresswell, P. Characteristics of peptide and major histocompatibility complex class I/β2microglobulin binding to the transporters associated with antigen processing (TAP1 and TAP2). *Proc. Natl. Acad. Sci. USA* **91**, 12716–12720 (1994).
10. Ptitsyn, O.B. Molten globule and protein folding. *Adv. Prot. Chem.* **47**, 83–229 (1995).
11. Christensen, H. & Pain, R.H. Molten globule intermediates and protein folding. *Eur. Biophys. J.* **19**, 221–229 (1991).
12. Creighton, T.E. How important is the molten globule for correct protein folding? *Trends Biochem. Sci.* **22**, 6–11 (1997).
13. Elliott, T., Cerundolo, V., Elvin, J. & Townsend, A. Peptide-induced conformational change of the class I heavy chain. *Nature* 402–406 (1991).
14. Townsend, A. *et al.* Association of class I major histocompatibility heavy and light chains induced by viral peptides. *Nature* **340**, 443–448 (1989).
15. Townsend, A. *et al.* Assembly of MHC class I molecules analyzed in vitro. *Cell* **62**, 285–295 (1990).
16. Lie, W.-R. *et al.* Peptide ligand-induced conformation and surface expression of the L^d class I MHC molecule. *Nature* **344**, 439–441 (1990).
17. Schumacher, T.N.M. *et al.* Direct binding of peptide to empty MHC class I molecules on intact cells and in vitro. *Cell* **62**, 563–567 (1990).
18. Ljunggren, H.G. *et al.* Empty MHC class I molecules come out in the cold. *Nature* **346**, 476–480 (1990).
19. Silver, M.L., Parker, K.C. & Wiley, D.C. Reconstitution by MHC-restricted peptides of HLA-A2 heavy chain with β2-microglobulin, *in vitro*. *Nature* **350**, 619–622 (1991).
20. Fahnestock, M.L., Tamir, I., Narhi, L. & Bjorkman, P.J. Thermal stability comparison of purified empty and peptide-filled forms of a class I MHC molecule. *Science* **258**, 1658–1662 (1992).
21. Fahnestock, M.L. *et al.* Effects of peptide length and composition in binding to an empty class I MHC heterodimer. *Biochemistry* **33**, 8149–8158 (1994).
22. Bouvier, M. & Wiley, D.C. Importance of peptide amino and carboxyl termini to the stability of MHC class I molecules. *Science* **265**, 398–402 (1994).
23. Beck, J.C., Hansen, T.H., Cullen, S.E. & Lee, D.R. Slower processing, weaker β2-M association, and lower surface expression of H-$2L^d$ are influenced by its amino terminus. *J. Immunol.* **137**, 916–923 (1986).
24. Neefjes, J.J. & Ploegh, H.L. Allele and locus-specific differences in cell surface expression and the association of HLA class I heavy chain with β2-microglobulin: differential effects of inhibition of glycosylation on class I subunit association. *European J. Immunol.* **18**, 801–810 (1988).
25. Huczko, E.L. et al. Characteristics of endogenous peptides eluted from the class I MHC molecule HLA-B7 determined by mass spectrometry and computer modeling. *J. Immunol.* **151**, 2572–2587 (1993).
26. DiBrino, M. *et al.* Endogenous peptides bound to HLA-A3 possess a specific combination of anchor residues that permit identification of potential antigenic peptides. *Proc. Natl. Acad. Sci. USA* **90**, 1508–1512 (1993).
27. Bouvier, M. & Wiley, D.C. Antigenic peptides containing large PEG loops designed to extend out of the HLA-A2 binding site form stable complexes with class I major histocompatibility complex molecules. *Proc. Natl. Acad. Sci. USA* **93**, 4583–4588 (1996).
28. Greenfield, N. & Fasman, G.D. Computed circular dichroism spectra for the evaluation of protein conformation. *Biochemistry* **8**, 4108–4116 (1969).
29. Reich, Z. *et al.* Stability of empty and peptide-loaded class II major histocompatibility complex molecules at neutral and endosomal pH: comparison to class I proteins *Proc. Natl. Acad. Sci. USA* **94**, 2495–2500 (1997).
30. Morgan, C.S., Holton, J.M., Olafson, B.D., Bjorkman, P.J. & Mayo, S.L. Circular dichroism determination of class I MHC-peptide equilibrium dissociation constants. *Prot. Sci.* **6**, 1771–1773 (1997).
31. Semisotnov, G.V. *et al.* Study of the "molten globule" intermediate state in protein folding by a hydrophobic fluorescent probe. *Biopolymers* **31**, 119–128 (1991).
32. Uversky, V.N., Winter, S. & Löber, G. Use of fluorescence decay times of 8-ANS-protein complexes to study the conformational transitions in proteins which unfold through the molten globule state. *Biophys. Chem.* **60**, 79–88 (1996).
33. Neefjes, J.J., Hämmerling, G.J. & Momburg, F. Folding and assembly of major histocompatibility complex class I heterodimers in the endoplasmic reticulum of intact cells precedes the binding of peptide. *J. Exp. Med.* **178**, 1971–1980 (1993).
34. Dornmair, K. & McConnell, H.M. Refolding and reassembly of separate α and β chains of class II molecules of the major histocompatibility complex leads to increased peptide-binding capacity *Proc. Natl. Acad. Sci. USA* **87**, 4134–4138 (1990).
35. Sadegh-Nasseri, S. & Germain, R.N. A role for peptide in determining MHC class II structure. *Nature* **353**, 167–170 (1991).
36. Stern, L.J. & Wiley, D.C. The human class II MHC protein HLA-DR1 assembles as empty αβ heterodimers in the absence of antigenic peptide. *Cell* **68**, 1–20 (1992).
37. Germain, R.N. & Rinker Jr, A.G. Peptide binding inhibits protein aggregation of invariant-chain free class II dimers and promotes surface expression of occupied molecules. *Nature* **363**, 725–728 (1993).
38. Heinrikson, R.L. Applications of thermolysin in protein structural analysis. *Meth. Enz.* **47**, 175–189 (1977).
39. Hecht, M.H., Sturtevant, J.M. & Sauer, R.T. Effect of single amino acid replacements on the thermal stability of the NH_2-terminal domain of phage-λ repressor. *Proc. Natl. Acad. Sci. USA* **81**, 5685–5689 (1984).
40. Bjorkman, P.J. *et al.* Structure of the human class I histocompatibility antigen, HLA-A2. *Nature* **329**, 506–512 (1987).
41. Madden, D.R., Garboczi, D.N. & Wiley, D.C. The antigenic identity of peptide-MHC complexes: a comparison of the conformations of five viral peptides presented by HLA-A2. *Cell* **75**, 693–708 (1993).
42. Elliott, T., Cerundolo, V. & Townsend, A. Short peptides assist the folding of free class I heavy chains in solution. *European J. Immunol.* **22**, 3121–3125 (1992).
43. Parham, P. Antigen presentation. Peptide feeding and cellular cookery. *Nature* **346**, 793–795 (1990).
44. Smith, J.D. *et al.* Disparate interaction of peptide ligand with nascent versus mature class I major histocompatibility complex molecules: Comparison of peptide binding to alternative forms of L^d in cell lysates and the cell surface. *J. Exp. Med.* **175**, 191–202 (1992).
45. Smith, J.D., Myers, N.B., Gorga, J. & Hansen, T.H. Model for the in vivo assembly of nascent L^d class I molecules and for the expression of unfolded L^d molecules at the cell surface. *J. Exp. Med.* **178**, 2035–2046 (1993).
46. Smith, J.D., Solheim, J.C., Carreno, B.M. & Hansen, T.H. Characterization of class I MHC folding intermediates and their diparate interactions with peptides and β2-microglobulin. *Mol. Immunol.* **32**, 531–540 (1995).
47. Elliott, T., Elvin, J., Cerundolo, V., Allen, H. & Townsend, A. Structural requirements for the peptide-induced conformational change of free major histocomopatibility complex class I heavy chains. *European J. Immunol.* **22**, 2085–2091 (1992).
48. Uversky, V.N. *et al.* Circularly permuted dihydrofolate reductase possesses all the properties of the molten globule state, but can resume functional tertiary structure by interaction with its ligands. *Prot. Sci.* **5**, 1844–1851 (1996).
49. Uversky, V.N., Narizhneva, N.V., Ivanova, T.V., Kirkitadze, M.D. & Tomashevski, A.Y. Ligand-free form of human α-fetoprotein: evidence for the molten globule state. *FEBS Lett.* **410**, 280–284 (1997).
50. Garboczi, D.N., Hung, D.T. & Wiley, D.C. HLA-A2-peptide complexes: Refolding and crystallization of molecules expressed in *Escherichia coli* and complexed with single antigenic peptides. *Proc. Natl. Acad. Sci. USA* **89**, 3429–3433 (1992).
51. Bjorkman, P.J. *et al.* The foreign antigen binding site and T cell recognition regions of class I histocompatibility antigens. *Nature* **329**, 512–518 (1987).