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Thermal Stability Comparison of Purified Empty and Peptide-Filled Forms of a Class I MHC Molecule

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A secreted form of a class I major histocompatibility complex (MHC) molecule was denatured and renatured in vitro in the absence of peptide. The resulting empty class I heterodimer was immunologically reactive and structurally similar to a heterodimer renatured in the presence of an appropriate restricted peptide. Thermal stability profiles indicated that the two forms of heterodimer differed in their resistance to denaturation by heat but that a significant portion of the empty class I heterodimers had a native conformation at physiological temperatures. Free energies calculated from these data gave a direct measure of the stabilization of the class I MHC molecule that resulted from peptide binding.

Class I MHC molecules bind short peptides derived from intracellular proteins that are transported along with the MHC molecule to the cell surface, where the complex is recognized by the antigen-specific receptor on a T cell (*1*). For most alleles, folding and surface expression of the class I heavy chain is dependent on the presence of its associated β_2-microglobulin (β_2M) light chain. Experiments in a mutant cell line in which class I surface expression was rescued by extracellular addition of peptide were initially interpreted to suggest that the peptide is required for proper folding and assembly of class I polypeptide chains; the peptide was hypothesized to act as a scaffold, without which the native class I structure could not form (*2*). However, empty class I molecules are assembled and expressed in mutant and nonmutant cell lines in the absence of added peptides (*3*, *4*) and are readily detectable on the cell surface if cells are grown at 26°C (*4*). At 37°C, empty class I molecules also reach the cell surface but rapidly become undetectable by conformationally sensitive antibodies unless stabilized by the binding of an exogenously added peptide or by the addition of excess β_2M (*4*, *5*). Although empty class I molecules are reported to be unstable at physiological temperatures (*4*, *5*), the thermodynamic stability of a purified empty class I molecule has not been directly compared with the same molecule in its peptide-filled form. Here, we compare the thermal denaturation profiles of the murine H-2K^d molecule, assembled in vitro from separated heavy and light chains in the presence and absence of a synthetic peptide, to K^d occupied with an endogenous mixture of peptides. From these data, we calculated the free energy contributed by the peptide to the stabilization of the K^d heterodimer and evaluated the portion of empty molecules that were folded at physiological temperatures. The stability assay used is a method for evaluating peptide binding to purified MHC molecules and can be used to compare the degree of stabilization conferred by peptides of different compositions and sizes.

A secreted form of K^d was efficiently expressed in Chinese hamster ovary (CHO) cells with a glutamine synthetase–based amplifiable expression system (*6*). A stop codon was inserted into the cDNA encoding the heavy chain of K^d after amino acid 284 by use of the polymerase chain reaction (*7*). The resulting modified cDNA was subcloned into an expression vector (*8*) that carried the glutamine synthetase gene as a selectable marker and as a means of gene amplification in the presence of the drug methionine sulfoximine (MSX) (*6*). The cDNAs encoding human or murine β_2M were subcloned into a similar expression vector (*8*) that lacked the glutamine synthetase gene. The heavy chain expression plasmid was transfected into CHO cells either alone or in combination with one of the β_2M expression plasmids, and cell lines resistant to high concentrations of MSX were selected. Supernatants from these CHO clones were tested for the presence of secreted K^d heterodimers by an enzyme-linked immunosorbent assay (ELISA) with antibodies against both heavy and light chains (*9*); the results were verified by immunoprecipitation with a K^d-specific monoclonal antibody (MAb) (*9*). The lines expressing the most K^d were those that had been transfected with the K^d and human β_2M combination (10 to 25 mg/liter), with undetectable amounts of heavy chain secreted in the absence of transfected β_2M cDNA and intermediate amounts detected in cells transfected with the K^d and murine β_2M combination (1 to 2 mg/liter). The increased recovery of the hybrid class I heterodimer over the completely murine heterodimer may reflect the greater stability reported for murine class I heavy chains that are complexed with human rather than with murine β_2M (*10*).

The highest expressing clone (K^d heavy chain and human β_2M combination) was introduced into a hollow fiber bioreactor device (*11*) in the presence of 100 μM MSX. Supernatants containing K^d were harvested daily and contained heterodimer up to 100 μg/ml, as quantitated by ELISA (*9*). Heterodimer was isolated from culture supernatants by immunoaffinity chromatography (*12*). Analysis by SDS–polyacrylamide gel electrophoresis (PAGE) of purified K^d showed several species migrating between 44 and 45 kD, corresponding to truncated heavy chain, and a sharp band at 12 kD, corresponding to β_2M. Digestion of purified K^d with peptide-*N*-glycosidase F (PNGase F) had no effect on the lower band but converted the upper band to a single sharp band migrating at 32 kD, the expected size of the 284–amino acid, truncated heavy chain, which indicates that the observed heterogeneity results from extensive N-linked glycosylation (Fig. 1). The complex eluted from a gel filtration column as a single species of 60 to 63 kD (*13*). NH_2-terminal sequencing of purified K^d yielded sequences in equimolar amounts that correspond to the first 20 residues of the K^d heavy chain and human β_2M (*13*). Hamster β_2M (*14*) and bovine β_2M sequences (*15*) were undetectable, which suggests that association with endogenous hamster β_2M and exchange with bovine β_2M in the medium (*16*) were minimal. Peptides associated with the hybrid heterodimer were isolated by acid elution (*17*) and sequenced. Tyrosine and proline predominated in the second and fourth posi-

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tions (*18*), consistent with defined characteristics of K^d-restricted peptides (*19*).

To obtain heterodimers that contained a single peptide species (peptide-filled) and no peptide at all (empty), we first denatured the heavy and light chains in guanidine hydrochloride and separated them from lower molecular mass material (including endogenous peptides) by gel filtration chromatography. Heavy and light chain peaks were pooled and renatured by dialysis in the presence and absence of a known K^d-restricted peptide (*20*). Quantitation of starting and final material indicated an overall recovery for both empty and peptide-filled molecules of 45 to 50% (*21*). The high yield of renatured K^d heterodimers in both peptide-filled and empty forms provides sufficient material for crystallization attempts. To verify the homogeneity and structural integrity of the renatured species, we compared empty and peptide-filled heterodimers with undenatured K^d starting material by gel filtration chromatography, NH_2-terminal sequencing, SDS-PAGE, immunoprecipitation with K^d- and β_2M-specific MAbs (*9*), and circular dichroism (CD). Both reassembled proteins were heterodimers (Fig. 1) that migrated as single peaks in the same elution volume as the starting K^d material on gel filtration columns (*13*). NH_2-terminal sequence analysis of peptide-filled K^d revealed heavy chain, light chain, and the K^d-specific peptide sequences in approximately equimolar amounts, whereas only the sequences of heavy and light chains were detected in an analysis of the empty K^d protein (*13*). The empty, peptide-filled, and starting K^d material were immunoprecipitated with equal efficiency with MAbs to the K^d heavy chain and β_2M (*9*) (Fig. 1).

The CD spectra of the three heterodimeric products in the far ultraviolet (UV) range were nearly identical, consistent with proteins containing a substantial amount of β sheet and with the spectra of class I molecules (*22*) (Fig. 2A). The strong similarity indicated successful renaturation of the empty and peptide-filled forms and suggested that the secondary structures of renatured products and the starting material had no major differences. CD analysis in the near UV range (Fig. 2B) revealed more obvious differences, which could result from localized changes in conformation or the presence of an additional tyrosine in the incorporated peptide. The spectrum of the starting K^d material most closely resembled that of the empty heterodimers. This information, coupled with the behavior of the K^d starting material in denaturation experiments, suggested that some of the protein purified from CHO cells had no stably associated peptide.

We monitored heat-induced unfolding of human β_2M and the empty, peptide-filled, and starting K^d material by recording CD at 223 nm while increasing the sample temperature from 28°C to 80°C (Fig. 3A). This wavelength is near ellipticity maxima for both α helix and β structure (*22*) and thus can be used to monitor changes in the amounts of both forms of secondary structure. In addition, changes in ellipticity at this wavelength resulting from denaturation of free β_2M are opposite in sign to those observed for denaturation of heterodimer, which introduces the possibility of distinguishing the contribution of the light chain in the unfolding of the K^d heterodimer. The melting curve of free β_2M showed a single transition temperature midpoint for unfolding during heat denaturation (T_m) at ~64°C. Two unfolding transitions were evident for empty K^d heterodimers. The first, with a T_m of ~45°C, we interpret as the simultaneous dissociation and unfolding of the K^d heavy chain. The second parallels the unfolding profile of free β_2M with a nearly identical T_m, which suggests that β_2M denaturation occurs independently of the heavy chain. The unfolding profile of peptide-filled K^d showed a transition with a T_m at ~57°C representing the dissociation and unfolding of the peptide-filled heavy chain. A clear transition for β_2M was not obvious in the unfolding profile of peptide-filled K^d, possibly being overlapped by the

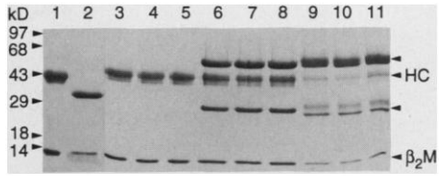
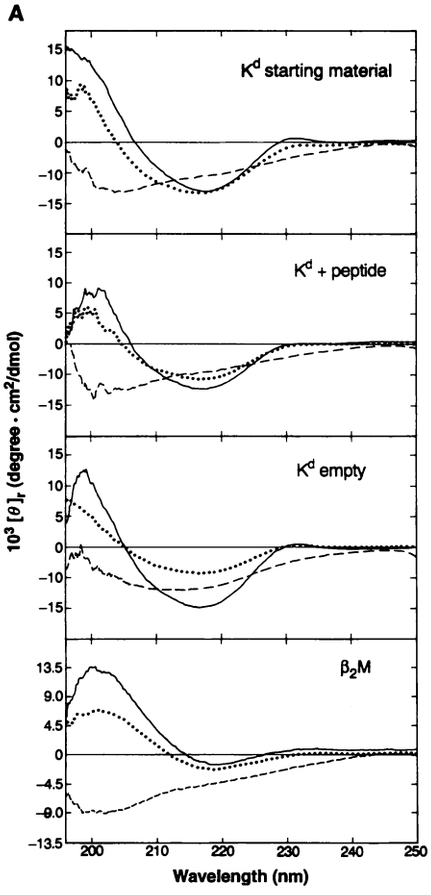


Fig. 1. SDS-PAGE (15%) comparison of the three forms of purified K^d protein and the effects of deglycosylation. Lanes 1 and 2, purified K^d, untreated (lane 1) and digested with PNGase F (lane 2; Boehringer Mannheim; for 20 hours at 37°C). Lanes 3 through 5, comparison of the three forms of K^d heterodimer. Lane 3, K^d starting material; lane 4, reassembled empty K^d; and lane 5, reassembled peptide-filled K^d. Lanes 6 through 11, comparative immunoprecipitations (*9*) of the three forms of K^d protein with MAbs 34-1-2 (lanes 6 through 8) and BBM1 (lanes 9 through 11). K^d starting material, lanes 6 and 9; empty K^d, lanes 7 and 10; and peptide-filled K^d, lanes 8 and 11. Unlabeled arrows mark the positions of antibody heavy and light chains. HC, position of K^d heavy chain; β_2M, position of light chain. Molecular size standards are shown at left.



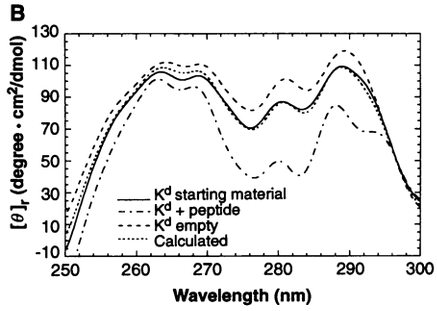


Fig. 2. (**A**) Far-UV CD spectra of the three forms of K^d heterodimer (0.45 mg/ml) and human β_2M (0.2 mg/ml) in 5 mM $NaPO_4$ (pH 7). Solid line, room-temperature scan; dashed line, 80°C scan; and dotted line, room-temperature scan after heating to 80°C. The measured CD signal is given as $[\theta]_r$, the molar ellipticity per residue. A spectropolarimeter (J-600, Jasco, Easton, Maryland) was used in the wavelength range of 194 to 250 nm with a 1.0-mm cell. Protein concentrations were calculated with extinction coefficients at 280 nm of 94,444 for the K^d heterodimers and 21,600 for β_2M. (**B**) Near-UV CD spectra (250 to 320 nm; 1.0-cm cell) of the three forms of K^d heterodimer (1.0 mg/ml in 5 mM $NaPO_4$, pH 7) and a spectrum calculated as the linear combination of the empty spectrum (72%) and peptide-filled spectrum (28%) that superimposes on the K^d starting material spectrum.

first transition. The large shift in T_m to a higher temperature in the peptide-filled K^d melting curve indicates that the presence of peptide stabilized the heavy chain structure, its association with β_2M, or both.

The melting curve for the K^d starting material was complex, with components of both the empty and peptide-filled unfolding curves. Three transitions were seen with T_m's corresponding to those of empty K^d, peptide-filled K^d, and free β_2M, which suggests that the K^d protein as secreted from CHO cells was a mixture of empty heterodimers and heterodimers that contained endogenous peptides. From the relative heights of the first two transitions in the K^d starting material melting curve, we estimate that 72% of the protein was empty and that 28% was occupied with endogenous peptides. These percentages were used to calculate a near UV CD spectrum that closely resembled the K^d starting material spectrum (Fig. 2B). After incubation of K^d starting material with the restricted peptide (*20*), the majority of the protein was converted to a form with a T_m corresponding to K^d reassembled with the same peptide, further evidence that many of the peptide-binding sites in the starting material were empty (*13*). The close agreement of the observed T_m for K^d reassembled with a single peptide with the second T_m of the starting K^d material, which was shown by sequencing of acid-eluted material to be occupied with a mixture of peptides (*18*), suggests that the degree of stabilization of the K^d heterodimer conferred by a stably bound peptide is relatively insensitive to the particular peptide sequence. However, small populations of peptide-filled K^d with different T_m's might not show detectable transitions in this assay. Experiments using K^d renatured with different defined peptides should clarify this issue.

Thermally induced denaturation of the three forms of K^d and of free β_2M were also monitored by the change in absorbance at 287 nm (Fig. 3B), yielding T_m's consistent with those derived from monitoring the CD signal, although the sensitivity of this method was insufficient for us to detect separate transitions for the empty and peptide-filled components of the starting K^d material. In the melting curve of empty K^d, both the heavy chain and β_2M transitions are apparent, whereas the peptide-filled K^d curve shows a strong cooperative transition for the heavy chain that overlaps the β_2M transition. After we repeated the heating cycle on the same samples, the melting curves were reproduced (Fig. 3B), which suggests that denaturation was at least partially reversible and that properly folded heterodimers or heterodimer-peptide complexes formed upon cooling.

We have used two methods to monitor thermal denaturation, which in combination are sensitive to both backbone structure and the environments of individual side chains. For reassembled empty and peptide-filled K^d, both methods showed only two transitions for what might be expected to be a three- or four-stage process: (i) dissociation of peptide (when present) from the heterodimer, (ii) dissociation of heavy and light chains, (iii) denaturation of heavy chain (with the possibility of separate transitions for the peptide-binding and α3 domains), and (iv) denaturation of β_2M. Besides the transition attributed to the denaturation of free β_2M, the presence of a single additional transition in the thermal denaturation of peptide-filled K^d suggests that loss of peptide and denaturation of the heavy chain occur simultaneously with the dissociation of the heterodimer.

To test the specificity of the stabilization of the K^d heterodimer by peptide, we compared the degree of stabilization conferred by the K^d-restricted peptide (*20*) with that conferred by a peptide that does not interact

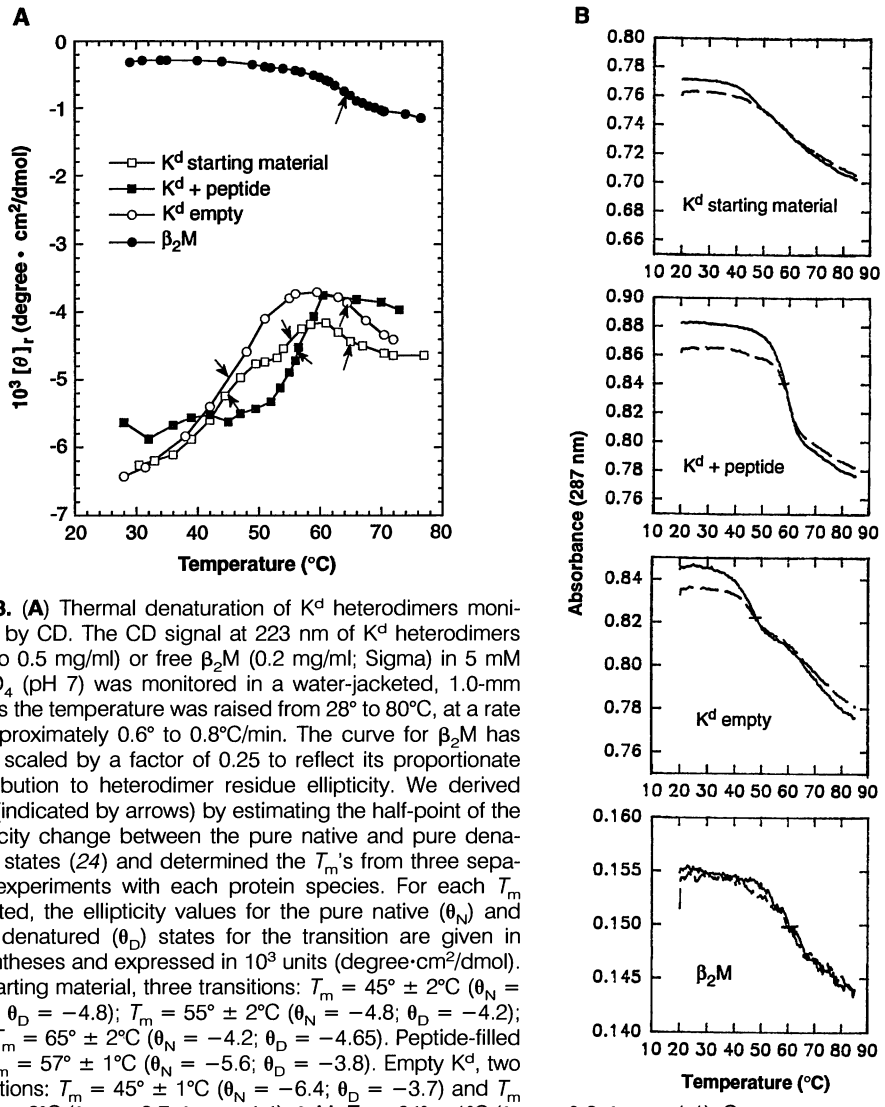


Fig. 3. (**A**) Thermal denaturation of K^d heterodimers monitored by CD. The CD signal at 223 nm of K^d heterodimers (0.4 to 0.5 mg/ml) or free β_2M (0.2 mg/ml; Sigma) in 5 mM $NaPO_4$ (pH 7) was monitored in a water-jacketed, 1.0-mm cell as the temperature was raised from 28° to 80°C, at a rate of approximately 0.6° to 0.8°C/min. The curve for β_2M has been scaled by a factor of 0.25 to reflect its proportionate contribution to heterodimer residue ellipticity. We derived T_m's (indicated by arrows) by estimating the half-point of the ellipticity change between the pure native and pure denatured states (*24*) and determined the T_m's from three separate experiments with each protein species. For each T_m reported, the ellipticity values for the pure native (θ_N) and pure denatured (θ_D) states for the transition are given in parentheses and expressed in 10^3 units (degree·cm²/dmol). K^d starting material, three transitions: T_m = 45° ± 2°C (θ_N = −6.3; θ_D = −4.8); T_m = 55° ± 2°C (θ_N = −4.8; θ_D = −4.2); and T_m = 65° ± 2°C (θ_N = −4.2; θ_D = −4.65). Peptide-filled K^d, T_m = 57° ± 1°C (θ_N = −5.6; θ_D = −3.8). Empty K^d, two transitions: T_m = 45° ± 1°C (θ_N = −6.4; θ_D = −3.7) and T_m = 64° ± 2°C (θ_N = −3.7; θ_D = −4.4). β_2M, T_m = 64° ± 1°C (θ_N = −0.3; θ_D = −1.1). On some curves, θ_N and θ_D were difficult to determine precisely, but all estimates gave the same T_m values to ± 1° to 2°C. (**B**) Thermal denaturation of K^d heterodimers monitored by change in absorbance at 287 nm. K^d samples (0.6 mg/ml) and free β_2M (0.3 mg/ml; Sigma) in 10 mM tris (pH 7.6), 50 mM NaCl, 0.5 mM EDTA, and 0.02% NaN_3 were monitored simultaneously in a spectrophotometer (Response II, Ciba Corning) as the temperature was raised from 20° to 80°C. Measurements were taken at 0.5°C intervals. Solid line, first heating cycle; dashed line, second heating cycle after cooling to 20°C. Horizontal bars on curves represent the T_m determined from two determinations of each curve as described in (A). K^d starting material; no T_m's were determined. The curve shows a single broad transition that may reflect multiple transitions that are not resolved with this method. Peptide-filled K^d, T_m = 59° ± 2°C. Empty K^d, T_m = 48° ± 2°C and a second T_m corresponding to the β_2M transition that was not marked on the curve because of difficulty in determining endpoints. β_2M, T_m = 61° ± 2°C.

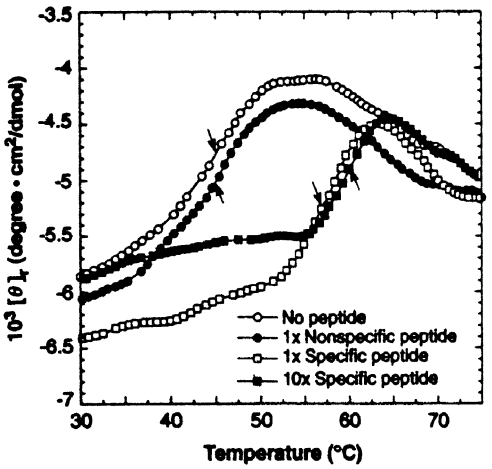


Fig. 4. Effect of peptide on the thermal stability of K^d heterodimers. Empty K^d heterodimers were heated to 80°C then cooled to room temperature in the presence of no peptide, a 1:1 or 10:1 ratio of a K^d-restricted peptide (*20*), or an equimolar ratio of a nonrestricted peptide (*23*). Cooled samples were passed through 0.2-μm filters, and thermal denaturation was monitored by CD at 223 nm as described in Fig. 3A. T_m's (indicated by arrows) were estimated as described in Fig. 3A. Cooled in the presence of no peptide, T_m = 45° ± 1°C. In the presence of nonspecific peptide, T_m = 45° ± 1°C. In the presence of 1× specific peptide, T_m = 57° ± 1°C. In the presence of 10× specific peptide, T_m = 60° ± 1°C.

with K^d (*23*). Empty K^d protein was heated to 80°C then incubated with no peptide or with an equimolar ratio of the K^d-restricted peptide or the nonrestricted peptide. After cooling the thermal unfolding curve was monitored by the change in molar ellipticity at 223 nm. In the absence of peptide or in the presence of the nonrestricted peptide, the T_m's of the transitions were unchanged (Fig. 4) relative to the unfolding curve measured during the first heating cycle (Fig. 3A). By contrast, after addition of the K^d-restricted peptide, the unfolding curve shifted (Fig. 4) to reflect the same degree of stabilization observed for the K^d protein assembled in the presence of the same peptide (Fig. 3A), which demonstrates that empty K^d can reassemble into a form capable of peptide binding after heating. The addition of excess restricted peptide shifted the T_m upwards slightly, perhaps reflecting the maximum stability of the heterodimer, but did not stabilize the protein beyond the T_m of β_2M.

To derive the degree of free energy stabilization of the K^d heterodimer caused by peptide, we subjected the CD thermal melting curves (Fig. 3A) to a thermodynamic analysis (*24*) that assumed a two-state unfolding model. At the physiological temperature (37°C), the peptide confers 4.4 kcal/mol more stability to the K^d heterodimer [change in free energy of the empty heterodimer (ΔG_{empty}) = 1.2 kcal/mol; change in free energy of the filled heterodimer (ΔG_{filled}) = 5.6 kcal/mol]. The equilibrium constant at 37°C derived (*24*) from the unfolding curve of the empty K^d suggests that 75% of the empty molecules were folded at physiological temperatures, and the derived change in free energy (ΔG) value (1.2 kcal/mol) suggests that the folded form was marginally more stable than the unfolded form.

We have renatured empty K^d with greater efficiency than reported for the renaturation of human leukocyte antigen–A2 (HLA-A2) in the absence of a restricted peptide (*25*), possibly because of allele differences or an increased stability of the human β_2M and murine heavy chain combination. In the system we used, peptide was not required for the formation of the K^d heterodimer, and a substantial portion of empty heterodimers were stable at physiological temperatures. Furthermore, secreted K^d protein that was purified as starting material for the reassembly experiments was mostly empty. This could result from loss of endogenous peptides during purification, a limiting peptide supply inside the transfected CHO cells, or inefficient recognition of the hybrid secreted class I form used here by mechanisms that normally govern transit to the cell surface (*26*). Peptide availability could perhaps be used in combination with differential regulation of the 88-kD protein that controls the exit of class I molecules from the endoplasmic reticulum (*27*) to modulate the expression of filled versus empty heterodimers at the cell surface.

A physiological role for empty MHC molecules could be to serve as the selecting elements during positive selection for self MHC in the thymus. As a way around the dilemma of the absence of foreign peptides during positive selection, it has been postulated that the foreign peptides to which the T cell receptor responds in the periphery resemble self-peptides that are bound to MHC molecules in the thymus (*28*). However, it is difficult to understand how a positively selected receptor could bind tightly enough to distinguish residues on a self MHC molecule from a nonself molecule yet apparently disregard interactions with any self peptides that are present in the peptide binding pocket. This problem is resolved if one assumes that the MHC molecules in the thymic cortical epithelium have empty peptide binding sites (*29*). Our results and the finding of stable empty murine class I molecules on the surface of cells at 37°C (*26*) suggest that at least some forms of empty class I molecules are stable under physiological conditions. In both studies, however, a murine heavy chain was complexed to a human β_2M molecule. In our attempts to produce secreted K^d from CHO cells transfected with the K^d heavy chain and murine β_2M genes, we find that the purified heterodimer contains a mixture of β_2M molecules that represent murine and bovine β_2M (*13*), which suggests that a significant amount of murine β_2M has been replaced by bovine β_2M that was present in the medium. Separation of the two forms of heterodimer would allow the recording of unfolding transitions for empty and peptide-filled K^d heavy chains complexed with murine β_2M. The measurement of stability conferred on a class I heterodimer by a peptide or nonpeptide ligand can be used as an assay for binding and to explore the effects of peptide length and composition on the binding to and the stabilization of different class I alleles.

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21. Purified K^d was diluted tenfold with buffered 6 M guanidine hydrochloride, concentrated with a Centricon-10 (Amicon), and passed over a fast protein liquid chromatography (FPLC) column (Superose 12; Pharmacia). Heavy and light chain peaks were pooled and dialyzed at 4°C against 8 M urea, 20 mM tris (pH 7.6), 150 mM NaCl, 1 mM EDTA, and 0.2 mM phenylmethylsulfonyl fluoride, then diluted to 0.1 mg/ml with buffered urea and dialyzed against two changes of buffer without urea, first at 4°C, then at room temperature. For renaturations with peptide, a 30-fold molar excess of peptide (*20*) was added at the time of dilution, and dialysis was performed in 500-dalton cutoff dialysis tubing. For renaturations of empty molecules, a 30% excess of human β_2M (Sigma) was added. Renatured material was concentrated eight- to tenfold in a pressure cell (10-kD cutoff; Filtron Omegacell, Northboro, MA) and passed over an FPLC column (Superdex; Pharmacia). Fractions corresponding to heterodimer were pooled and concentrated.
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24. For a two-state unfolding process, the equilibrium constant $K(T)$ can be evaluated at a particular temperature from the limiting ellipticity values of the pure native (N) and denatured (D) states, θ_N and θ_D, respectively, and the ellipticity at T, $\theta(T)$, as follows:
$$K(T) = \frac{\theta_N - \theta(T)}{\theta(T) - \theta_D}$$
ΔG, the change in enthalpy (ΔH), and the change in entropy (ΔS) can then be derived from $K(T)$ by standard thermodynamic relationships. $\Delta G = -RT\ln K$, where R is the gas constant, and T is the temperature in kelvin. $\Delta H = RT^2(\delta \ln K/\delta T)$, and $\Delta S = (\Delta H - \Delta G)/T$. For these and the following equations, T is in kelvin. To evaluate ΔG at temperatures below the transition region where $K(T)$ cannot be directly determined experimentally, we used the following equation [W. J. Becktel and J. A. Schellman, *Biopolymers* **26**, 1859 (1987)]:
$$\Delta G = \Delta H_m \left(\frac{T_m - T}{T_m}\right) - \Delta C_P \left\{T_m - T\left[1 - \ln\left(\frac{T}{T_m}\right)\right]\right\}$$
where the heat capacity change, ΔC_P, is assumed to be temperature-independent [P. L. Privalov and S. J. Gill, *Adv. Protein Chem.* **39**, 191 (1988)] and derived as $\Delta C_P = (\delta \Delta H/\delta \Delta T)_P$ (pressure held constant); T_m is the transition temperature for unfolding during heat denaturation (the temperature at which $\Delta G = 0$); and ΔH_m is the enthalpy change at T_m. We used the following values for these parameters to calculate ΔG(37°C) for empty and peptide-filled K^d from the melting curves shown in Fig. 3A: $\Delta C_{P(empty)} = 0.3$ kcal/mol per degree; $T_{m(empty)} = 45$°C; $\Delta H_{m(empty)} = 48$ kcal/mol; $\Delta C_{P(filled)} = 0.9$ kcal/mol per degree; $T_{m(filled)} = 56$°C; and $\Delta H_{m(filled)} = 107$ kcal/mol. In principle, the standard free energy change for any dissociation reaction is dependent on the concentration at which the equilibrium is measured. Therefore, all calculated values in this paper are strictly applicable only in the concentration ranges reported here. For the large ΔH_m values derived, however, ΔG is relatively independent of concentration.
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30. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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Localization of Targets for Anti-Ulcer Drugs in Cells of the Immune System

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The gastric mucosa consists of the epithelium, which lines the lumen, the lamina propria, and the muscularis mucosae. The targets of drugs used to treat stomach and duodenal ulcers are thought to be the acid-secreting parietal cells of the epithelium. However, immune cells in the lamina propria are the only cells that showed detectable messenger RNAs for histamine, muscarinic, gastrin, and dopamine receptors by in situ hybridization histochemistry. None of the epithelial cells expressed any of these messenger RNAs. Thus, the targets of antiulcer drugs seem to be cells of the immune system in the gut and not parietal cells, as generally believed. This conclusion may revise the thinking about ulcer formation and may shed light on the etiology of such chronic small intestinal diseases as Crohn's disease.

Acid-secreting parietal cells are located among the epithelial cells of the gastric mucosa. Dysfunction of these cells has been thought to be the leading cause of ulcer disease. For centuries agents that neutralize acid were used to treat dyspepsia and gastric ulcers. In the 1950s vagotomy (severing the parasympathetic innervation of the stomach, the vagus nerve) was introduced as a method to treat ulcer disease unresponsive to antacids. Drugs that block the action of the vagal neurotransmitter, acetylcholine, at muscarinic receptors were also employed. Unfortunately, side effects limited the utility of these muscarinic antagonists (*1*).

In the early 1970s histamine H2 receptor antagonists began to be used to heal and protect against peptic ulcers (*2*, *3*). Dopamine also modulates gastric acid secretion (*4*), and dopamine antagonists prevent ulcer relapse (*5*). Administration of tyrosine, which is converted into dopamine by the actions of tyrosine hydroxylase and dopa decarboxylase, also prevents experimental ulcer formation (*6*).

Because the parietal cells of the stomach secrete acid, it has been assumed that all of the drugs mentioned above act on these cells. Gastrin, a peptide hormone produced by antral cells in the stomach, is also thought to stimulate gastric acid secretion by acting on parietal cells (*7*). We have used in situ hybridization histochemistry to visualize histamine (H2), muscarinic acetylcholine (M1 to M5), gastrin (GR), and dopamine (D1 to D5) receptors in the stomach (*8*).

There were numerous cells in the lamina propria of the stomach containing mRNA for H2 (Fig. 1, A to C), M1 (Fig. 1D) to M5, GR (Fig. 1E), and D1 to D5 (Fig. 1F) receptors. The probes directed to D3, D4,

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