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Assembly and Dissociation of Human Leukocyte Antigen (HLA)-A2 Studied by Real-Time Fluorescence Resonance Energy Transfer†

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ABSTRACT: Class I major histocompatibility complex (MHC) heterodimer, composed of human leukocyte antigen (HLA)-A2 heavy chain and human β_2-microglobulin (β_2m), was produced by denaturation and gel filtration of the recombinant water-soluble HLA-A2/β_2m/peptide ternary complex in 8 M urea Tris−HCl buffer, followed by refolding of the separated chains without peptide. Peptide affinity and kinetics of the ternary complex formation and dissociation were investigated in real time by monitoring the fluorescence resonance energy transfer (FRET) from intrinsic HLA-A2 heavy-chain tryptophans to a dansyl fluorophore conjugated to the bound peptide. Peptide binding to the heterodimer was a second order process with rate constants linearly dependent upon temperature in Arrhenius coordinates over 0−20 °C. The binding rate constant of pRT6C-dansyl [ILKEPC(dansyl)HGV] at 37 °C evaluated by extrapolation of the Arrhenius plot was $(2.0 \pm 0.5) \times 10^6$ M^{-1} s^{-1}. Association of the heavy chain with β_2m was a first order process, apparently controlled by a conformational transition in the heavy chain. One of these conformations bound to β_2m to form the heavy chain/β_2m heterodimer whereas the second conformer oligomerized. Peptide dissociation from the ternary complex was a first-order reaction over the temperature range 20−37 °C, suggesting that the ternary complex also exists in two conformations. Taken together, the present data suggest that association of β_2m changes the HLA-A2 heavy-chain conformation thereby promoting peptide binding. Peptide dissociation from the ternary complex induces dissociation of the heavy-chain/β_2m heterodimer thereby causing oligomerization of the heavy chain. The lability of the HLA-A2/β_2m heterodimer and the strong tendency of the "free" heavy chain to oligomerize may provide an efficient mechanism for control of antigen presentation under physiological conditions by reducing the direct loading of HLA with exogenous peptide at the cell surface.

Interaction of class I MHC[1] ternary complexes with T-cell receptors (TCRs) plays a key role in adaptive immune responses. T cell cytotoxicity is controlled by the number of expressed MHC complexes presenting the antigenic peptide and their affinity to TCR, both of which are determined by the nature of antigenic peptide and MHC molecule (*1*). It has become clear that slight differences in the sequence of antigenic peptide change the conformation of the MHC/peptide complex leading to qualitative changes in T-cell responses that may be either agonistic or antagonistic (*2*−*4*). Therefore, it is important to resolve the mechanism of the peptide−MHC interaction and quantify the relevant thermodynamic and kinetic parameters. Significant progress has been achieved by elucidation of peptide binding motifs (*5*, *6*) and the peptide binding constants for those MHC molecules which possess relatively stable heavy chain/β_2m complexes for several class I alleles, H-2K^d, H-2D^d, H-2K^b, HLA-B27, and HLA-Aw68 (*7*−*12*). However, kinetic and thermodynamic data on peptide interaction with HLA-A2/β_2m heterodimer are not available so far, apparently due to its lability.

The yield of HLA-A2 ternary complex refolded in vitro from recombinant heavy chain and β_2m was a function of peptide structure, and the complex refolding lasted for several days (*13*). The equilibrium peptide dissociation constants were estimated from the ternary complex refolding experiments to be in the micromolar range. This is in stark contrast with the equilibrium peptide dissociation constants for the mouse class I MHC proteins H-2K^d or H-2K^b, which are in the nanomolar or even picomolar range (*9*, *14*).

In the present study, we first describe a protocol for the production of HLA-A2/β_2m heterodimer, i.e., peptide-free or "empty" HLA-A2, and then characterize its interaction with peptides. Moreover, we found that the previously established mechanism for assembly/dissociation of H-2K^d and H-2K^b/β_2m/peptide ternary complexes (*9*, *14*) is also

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[1] Abbreviations: MHC-I, class I major histocompatibility complex; HLA, human leukocyte antigen; APC, antigen presenting cell; TCR, T-cell receptor; FRET, fluorescence resonance energy transfer; hc, heavy chain; β_2m, microglobulin; p, peptide; pRT6C, ILKEPCHGV; pRT8C, ILKEPVHCV.

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operating for the HLA-A2 ternary complexes and that its assembly takes place predominantly via an intermediate complex of the heavy chain with β_2m.

MATERIALS AND METHODS

Peptides. All chemicals were purchased from Sigma unless otherwise stated. Dansyl aziridine (5-dimethylaminonaphthalene-1-sulfonyl aziridine) was purchased from Molecular Probes. Influenza virus matrix M1 peptide (residues 58−66, GILGFVFTL) (matrix) and peptides from human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (residues 197−205, ILKEPVHGV) and cysteine substituted variants ILKEPCHGV (pRT6C) and ILKEPVHCV (pRT8C) were synthesized by an automated solid-phase method on an Applied Biosystems model 432A synthesizer using the manufacturer's standard Fmoc protocol. The cysteines in peptides pRT6C and pRT8C were dansylated to yield the respective derivatives, ILKEPC(dansyl)HGV (pRT6C-dansyl) and ILKEPVHC(dansyl)V (pRT8C-dansyl) by the following protocol: 0.3 mL of 10-fold molar excess of dansyl aziridine in dimethylformamide was added to 1 mL of peptide solutions (3 mg/mL) in 0.1 M bicarbonate buffer, pH 8.2 and allowed to react for 2 h at room temperature in the dark. Labeled peptides were purified by HPLC to essentially 100% purity. Fluorescence emission and absorption spectra as well as fluorescence and excitation anisotropy spectra of the peptides in water and glycerol did not reveal any heterogeneous labeling (data not shown). Concentrations of peptides and proteins were determined spectrophotometrically using the extinction coefficients 1.42×10^3 M^{-1} cm^{-1} for tyrosine at 274 nm, 1.97×10^2 M^{-1} cm^{-1} for phenylalanine at 257 nm, (*15*), and 4.57×10^3 M^{-1} cm^{-1} for dansyl at 335 nm (*16*).

Preparation of HLA-A2/β_2m Heterodimer. HLA-A2 and β_2m molecules were produced by recombinant DNA technology and refolded in the presence of a chemically synthesized matrix peptide (*17*). This isolated ternary complex was denatured by addition urea (8 M final concentration) and its components separated by gel filtration on a Superose 12 column equilibrated with 8 M urea, 20 mM Tris, and 150 mM NaCl buffer, pH 7.5. The gel filtration profile of the ternary complex exhibited two peaks eluted at about 37.2′ and 43.3′ with a ratio of 3.5 at 0.3 mL/min flow rate, in agreement with the 1:1 stoichiometry of the heavy and light chains in the complex and their respective extinction coefficients. The collected fractions of the heavy chain and β_2m were dialyzed overnight at 4 °C with cutoff of 6 kDa, either separately or together against the same buffer without urea. Samples of the heavy chain/β_2m heterodimer were concentrated to 2−5 mM and stored at 4 °C not longer than 5−7 days before experiments. The yield of HLA-A2/β_2m heterodimer was about 80−90% as evaluated by the equilibrium peptide binding assay. Further purification of the HLA-A2/β_2m heterodimer by gel filtration was impossible due to its limited stability. Samples of "free" heavy chains were concentrated to ≤1 mM and used immediately for binding assays.

Extinction coefficients of HLA-A2/β_2m and β_2m molecules were calculated as 9.2×10^4 M^{-1} cm^{-1} and 2.1×10 M^{-1} cm^{-1} at 280 nm respectively, based on the extinction coefficients of tryptophan and tyrosine.

Fluorescence Binding Assays. Fluorescence emission was recorded on a PTI spectrofluorimeter with a single photon counting registration system. The sample holder was thermostated with an accuracy of ±0.5 °C. All experiments were carried out in a 4×4 mm magnetically stirred, quartz optical cuvette. Excitation and emission wavelengths of 290 nm (slit width 4 nm) and 530 nm (slit width 16 nm) were used, respectively. All experiments were carried out in 20 mM Tris, 150 mM NaCl, pH 7.5 buffer.

Peptide affinity and kinetics of the ternary complex assembly and dissociation were investigated in real time by monitoring the fluorescence energy transfer from intrinsic tryptophans in the HLA-A2 heavy chain to the dansyl conjugated to the peptide. Peptide binding kinetics were initiated by addition of a small volume (1−10 μL) of one solution (usually 1−4 mM stock of the heterodimer) to 140 μL of a continuously mixed solution of peptide by a micrometric syringe (Hamilton). Peptide dissociation from the ternary complex was induced by the addition of large excess (final concentration 50−60 μM) of unlabeled M1 peptide, to block the rebinding of the dissociated labeled peptide.

Peptide binding and dissociation kinetics were processed by the Global Analysis software which fits the data set to an arbitrary model, in this case, a sum of one, two, or three exponential components and background

$$y(t) = a_0 + \sum_{i=1}^{3} a_i \cdot \exp(-t/\tau_i)$$

RESULTS

Equilibrium Binding Titrations. Preparation of HLA-A2/β_2m heterodimer was started from denaturation of the heavy chains/β_2m/peptide trimer as described in Preparation of HLA-A2/β_2m heterodimer. The gel filtration profile of the HLA-A2/β_2m/matrix peptide complex recorded at 0.3 mL/min exhibited two peaks with a ratio of 3.5, in agreement with the 1:1 stoichiometry of the heavy and light chains. To refold the proteins, the eluted fractions were dialyzed against the same Tris−NaCl buffer without urea. Refolded β_2m was purified by gel filtration and kept at 4 °C as stock solution (10−20 μM). During the overnight dialysis and reduction of urea concentration, the "free" heavy chain exhibited a high tendency to oligomerize**.** Nevertheless, when both eluted fractions were incubated together and dialyzed overnight at 4 °C, the yield of the HLA-A2 heavy chain/β_2m heterodimer was found to be about 80−90%. This was checked by the equilibrium peptide binding assay as well as by the size exclusion chromatography of the products of the peptide binding reaction to the "empty" heterodimer. We found that the elution time of the complex produced by the peptide binding to the "empty" heterodimer was the same as that of the HLA-A2 heavy chain/β_2m/matrix peptide ternary complex used for the heterodimer preparation. This result demonstrates that the ternary complexes produced by peptide binding to the "empty" heterodimer and the complex used for preparation of the heterodimer had the same size and therefore the same stoichiometry. Thus, this procedure establishes a method for production of "empty" HLA-A2/β_2m heterodimers**.**

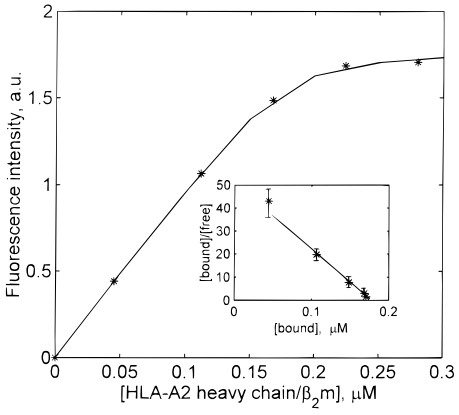


FIGURE 1: Equilibrium binding curve calculated from results of an equilibrium titration experiment and its Scatchard analysis (inset): five 5 μL aliquots of HLA-A2/β_2m stock solution (0.6 μM) were added to 150 μL of pRT6C-dansyl solution in 20 mM Tris 150 mM NaCl buffer, pH 7.5 (0.178 mM) at 25 °C. Fluorescence intensity ($\lambda_{em} = 530$ nm, $\lambda_{ex} = 280$ nm) was corrected for dilution. The equilibrium constant of peptide pRT6C-dansyl dissociation from HLA-A2 molecule at 25 °C was calculated as $(3.5 \pm 1.5) \times 10^8$ M^{-1}.

An equilibrium binding curve calculated from titration of dRT6C-dansyl (0.18 μM) at 20 °C with the HLA-A2/β_2m heterodimer is shown in Figure 1. The equilibrium dissociation constant, K_d, calculated from the Scatchard analysis (Figure 1, insert) was $(3.5 \pm 1.5) \times 10^{-8}$ M^{-1}.

Kinetics of Peptide Binding to the HLA-A2/β_2m Heterodimer. Binding of 0.06 μM pRT6C-dansyl and 0.02 μM heavy chain/β_2m heterodimer at 0, 10, and 20 °C is shown in Figure 2a. The binding time course of pRT6C-dansyl and pRT8C-dansyl to the HLA-A2/β_2m heterodimer were biphasic in the 0−20 °C temperature range. Although both phases of peptide binding were temperature dependent, the fast binding phase exhibited greater dependence than the slow one. Figure 2b shows the concentration dependence of peptide-binding time course at 0 °C. Importantly, the total amplitudes of the peptide-binding kinetics depended on the binding rates, i.e., the faster the peptide bound to the HLA-A2/β_2m heterodimer, the larger was its amplitude. Parameters of a biexponential fit to the normalized peptide binding kinetics and the peptide binding rate constants, calculated as the ratio of reciprocal binding time constants of the fast phase to peptide concentrations for 0, 10, and 20 °C, are listed in Table 1. These data show that the time constants of the fast binding phase were linearly dependent on peptide concentration, whereas those of the slow phase were independent. An Arrhenius plot of the binding rate constants calculated for the fast binding phase is shown in the inset to Figure 2a. The rate constant of pRT6C-dansyl [ILKEPC-(dansyl)HGV] binding to the heterodimer at 37 °C and the binding activation energy evaluated from the Arrhenius plot were $(2.0 \pm 0.5) \times 10^6$ M^{-1} s^{-1} and (14.7 ± 0.8) kcal M^{-1} correspondingly.

To examine the nature of the slow kinetic phase, we carried out several experiments using different concentrations of the heterodimer and peptide or an excess of β_2m. Results of these are illustrated by three kinetic profiles recorded at 10 °C, using 0.32 μM pRT6C-dansyl and three concentrations of the HLA-A2 heterodimer [0.05 μM (1), 0.1 μM (2), and 0.2 mM (3)] (Figure 2A, inset). Neither the 4-fold variation in heterodimer concentration nor addition of 3 μM excess β_2m

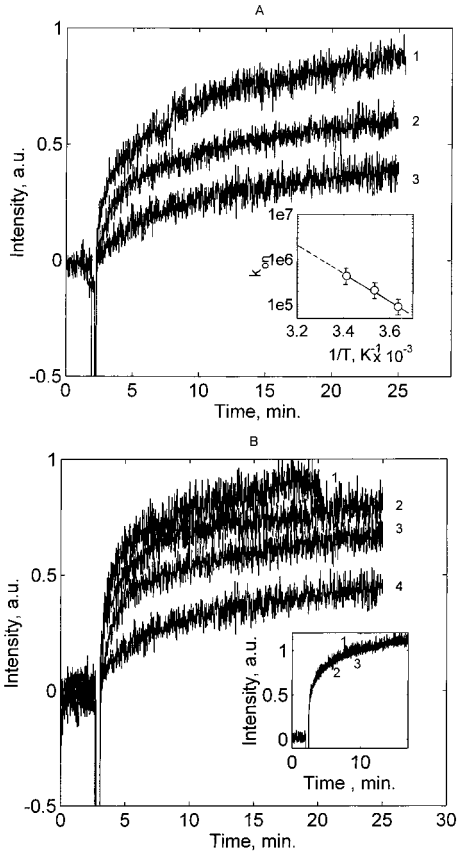


FIGURE 2: (A) Temperature dependence of pRT6C-dansyl (0.06 μM) binding kinetics to HLA-A2/β_2m (0.02 μM): 20 °C (1), 10 °C (2), 0 °C (3). The other experimental conditions are the same as in the legend to Figure 1. (Inset) Arrhenius plot of the pRT6C-dansyl binding rate constants to the HLA-A2/β_2m heterodimer. The rate constants were calculated from the fast phase binding time constants. (B) Peptide binding time-courses to HLA-A2/β_2m heterodimer (0 °C) at different concentrations of pRT6C-dansyl: (1) 0.23, (2) 0.17, (3) 0.11, (4) 0.06 μM. Concentration of HLA-A2/β_2m was 0.04 μM in all experiments. (Inset) Time-courses of pRT6C-dansyl (0.32 μM) binding to HLA-A2/β_2m heterodimer recorded by emission at 530 nm, excitation 280 nm, at 10 °C. [HLA-A2/β_2m] = 0.05 μM (1), 0.1 μM (2), 0.2 μM (3).

(data not shown) affected the rate of the slow phase of peptide binding. Therefore, this phase in the peptide binding kinetics being independent of concentrations of the heterodimer, β_2m or peptide is a monomolecular process.

Ternary Complex Assembly from Heavy Chain, b2m, and Peptide. To resolve different pathways of the ternary complex assembly, we studied the interaction among all three complex constituents. First, we incubated the RT6C-dansyl peptide with "free" heavy chain and resolved a very limited, yet specific binding (Figure 3). As this figure shows, addition of β_2m to "free" heavy chain−peptide mixture dramatically changed the reaction time course. The mechanism of the heavy chain−β_2m interaction was studied by running the reaction at 20 °C with 2.6 or 5.2 mM β_2m and 1 μM peptide (Figure 3, inset). The reaction time course was biphasic. Since concentration of the monomeric heavy chain could not be accurately determined spectroscopicaly due to its tendency to oligomerize (the upper limit for the "free" heavy-chain monomers concentration determined by optical density was 0.5 mM), β_2m concentration was varied. Within limits of the experimental error (10−15%) the ternary complex assembly kinetics were independent of β_2m concentration,

Table 1: Analysis of Peptide (pRT6C-Dansyl) Binding Kinetics to HLA-A2 Heavy Chain/β_2m Heterodimer as a Function of Peptide Concentration at 0, 10, and 20 °C[a]

t (°C)	[pRT6C-dansyl] (μM)	a_1	τ_1 (s)	k_{on} (10^5 M^{-1} s^{-1})	τ_2 (s)
0	0.06	0.31 ± 0.05	138 ± 30	1.21 ± 0.25	856 ± 180
0	0.11	0.27 ± 0.05	88 ± 20	1.05 ± 0.20	900 ± 180
0	0.17	0.56 ± 0.08	49 ± 10	1.20 ± 0.25	775 ± 150
0	0.23	0.52 ± 0.08	33 ± 6	1.30 ± 0.20	405 ± 80
10	0.06	0.45 ± 0.08	52 ± 10	3.20 ± 0.60	719 ± 150
10	0.11	0.53 ± 0.08	36 ± 6	2.50 ± 0.50	554 ± 100
10	0.17	0.52 ± 0.08	27 ± 5	2.20 ± 0.50	385 ± 80
10	0.23	0.54 ± 0.08	18 ± 4	2.40 ± 0.50	373 ± 80
20	0.06	0.29 ± 0.05	34 ± 6	4.90 ± 1.00	510 ± 100
20	0.11	0.41 ± 0.07	16 ± 4	5.70 ± 1.00	295 ± 600

[a]Heterodimer concentration was ≤0.02 mM. The normalized peptide binding time courses were fitted to a biexponential function $y(t) = 1 - a_1\exp(-t/t_1) - a_2\exp(-t/t_2)$. The peptide binding rate constants to the heterodimer were calculated from the fast time component assuming pseudo-first order binding kinetics

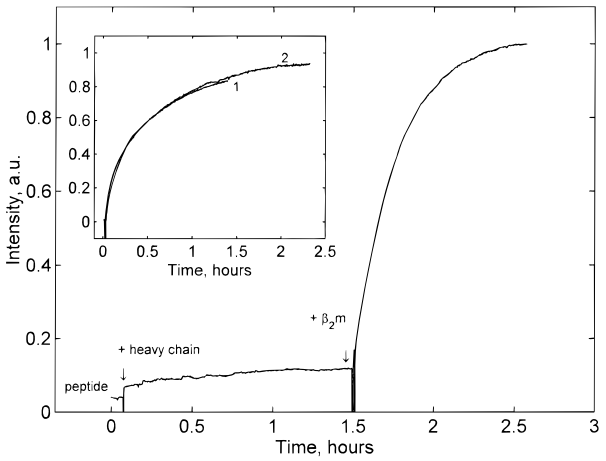


FIGURE 3: Time-course of the ternary complex assembly from HLA-A2 heavy chain, β_2m and pRT6C-dansyl at 20 °C. The reaction was initiated by dilution of "free" heavy chains (final concentration ≤ 0.5 μM) to 1 μM pRT6C-dansyl solution. β_2m (final concentration 1 μM) was added to the sample at the second stage of the reaction. Vertical arrows indicate addition of heavy chain and β_2m. Inset: ternary complex assembly time-courses as a function of β_2m concentration [(1) 2.6, (2) 5.2 μM] recorded under similar experimental condition.

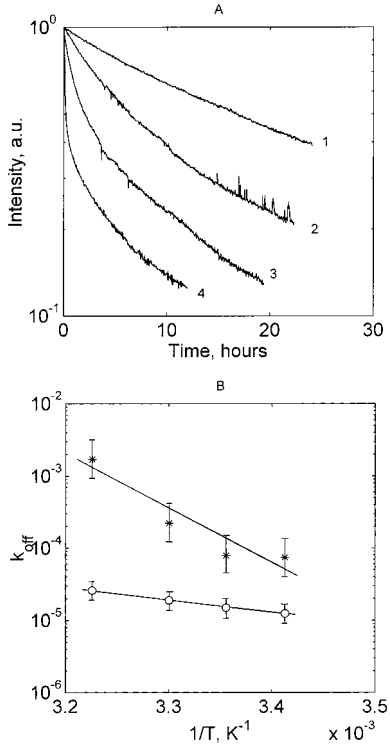


FIGURE 4: (A) Kinetic profiles of peptide dissociation from HLA-A2/β_2m/pRT6C-dansyl ternary complex at 37 (1), 30 (2), 25 (3), 20 °C (4). The reaction was initiated by addition of 30 μM peptide to 0.2 μM solution of the ternary complex. The reaction time-course was monitored at 530 nm upon excitation at 280 nm in the following sampling mode: the sample illumination phase of 10 s was repeatedly followed by the dark phase of 190 s so that the total time of the sample excitation was ≤1.2 h during the course the dissociation experiments. (B) Arrhenius plots of the peptide dissociation rate constants calculated from the kinetic profiles shown in panel A. The curves were processed by fitting to biexponential model: $y(t) = a_1 \exp(-t/\tau_1) + a_2 \exp(-t/\tau_2)$, and the fast (*) and slow (○) rate constants were calculated as reciprocal values of decay time constants τ_1 and τ_2.

suggesting that the assembly is a zero order process. The above approximation of these experimental results with the two-exponential model was satisfactory by the statistical criterion of the employed data processing method, i.e., the residuals did not show any systematic variations. Since the molecular nature of these kinetic conformers is unclear, and similar statistical validity could be obtained for example with a continuous distribution of rate constants (not shown), we characterized this zero order process with an average rate constant $k_{hd} = (7.0 \pm 2.0) \times 10^{-4}$ s^{-1} (20 °C). This shows that the rate limiting step in the ternary complex assembly from separated heavy chain, β_2m, and peptide is association of the heavy chain with β_2m, followed by the relatively fast peptide binding to the heterodimer.

Ternary Complex Dissociation. Peptide dissociation from the ternary complex was initiated by addition of a 60-fold excess of the nuclear protein matrix peptide. A biphasic rate of peptide dissociation from the ternary complex was observed at 20−37 °C (Figure 4A). In analogy with the results of H-2K^d or H-2K^b/human β_2m/peptide dissociation kinetics, the two phases observed for the HLA-A2 are apparently due to existence of the ternary complex in two conformations. The amplitude of the fast component decreased from 63 to 14% upon cooling from 37 to 20 °C. The rate constant of the fast component exhibited stronger temperature dependence than the slow one as illustrated by the Arrhenius plots (Figure 4B).

Dissociation of the "Empty" HLA-A2/β_2m Heterodimer. As shown above, the rate of peptide binding to the HLA-A2/β_2m heterodimer was biphasic. The amplitude of the fast phase was found to be proportional to concentration of the heterodimer in the "open" i.e., peptide-binding conformation.

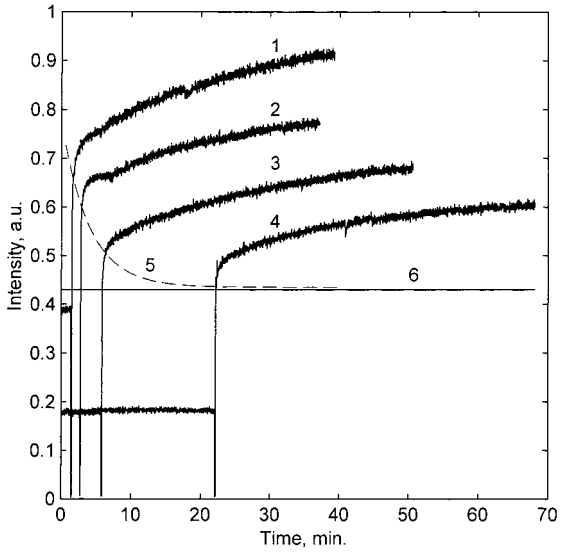


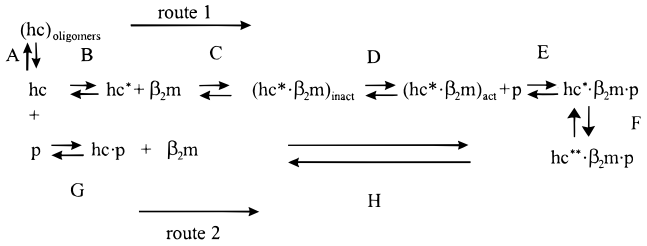
FIGURE 5: Dissociation of the "open" conformation of the HLA-A2 heavy chain/β_2m heterodimer at 20 °C was studied by monitoring pRT6C-dansyl binding. The sample was excited at 290 nm (10 nm slit) and emission was monitored at 510 nm (20 nm slit). An aliquot (15 μL) of 4 μM heterodimer stock solution was dissolved in 150 μL of buffer. Dansylated peptide pRT6C-dansyl (final concentration 2.2 μM) was added to the sample at the following different time points after protein dilution: 0 (1), 1 (2), 5 (3), and 21 min (4). The fast binding phase in curves 1−4 reflects peptide binding to the "open" conformation of the heterodimer. The decay curve of the "open" conformation (5) was calculated from the values of the fast binding phase preexponential coefficients. Line 6 represents the background fluorescence intensity level due to the dansyl direct excitation and the tryptophan fluorescence of the heterodimer.

Therefore, this amplitude can be used to monitor this conformation dissociation time course. Its concentration can be estimated from the value of the preexponential coefficient of the fast exponential term of the binding kinetics. The following experiment illustrates evaluation of the heterodimer dissociation time-course: 15 μL aliquots of 4 μM heavy chain/β_2m stock were diluted in 125 μL of buffer and then 10 μL aliquots of pRT6C-dansyl (34 μM) were added to the sample, either immediately or with delays of 0, 1, 5, and 21 min. (Figure 5). The heterodimer dissociation time constant at 20 °C was calculated from these data to be 4 ± 1 min.

DISCUSSION

In this study, we have first established a methodology for preparation of a peptide-free class I MHC heterodimer composed of human leukocyte antigen HLA-A2 heavy chain and human β_2m and then characterized its interactions with specific peptides. This enabled to study for the first time the thermodynamic and kinetic properties of soluble human class I MHC heavy-chain/β_2m/peptide ternary complexes. Moreover, it extends our previous analysis of the interactions of mouse heavy chains with human β_2m and peptides (*9*, *14*). The use of human β_2m, which was required to attain increased stability of the heterodimers with mouse H-2K^d or H-2K^b heavy chains, provides a species homogeneous stable enough system to be studied by the real-time binding assay. Our results show that the protocol developed for preparing mouse H-2K^d/β_2m (*18*) and H-2K^b/β_2m (*14*) heterodimer complexes may also be used for preparing human HLA-A2/β_2m heterodimer. It was found however, that HLA-A2 heavy chains exhibit a much more pronounced tendency to oligomerize. This was a major obstacle in preparing the peptide-free heterodimer.

Scheme 1



In accordance with our previous results for the H-2K^d and H-2K^b molecules (*9*, *14*), the mechanism of the HLA-A2 complex assembly and dissociation is described in Scheme 1. One pathway starts by association of the "free" heavy chain with β_2m (steps B and C) while the other minor pathway begins with peptide binding to the heavy chain (step G). Heavy-chain association with β_2m alters the heavy-chain peptide-binding site conformation yielding peptide affinities in the nano- or subnanomolar range at 20 °C. Although the "open" conformation of the heavy-chain/β_2m heterodimer is unstable and dissociates within several minutes at physiological temperature, bound peptide slows down β_2m dissociation. Peptide dissociation (step E) lowers the affinity of the heterodimer and leads to β_2m dissociation (step C). The heavy chain, in turn, loses its high peptide-binding affinity upon β_2m dissociation. These allosteric affinity modulations are most likely related to structural changes that occur in the heavy chain upon interaction with β_2m and peptides.

The biphasic time course of peptide binding to the heterodimer and its independence on the addition of excess β_2m strongly suggest that the heterodimers can exist in two conformations, one of which does not bind peptides. The conversion of the inactive to the active conformation constitutes the rate-limiting step in the peptide binding to the heterodimer. It is interesting that the equilibrium dissociation constant calculated from the equilibrium titrations $K_d^{titr} = (5.5 \pm 2.0) \times 10^{-8}$ M^{-1} and the "kinetic" equilibrium dissociation constant calculated using the ratio of the average peptide binding $\langle k_{on} \rangle$ and dissociation $\langle k_{off} \rangle$ rate constants, calculated from the biexponential kinetic curves as 7.6×10^3 M^{-1} s^{-1} and 4.2×10^{-5} s^{-1}, respectively, $K_d^{kin} = (3.5 \pm 1.5) \times 10^{-8}$ M (all at 20 °C) are in good agreement. Still, since two phases were observed in the complex assembly and in its dissociation, with distinct dissociation rate constants for each step, peptide interaction with the heterodimer cannot be adequately characterized by a single equilibrium dissociation constant. Assuming that the "open" conformation is dominant, we characterize peptide interaction with the heterodimer by two equilibrium dissociation constants, i.e., $= K_d' = k_{off}^1/k_{on} = 1.4 \times 10^{-10}$ M^{-1} and $K_d'' = k_{off}^2/k_{on} = 2.7 \times 10^{-11}$ M^{-1}, where k_{on} is the peptide binding rate constant to the "open" heterodimer while k_{off}^1 and k_{off}^2 are the peptide dissociation rate constants from two distinct conformations of the ternary complex. Suggesting that only the more stable ternary complex conformation is of physiological relevance, we attribute the constant to the peptide equilibrium dissociation constant. In addition, the equilibrium dissociation

Table 2: Comparison of Peptide Binding and Dissociation Rate Constants for Mouse H-2K^d (*9*), H-2k^b (*14*), and Human HLA-A2 MHC Class I Molecules[a]

peptide	MHC-I	k_{on} ($\times 10^5$ M^{-1} s^{-1})	k_{off} (s^{-1})
OVA3C-dansyl	H-2K^b	2.3 ± 0.8	$(3.0 \pm 1.0) \times 10^{-5}$
OVA7C-dansyl	H-2K^b	2.5 ± 0.8	$(8.1 \pm 3.0) \times 10^{-6}$
dNP19	H-2K^d	3.3 ± 1.0	$(1.0 \pm 0.2) \times 10^{-4}$
dNP20	H-2K^d	3.0 ± 1.0	$(8.2 \pm 1.0) \times 10^{-5}$
dNP21	H-2K^d	3.3 ± 1.0	$(4.0 \pm 0.5) \times 10^{-3}$
dNP22	H-2K^d	3.8 ± 1.0	$(5.8 \pm 0.6) \times 10^{-4}$
pRT6C-dansyl	HLA-A2	4.3 ± 1.3	$(4.7 \pm 0.5) \times 10^{-5}$
pRT8C-dansyl	HLA-A2	3.8 ± 1.2	$(7.8 \pm 1.0) \times 10^{-6}$

[a] All measurements were carried out at 20 °C in Tris−HCl buffer, pH 7.5.

constant determined from equilibrium titrations, may be affected by dissociation of the heterodimer. As a result, the peptide binding rate constant derived from the equilibrium titrations reflects the different pathways of the ternary complex assembly which may proceed via peptide binding to the heterodimer which exists in the "open" and "closed" conformation as well as via assembly of its separated three components (see Scheme 1). Therefore, in case of multistep reactions it is preferable to try and determine the affinity constants from the constitutive elementary kinetic rate constants.

The presented experimental results show that together with the fast peptide binding to the heterodimer there are two other reaction paths leading to the ternary complex formation. One is due to association of "free" heavy chains with β_2m and, proceeds on hours time domain following peptide binding, (Figure 3). Apparently this process always takes place in peptide-binding experiments; however, its contribution to the overall reaction yield obviously depends on the initial concentrations of "free" heavy chain and β_2m. The second process is illustrated by the slow phases in the reactions shown in Figure 2. This reaction proceeds within minutes at the same temperature (20 °C). Since the rate constants of the slow phase in the peptide binding kinetics shown in Figure 2 was higher that that of the ternary complex assembly reaction from the "free" heavy chain, β_2m, and peptide (Figure 3) and was independent of their concentrations, and the heterodimer was free of any low affinity peptides, we reason that it is a result of an intraheterodimer process. We attribute this process to a conformational transition in the heterodimer from an "open", peptide-binding state, to a "closed" one, which cannot bind peptides.

A close examination of the kinetic traces presented in the recent study of peptide binding to H-2D^d/β_2m heterodimer (*12*) suggests that there is also evidence for a biphasic time course rather than a "baseline drift". Thus, consideration of an additional phase in the reaction may eliminate the discrepancy between the equilibrium dissociation constants derived by these authors from kinetic and equilibrium affinity measurements.

The bimolecular rate constants of peptide binding to the human HLA-A2 heterodimer are similar to those observed for the analogous reactions with mouse H-2K^d/β_2m (*9*) and H-2K/β_2m (*14*) heterodimers (Table 2). Namely they are within the limits of experimental accuracy in the range of 10^5-10^6 M^{-1} s^{-1}. As elaborated earlier in more detail (*19*), the diffusion controlled rate constant for binding of a low molecular weight ligand to its specific site on a protein is limited by its "approach cone" and an orientation factor. These reduce the reaction rate constants to $\sim 10^7$ M^{-1} s^{-1}. The flexible nature of oligopeptides or oligosuccharides was found to bring it further down by one or 2 orders of magnitude, i.e., 10^5-10^6 M^{-1} s^{-1} as also found in the present case. The peptide binding rate constants were not found to vary much with allele or species, probably due to similarities of the overall protein and the groove structures as well as sizes of antigenic peptides.

The rate of the HLA-A2 ternary complex assembly from separated heavy chain, β_2m and peptide was found to be controlled by association of the heavy chain and β_2m. This is similar to our observations for the mouse H-2K^d and H-2K^b heavy chains assembly with the human β_2m. The heterodimer association rate was independent of β_2m concentration and hence is apparently controlled by conformational changes in the heavy chain that either enable β_2m binding or lead to its oligomerization. This oligomerization process explains why the final yield of ternary complexes in the reaction between the heavy chain/β_2m heterodimer and peptide was a function of the peptide-binding rate (Figure 2). Indeed, upon dilution of the heterodimer stock solution into a peptide containing solution, the heterodimer dissociation and peptide binding proceed in parallel. Therefore, the lower the rate of peptide binding, the larger is the fraction of the dissociated heterodimer. Due to the irreversible oligomerization of the dissociated heavy chains, the amplitude of the peptide-binding time-course exhibited a pronounced dependence of the peptide-binding rate and therefore on peptide concentration.

Independence of the rate of "free" heavy-chain binding to β_2m on the peptide concentration indicates that the rate of the heavy chain conformational transformation is independent of its interaction with peptides. Therefore, peptides do not play a role of a chaperon molecule in the heavy-chain folding. On the other hand, the limited peptide binding to the "free" HLA-A2 heavy chain is significantly enhanced upon β_2m addition. This clearly shows that β_2m association with the heavy chain alters the peptide binding site of the heavy chain, probably by a conformation change. This makes the association of β_2m and heavy chain followed by peptide binding, depicted as route 1 in the assembly scheme, a dominant pathway in the ternary complex assembly. The peptide binding to the heavy chain is, in turn, increasing the stability of the heavy-chain−β_2m interaction.

The present results show that peptide affinities for the mouse or human class I heterodimers may differ by more than 2 orders of magnitude, yet the binding rate constants for all optimal length peptides and class I heterodimers are similar. Therefore, the differences in peptide affinity are mainly due to differences in the peptide dissociation rate constants. This is a result of different number interactions contributing to the binding energy, e.g., of hydrogen bonds between peptides and residues in the MHC heavy chain groove. Although the stability of the HLA-A2/β_2m heterodimer is lower than that of H-2K/β_2m or H-2K/β_2m, lifetimes of HLA-A2 ternary complexes, determined by peptide dissociation, were found to be at least the same or longer. In addition, as HLA-A2 heavy chains exhibit a stronger tendency to oligomerize than H-2K^d or H-2K^b heavy chains, HLA-A2 expressing cells are expected to be less susceptible to directly binding exogenous peptide at the cell

surface than H-2K^d or H-2K^b expressing cells. However, it must be noted that recent evidence suggests that exogenous peptides may be loaded by the spontaneous recycling of HLA-A2 (*20*, *21*).

Oligomerization of newly synthesized heavy chains in endoplasmic reticulum would obviously impede the in vivo complex expression and therefore chaperones are likely assisting the heavy chain in its transformation into an appropriate conformation for association with β_2m (*22*). Indeed, the class I MHC heterodimer was shown to be associated with various chaperones and transport associated protein (TAP1) leading to peptide binding (*23*). The present results suggest that stability of the heavy chain/β_2m heterodimer of all alleles examined so far significantly increases upon peptide binding apparently due to conformational changes in heavy chain (Scheme 1, F). Therefore, it is likely that the peptide binding not only stabilizes the complex and produces the final shape of its agretop, but it may also cause the ternary complex dissociation from TAP1 and allow its transport to the cell surface. The biphasic dissociation kinetics of peptides from the ternary complex supports the existence of the ternary complex in at least two distinct conformations, but likely only the stable one is expressed on the cell surface.

In summary, evidence has been presented here that an allosteric mechanism is controlling the interaction among the human class I ternary complex components, i.e., the conformational transitions in the heavy chain, induced upon its association with β_2m or peptide binding, govern its assembly and dissociation.

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