Subunit Interactions of Class I Histocompatibility Antigens†

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ABSTRACT: The kinetics of dissociation of iodinated β_2-microglobulin (β_2m) from the papain-solubilized class I histocompatibility antigen HLA-B7 have been investigated. In the presence of unlabeled β_2m, most of the HLA dissociates according to a single rate constant, whereas in the absence of unlabeled β_2m, the system approaches an equilibrium dependent upon the initial HLA concentration. When iodinated β_2m is incubated with unlabeled HLA-B7, the rate of incorporation of β_2m into the complex is much less dependent on the concentration than is expected for a simple association/dissociation system; instead, the system behaves as if the "activity" (in a thermodynamic sense) of the HLA heavy-chain intermediate cannot surpass a critical concentration. The dissociation rate for each class I specificity is a function of temperature, ionic strength, pH, and the status of the heavy chain (papain solubilized vs. detergent solubilized). High temperature, high ionic strength, and extremes of pH promote dissociation. The intact molecule dissociates about 10 times more slowly than the papain-solubilized molecule. In contrast, the rate of dissociation of all papain-solubilized class I antigens tested falls within the range of about a factor of 2. The presence of the carbohydrate has no effect on the rate of dissociation. The possibility that HLA class I antigen dissociation may occur in vivo within acidic internal vesicles is discussed.

HLA class I histocompatibility antigens are heterodimeric glycoproteins that are the major barrier to tissue transplantation in man (Ploegh et al., 1981a), as well as an obligatory component of the antigen recognition apparatus of cytotoxic T lymphocytes (Zinkernagel & Doherty, 1979; Cohn, 1983). The polymorphic, glycosylated, 44-kilodalton heavy chain is inserted in the plasma membrane so that the bulk of the molecule is exposed to the extracellular space, while only about 30 amino acids are exposed to the cytoplasm. The nonpolymorphic, nonglycosylated, 12-kilodalton light chain, which is also known as β_2-microglobulin (β_2m),[1] binds the extracellular part of the heavy chain and is also found free in blood and urine. Papain can be used to cleave off the membrane binding region and the cytoplasmic domain of the heavy chain, leaving a complex consisting of 270–273 amino acids of the heavy chain and β_2m (HLA_{pap}).

Retention of antigenic activity serves as a simple criterion for native class I antigen structure. In the human system, antisera specific for single alleles generally do not recognize the heavy chain alone but instead recognize the heavy chain only in association with the light chain (Krangel et al., 1979). A second criterion of native structure is the ability to participate in in vitro cytolytic T cell (CTL) assays, such as the xenogenetic recognition assay (Engelhard et al., 1978) and the virus recognition assay (Finberg et al., 1978).

A third criterion for native structure addresses directly the biological significance of the noncovalent linkage between the two subunits. It has been observed that when iodinated β_2m is incubated with papain-solubilized class I complex, the β_2m in the complex equilibrates with iodinated β_2m (Hyafil & Strominger, 1979), presumably through a free heavy-chain intermediate. β_2m exchange also occurs on the surface of living cells in vivo (Kimura et al., 1983). The binding site on the heavy chain for β_2m is highly conserved throughout mammalian evolution, since murine β_2m associates with the human heavy chain upon cell fusion (Jones et al., 1976) as well as upon transfection of class I human genes into mouse cells (Barbosa et al., 1982; Lemonnier et al., 1982), thus allowing cell surface expression of the class I complex. Very extensive exchange can also be demonstrated in tissue culture in the case of the class I-like molecule T6, where endogenous β_2m is completely replaced by bovine β_2m from the medium used to grow the cells (Bernabeu et al., 1984; Kefford et al., 1984).

In this paper, the exchange and dissociation reactions of class I antigens are investigated by using papain-solubilized HLA-B7 ($HLA\text{-}B7_{pap}$) as a model compound to avoid the complications caused by the presence of the detergent-binding domain. Of primary interest in this research is the nature of the free heavy-chain intermediate. ^{125}I-β_2m (referred to herein as I-β_2m) can be prepared that is labeled only at tyrosine-67 (Parker & Strominger, 1983), and this modified β_2m is capable of substituting for unmodified β_2m in $HLA\text{-}B7_{pap}$. It is proposed that class I antigen must be able to participate in the β_2m exchange reaction in order to be considered native.

Since the mechanism by which class I antigens participate in CTL recognition of antigen is unknown, it is possible that the subunit exchange reaction plays an important role. At this time it is not clear what form of class I antigens is physiologically important for T cell recognition. Normally, class I antigens are present not only on the cell surface but also in the membranes of intracellular organelles, with several different carbohydrate structures (Ploegh et al., 1981b). Moreover, HLA complex that has already been expressed at the cell surface can be internalized into subcellular organelles, especially upon T cell activation (Tse & Purnis, 1984).

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[1] Abbreviations: β_2m, β_2-microglobulin; buffer A, 10 mM Tris-HCl, pH 7.4, 140 mM NaCl, and 0.1 mg/mL lysozyme; cpm, counts per minute; CTL, cytotoxic T lymphocyte; EXP, experimental percent cpm of I-HLA; HLA, human class I lymphocyte antigens; $HLA\text{-}B7_{det}$, detergent-solubilized HLA-B7; $HLA\text{-}B7_{pap}$, papain-solubilized HLA-B7 (p34,12); H, papain-solubilized heavy chain; I-β_2m, β_2m iodinated at tyrosine-67; I-HLA, HLA containing I-β_2m; OBS, observed percent cpm of I-HLA; P_0, percent cpm of I-HLA already dissociated at time = 0; P_{max}, percent cpm of I-β_2m capable of associating with HLA; P_ω, percent cpm of I-HLA that dissociates much more slowly than defined by k_2; THEO, theoretical percent cpm of I-HLA; Tris, tris(hydroxymethyl)-aminomethane; SDS, sodium dodecyl sulfate; HPLC, high-performance liquid chromatography.

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Therefore, a comparison of dissociation rates of individual class I antigen specificities under a range of conditions might provide insight into class I antigen function. This study analyzes how the rate of dissociation of different HLA alleles is affected by ionic strength, pH, and temperature. It is possible that the rate of dissociation of β_2m from the heavy chain is physiologically relevant, since dissociation may occur to some degree during recycling of class I antigen through acidic compartments in vivo. Since cytotoxic T cells recognize antigen only in the presence of the correct class I antigen allele, study of HLA dissociation and exchange provides a foundation for understanding the chemistry of one of the major components of T cell antigen recognition.

Materials and Methods

Materials. Human urinary β_2m was a gift from Arnold Sanderson. Papain-solubilized HLA-A2, HLA-B7, HLA-A28, and HLA-Bw40 were obtained from the cell lines JY and LB as described (Lopez de Castro et al., 1979). Detergent-solubilized HLA-A2 (HLA-$A2_{det}$) and HLA-B7 (HLA-$B7_{det}$) were a gift from Braydon Guild and Michael Greenburg; they were obtained from the cell line JY by Nonidet P-40 solubilization and affinity chromatography with the monoclonal antibodies PA2.1 and BB7.1 (Parham, 1979). Endoglycosidase F was a gift from Steven Alexander. Agarose-bound *Lens culinaris* agglutinin was obtained from Vector Laboratories. The nonionic detergent Renex 30 was from Accurate Chemical and Scientific Corp., Westbury, NY.

Preparation of I-β_2m. I-β_2m iodinated primarily at tyrosine-67 was prepared as described (Parker & Strominger, 1983). Typically, between 60% and 90% of this preparation could be exchanged into HLA-$B7_{pap}$, compared to about 5–40% of directly iodinated I-β_2m.

Preparative Exchange. A 10-μL aliquot of HLA-$B7_{pap}$ (22 μM) was incubated with 200 μL of I-β_2m (93 nM, 1.3×10^{16} cpm/mol) in buffer A (10 mM Tris-HCl, pH 7.4, 140 mM NaCl, and 0.1 mg/mL lysozyme) at 37 °C for about 12 h. The sample was then separated on a 1.2 × 40 cm Sephadex G-75 column in buffer A at a flow rate of 0.1 mL/min at 4 °C. The fractions containing ^{125}I-HLA-$B7_{pap}$ (referred to herein as I-HLA) were pooled and stored on ice until use. The concentration of HLA-B7 in this pool was 56 nM.

Dissociation. At zero time, 10 μL of β_2m (83 μM) and 10 μL of 10 mg/mL ovalbumin were added to 500 μL of I-HLA or dilutions of I-HLA in buffer A. To this was added 125 μL of 500 mM sodium phosphate, pH 7.5, and the solution was filtered through a Millipore type HV 0.45-μm syringe filter and incubated at the desired temperature. Aliquots of 50–100 μL were injected onto a Bio-Rad TSK-250 sizing column attached to a Waters Associates HPLC system consisting of a U6K injector and a 6000A pump. A Waters Associates Model 440 flow cell operating at 280 nm and a Waters Associates data module were often used to check for both peak sharpness and column equilibration. The running buffer was either 500 mM NaCl–200 mM Tris-HCl, pH 7.4, or 100 mM Tris–acetate, pH 7.1, with or without 0.05% Renex 30; the samples were eluted at a flow rate of 1 mL/min. The exact identity of the running buffer and the concentration of ovalbumin and/or lysozyme in the incubation solution had no detectable influence on the rate constants or equilibrium constants obtained, although they did influence the fraction of radioactivity recovered, which usually was near 100%. The Tris–acetate buffer was eventually deemed the buffer of choice because of the corrosive action of chloride ion on the Waters 6000A pumps. In the case of detergent-soluble HLA, Renex 30 was used to maintain solubility of the HLA.

The radioactivity eluted from the column in two peaks; the first peak contained I-HLA, and the second peak contained I-β_2m. The time length of the fractions was adjusted during each run so that only 12 fractions had to be collected for complete peak separation. Each fraction was counted in a Searle Model 1185 γ counter. The cpm of I-HLA and the cpm of I-β_2m were calculated for each injection and corrected for background. Background was typically 26 cpm per fraction. When the data were tabulated as percent recovered cpm of I-HLA, reproducibility between identical samples was usually within 1%.

Association. To 1.0 mL of HLA-$B7_{pap}$ at various concentrations was added 250 μL of 500 mM sodium phosphate, pH 7.5, and 20 μL of I-β_2m (44 nM, 2.8×10^{17} cpm/mol). Even at the lowest HLA concentrations, HLA was in about 10-fold excess over I-β_2m. Aliquots were injected as described above.

Chain Separation. Two aliquots of 200 μL each of HLA-$B7_{pap}$ (1 mg/mL) mixed with tracer I-β_2m were incubated for 90 min at 23 °C in 6 M guanidine hydrochloride and 10 mM Tris-HCl, pH 7.4. One aliquot was loaded onto a 1.2 × 40 cm Bio-Gel P-300 column equilibrated in 6 M guanidine hydrochloride and 10 mM Tris-HCl, pH 7.4. The profiles of absorbance and radioactivity of each fraction confirmed that separation of the HLA heavy chain and the I-β_2m was almost complete. The concentrations of HLA heavy chain and HLA-$B7_{pap}$ complex were determined by using extinction coefficients at 280 nm of 67 000 and 88 000 M^{-1} cm^{-1}, respectively. The second aliquot was diluted to the same volume as the heavy-chain pool, and both aliquots were dialyzed against buffer A. The concentrations of each sample were measured again after dialysis.

Ionic Strength Dependence of Dissociation. Solid NaCl was dissolved in buffer A so that the desired concentration of NaCl was obtained after addition of I-HLA_{pap}. Time courses were carried out as described above.

Glycosidase Treatment of HLA. An aliquot of 20 μM HLA-$B7_{pap}$ (100 μL) in buffer A was incubated with 10 μL of 500 mM sodium phosphate and 25 mM EDTA, pH 6.1. To this solution was added 20 μL of endoglycosidase F in 50% glycerol, and the mixture was incubated at 37 °C. After 16 h, another 10-μL aliquot of endoglycosidase F was added. After 38 h, 40 μL of 0.5 M Tris-HCl, pH 7.4, and 10 mg/mL bovine serum albumin was added. This solution was loaded on a 1-mL *Lens culinaris* agglutinin–agarose column equilibrated in buffer A containing 0.1 mM $CaCl_2$ and 0.1 mM $MnCl_2$ and incubated for 4 h at 4 °C. SDS gel analysis of the flow through indicated that most of the HLA-$B7_{pap}$ migrated at the expected position of deglycosylated HLA-$B7_{pap}$ (data not shown).

pH Dependence of Dissociation. I-HLA_{pap} was diluted into buffer A containing 63 mM sodium phosphate at pH 5.8, 6.7, or 7.4 or 63 mM sodium acetate, pH 5.0. High pH experiments were done in buffer A containing 63 mM Tris-HCl, pH 8.5, 63 mM sodium carbonate, pH 9.5, or 63 mM sodium phosphate, pH 10.5.

pH Dependence of Association. On ice, a 50-μL aliquot of I-β_2m (100 nM, 2×10^{16} cpm/mol) was mixed with 350 μL of buffer A containing 0.05% NaN_3, 70 mM sodium phosphate, pH 5.8 or 6.7, and 0.55 μM HLA-$B7_{pap}$. After filtration at zero time, the solution was incubated at 32.5 °C, and aliquots were removed at various times and immediately injected onto the HPLC as described above.

Data Analysis

Minimization Routine. The total number of counts (corrected for background) in the I-HLA and I-β_2m peaks for each

time point were entered into a computer program that calculates the observed percentage cpm of I-HLA (OBS) as follows:

$$\text{OBS} = \frac{100(\text{cpm of I-HLA})}{\text{cpm of I-}\beta_2\text{m} + \text{cpm of I-HLA}} \quad (1)$$

The computer program then calculates how well the experimental data fit theoretical values by calculating the variance:

$$\text{variance} = \frac{\text{sum for all time points of (EXP} - \text{THEO)}^2}{\text{number of time points}} \quad (2)$$

where EXP = experimental percentage of cpm of I-HLA, which is a function of OBS, and THEO = theoretical percentage of cpm of I-HLA, as defined below. The program then minimizes the variance by optimizing the values of each of the independent variables by using an algorithm developed by W. C. Davidon, adapted for HP-Basic by J. Granlund and V. Granlund, and translated into Fortran 77 by K. Parker.

Simple Dissociation. For each time course, the rate constant of dissociation, k_2, and the two boundary condition variables, P_ω and P_0, were solved for as independent variables according to the following equations. P_0 corresponds to I-HLA that has already dissociated prior to the start of the experiment, whereas P_ω corresponds to I-HLA that dissociates with a rate constant much smaller than k_2:

$$\text{EXP} = 100\frac{\text{OBS} - P_\omega}{100 - P_\omega - P_0} \quad (3)$$

Under these conditions, the concentration of I-HLA decreases according to a single rate constant:

$$\text{THEO} = 100e^{-(k_2\text{time})} \quad (4)$$

See Results and Discussion for the significance of P_0 and P_ω.

Dissociation to Equilibrium. The equilibrium concentration of HLA–B7 complex (C_{eq}) was calculated from the definition of the equilibrium constant (K):

$$K = \frac{(C_{tot} - C_{eq})^2}{C_{eq}} \quad (5)$$

where C_{tot} is the total concentration of HLA–B7 in all forms. After solving for C_{eq}

$$C_{eq} = \frac{2C_{tot} + K - (4.0C_{tot}K + K^2)^{0.5}}{2.0} \quad (6)$$

In this case, the theoretical percentage of cpm of I-HLA as a function of time is given by the equation

$$\text{THEO} = 100[C_{eq} + C_{tot}e^{-(k_2\text{time})[(C_{tot}+C_{eq})/(C_{tot}-C_{eq})]}/[C_{tot} + C_{eq}e^{-(k_2\text{time})[(C_{tot}+C_{eq})/(C_{tot}-C_{eq})]}]] \quad (7)$$

This equation can be derived upon integration of the rate equation describing opposing reactions in the system $C \rightleftharpoons A + B$ (see, e.g., Bamford & Tipper (1969), p 37).

Association of I-β_2m with HLA–B7 at Equilibrium. Since the heavy-chain concentration H should be constant with time, integration of the equation

$$dC/dt = k_1(C_{tot} - C)H - k_2C \quad (8)$$

where C is the concentration of iodinated HLA complex as a function of time yields

$$\text{THEO} = 100 - \frac{100(k_1He^{-[k_2+(k_1H)]\text{time}} + k_2)}{k_2 + k_1H} \quad (9)$$

where

$$H = 0.5[-K + (K^2 + 4.0K(C_{tot}))^{0.5}] \quad (10)$$

EXP becomes a function of P_{max}, instead of P_0 and P_ω, as follows:

$$\text{EXP} = 100(\text{OBS}/P_{max}) \quad (11)$$

where P_{max} is the percentage of the I-β_2m capable of associating with HLA–B7.

Results

Dissociation of I-HLA. As was first observed by Hyafil & Strominger (1979), the dissociation of β_2m from papain-solubilized HLA obeys first-order kinetics when unlabeled β_2m is added to block the reverse reaction. In order to prove that HLA dissociation is entirely independent of the initial HLA concentration, I-HLA_{pap} was diluted to 22, 11, 5.5, and 2.8 nM and incubated with 1.3 μM β_2m at 23 °C. For each time point, an aliquot was injected onto the HPLC, and the percentage of the recovered radioactivity that migrated as I-HLA_{pap} was calculated. This technique is much more rapid than earlier experiments that relied on classical gel filtration to achieve subunit separation [see Hyafil & Strominger (1979) and Parham (1983)]. It is also more reliable than procedures that rely on monoclonal antibodies to separate I-HLA from I-β_2m (Church et al., 1982), since antibodies have been shown to influence the thermodynamics of subunit association (Parham, 1983).

As expected, all four time courses are superimposable, as must be the case for a first-order reaction. The rate constant was determined to be 7.9×10^{-4} min^{-1} at 23 °C, which compares with the value of 8.4×10^{-3} min^{-1} at 37 °C obtained by Hyafil and Strominger. The difference can be attributed mostly to the temperature and slightly to the differences in buffers used (see below).

Two more variables must be introduced before a plot of ln (percent cpm of I-HLA) vs. time is a straight line (see Materials and Methods). These two variables reflect deviation from ideal behavior either at initial conditions or after several half-lives of dissociation. P_0 is defined as the percentage of the I-HLA that has already dissociated, or which by an experimental artifact appears to have dissociated prior to the start of the time course, and usually amounts to about 3–10% of total I-HLA. P_ω is defined as the percentage of I-HLA that has not dissociated after many half-lives and is typically in the range 10–40% of the total isotope. P_ω corresponds mostly to material that dissociates more slowly than the majority of the I-HLA, since after exceedingly long periods of time only about 5% of the label remains in the I-HLA peak (data not shown).

Dissociation of I-HLA to Equilibrium. As Hyafil and Strominger observed earlier, when the above experiment is repeated in the absence of β_2m, the system approaches equilibrium with the same rate constant of dissociation (Figure 1). The reaction is no longer first order but instead is concentration dependent, so that the equilibrium reached is dependent upon the initial concentration of I-HLA_{pap}. The continuous lines in Figure 1 represent the best fit of the experimental data to the theoretical calculations. Since the reverse reaction now becomes important, the data can be used to calculate both the rate constant of association and the equilibrium constant. The value of the equilibrium constant obtained (0.65 nM at 23 °C) is consistent with that obtained by Hyafil and Strominger (4 nM at 37 °C) when the difference in temperature and buffer is taken into account.

Association of I-β_2m with HLA–B7. HLA-$B7_{pap}$ was diluted to several different concentrations in the same buffer as used above and preequilibrated overnight at 23.5 or 32 °C. Upon addition of I-β_2m, the incorporation of radioactivity into

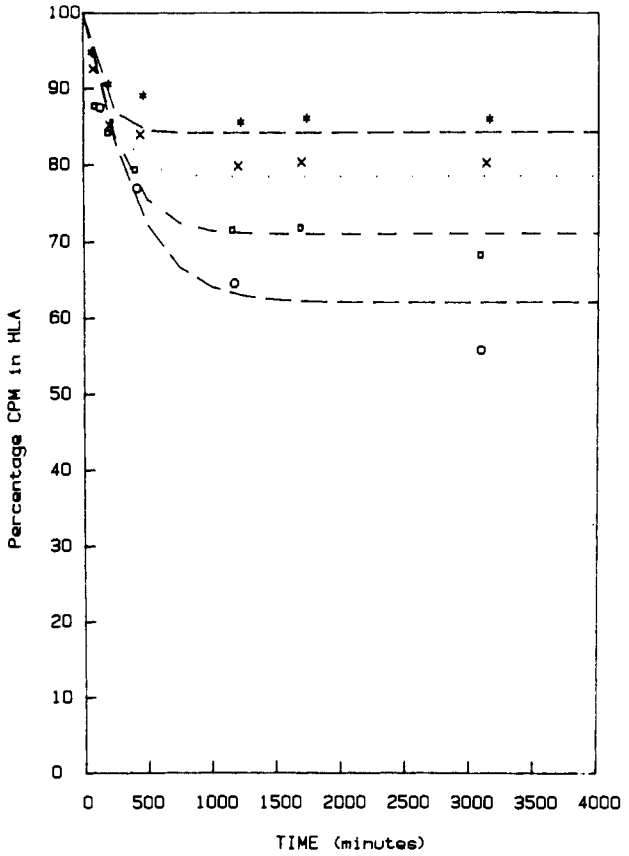

FIGURE 1: Dissociation of I-HLA–B7 to equilibrium. Iodinated HLA–B7 was diluted to 22 (asterisk), 11 (×), 5.5 (□), or 2.8 nM (○) and the formation of free β_2m followed as a function of time at 23 °C. The theoretical percent I-HLA at each dilution (plotted curves) was matched to the experimental percent I-HLA with $k_2 = 7.86 \times 10^{-4}$ min^{-1}, $P_0 = 4.52\%$, $P_\infty = 17.5\%$, $K = 0.65$ nM, and variance = 5.89.

HLA was followed by using the HPLC assay (Figure 2). Even at the highest HLA concentrations, a substantial amount of the isotope did not incorporate; therefore, the percentage of incorporable I-β_2m was calculated as the independent variable P_{max}. The older the preparation of I-β_2m and the higher the specific activity, the lower the value of P_{max} (data not shown). At low concentrations, the rate of association was described well by k_2 and K as determined above. At concentrations equal to or higher than K the rate of incorporation was much slower than expected. Since all of the variables necessary to describe the system had already been calculated, the apparent value of the concentration of free heavy chain (H) was calculated for each time course from eq 7 (see Data Analysis) and compared to the value calculated directly from K and the HLA concentration by using eq 8 (Table I). The amount of H detectable by the association data in Figure 2 was much smaller than that predicted. This large discrepancy will be discussed below.

Association of Heavy Chain Purified by 6 M Guanidine. HLA_{pap} heavy chain was purified on a Bio-Gel P-300 sizing column in 6 M guanidine as described previously (Terhorst et al., 1976). Following addition of the nonionic detergent Renex 30 to help prevent aggregation, dialysis to remove guanidine, and addition of I-β_2m, formation of I-HLA_{pap} was completely undetectable by the HPLC assay under a variety of incubation conditions and after several days of incubation (Figure 3). A control aliquot of $HLA–B7_{pap}$ that had been treated with the guanidine solution in parallel for the same period of time, but not run over the sizing column, had at most

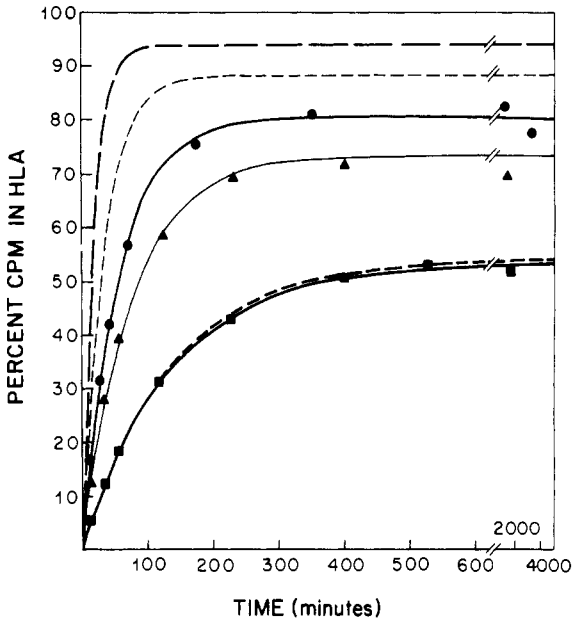

FIGURE 2: Exchange of I-β_2m into HLA–B7 at equilibrium. HLA–B7 was preequilibrated at 32 °C at 9.4 (■), 240 (▲), or 940 nM (●), and I-β_2m was added at $t = 0$. Formation of I-HLA with time was followed at 32 °C. The theoretical percent I-HLA was matched to the experimental percent I-HLA with $k_2 = 3.5 \times 10^{-3}$ min^{-1}, $P_{max} = 84.2\%$, $K = 3.73$ nM, and variance = 1.21 when H is taken from the "experimental" column of Table I. If H is taken from the "theoretical" column of Table I, for each time course the discontinuous line of corresponding thickness is obtained. The variance calculated by using the data and the three discontinuous lines is 206.4.

Table I: Comparison of Theoretical and Experimental Heavy Chain Concentrations

HLA concn[a]	H (theor)[b]	H (exptl)[c]
	Experiment 1[d]	
9.4	4.4	4.3
240	28	10
940	57	15
	Experiment 2[e]	
14	2.7	1.8
29	4.0	2.5
57	5.8	2.7
110	8.3	3.3
230	12	3.4
2300	38	4.0

[a] All concentrations are expressed in nanomolar. [b] The value of H in this column was calculated by using eq 8. [c] The value of H in this column was calculated by eq 7 and the data from the time course. [d] The temperature in this experiment was 32 °C; $k_2 = 3.5 \times 10^{-3}$ min^{-1}; $K = 3.73$ nM; $P_{max} = 84.2\%$. [e] The temperature is this experiment was 23 °C; $k_2 = 7.86 \times 10^{-4}$ min^{-1}; $K = 0.649$ nM; $P_{max} = 73.0\%$.

20% of the exchange activity of a comparable amount of nondenatured material. (Note that the guanidine-treated sample is almost 5 times more concentrated than the control.) Results similar to these have been obtained in many labs using antigenic activity as an assay for native HLA structure.

All efforts to isolate the free papain-solubilized heavy chain in a form capable of reassociation with β_2m using milder conditions were unsuccessful, including chain separation at pH 5.0 or pH 11.0 followed by ion-exchange chromatography (60-min separation time), gel filtration (20-min separation time), or affinity chromatography.

Ionic Strength Dependence of Dissociation Rate. In order to determine how ionic strength influences the stability of the $HLA–B7_{pap}$ complex, labeled complex was prepared in which all of the label was in the light chain (Parker & Strominger, 1983). This material was diluted into a buffer of the desired

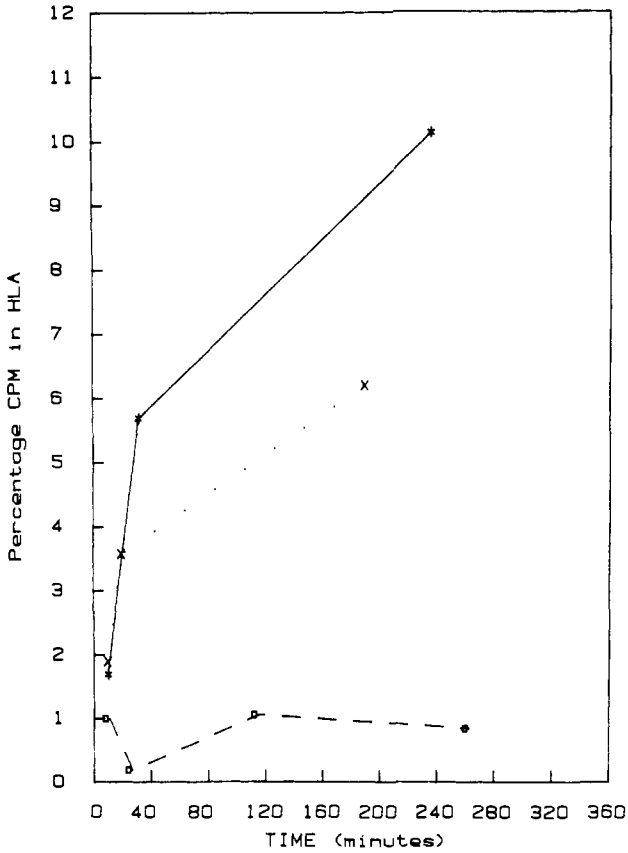

FIGURE 3: Exchange of I-β_2m into guanidine-treated HLA–B7. I-β_2m was incubated at 37 °C with 62 nM HLA–B7 in buffer A (asterisk), with 290 nM HLA–B7 that had been dialyzed into 6 M guanidine hydrochloride before dialysis back into buffer A (×) or with 220 nM HLA–B7 heavy chain in buffer A (□).

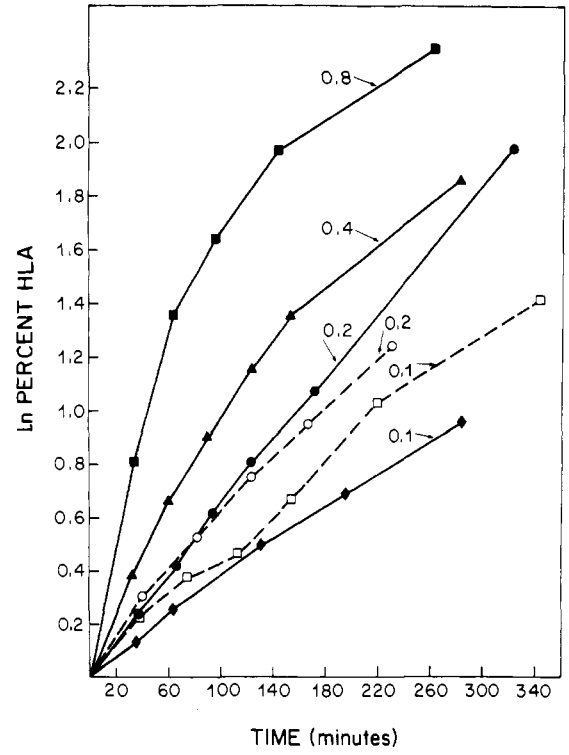

FIGURE 4: β_2m dissociation from HLA–B7$_{pap}$: influence of ionic strength and glycosylation. For each time course, aliquots were removed as a function of time at 37 °C and injected onto a Bio-Rad TSK-250 gel filtration column. The percent of recovered counts that migrated as I-HLA was calculated for each time point. For each time course, the data were corrected by using P_0 and P_∞. Time courses were carried out in 0.1 M NaCl (♦), 0.2 M NaCl (●), 0.4 M NaCl (▲), and 0.8 M NaCl (■). Time courses of deglycosylated HLA–B7$_{pap}$ were carried out in 0.1 M NaCl (□) and 0.2 M NaCl (○).

salt concentration at the start of the experiment. The initial rate of dissociation was found to be first order in the presence of excess unlabeled β_2m at all ionic strengths tested, as expected (Figure 4). The rate of dissociation was found to be nearly proportional to the NaCl concentration.

Effect of Deglycosylation on Dissociation Rate. In order to determine whether the carbohydrate plays any significant role in subunit interactions, the carbohydrate on class I antigen was removed by digestion with endoglycosidase F, which leaves only a single *N*-acetylglucosamine residue attached to the protein (Elder & Alexander, 1982). The HLA heavy chain of this material migrated as a single band by SDS gel electrophoresis, of lower molecular weight than the native heavy chain. The rate of dissociation of deglycosylated HLA–B7$_{pap}$ was found to be identical with the control at both 100 and 200 mM NaCl (Figure 4). Incubation at HLA–B7$_{pap}$ in the deglycosylation buffer and other buffers prior to the exchange experiment resulted in no significant difference in the subsequent rate of exchange (data not shown).

pH Dependence. To determine how pH influences the stability of the HLA–B7$_{pap}$ complex, the rate of dissociation of the HLA–B7$_{pap}$ complex was determined after dilution into solutions of the appropriate pH (Figure 5). The rate of dissociation was found to increase by a factor of at least 100 over fairly small changes in pH. To determine whether these changes were primarily the result of denaturation of the HLA–B7$_{pap}$ complex instead of dissociation, the rate of formation of iodinated HLA–B7$_{pap}$ complex was studied at pH 5.8 and 6.7. A significant amount of labeled HLA–B7$_{pap}$ complex was formed even at pH 5.8 (Figure 6). After moderately long incubation times, the total amount of iodinated HLA–B7$_{pap}$ complex began to decrease at the lower pH. This could be attributed to irreversible denaturation, but the kinetics are slow enough that it is apparent that denaturation does not contribute significantly to the increased dissociation rate observed in Figure 5. In fact, the rate of formation of I-HLA–B7$_{pap}$ complex is significantly faster at the lower pH. Together, the data in Figures 5 and 6 suggest that the primary

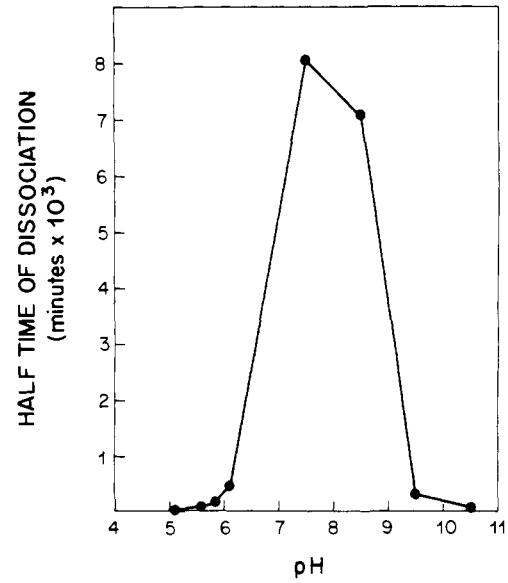

FIGURE 5: Effect of pH on half-time of dissociation. Time courses were carried out at 0 °C in buffers of different pHs as described under Materials and Methods, and the rate constant was determined by using the same analysis as described in the legend to Figure 4.

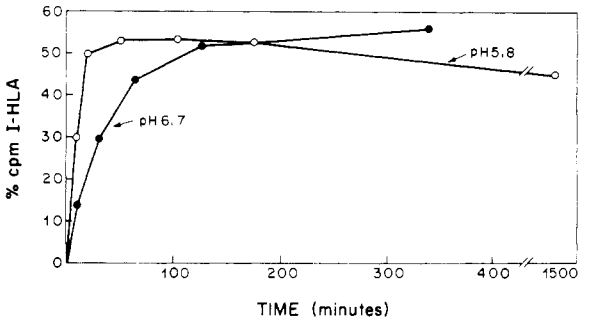

FIGURE 6: Effect of pH on the rate of association of I-β_2m into HLA_{pap}. Time courses were carried out at 32.5 °C with 550 nM $HLA-B7_{pap}$ at pH 5.8 (○) or 6.7 (●). In this figure, no correction was made for I-β_2m that is unable to associate with $HLA-B7_{pap}$.

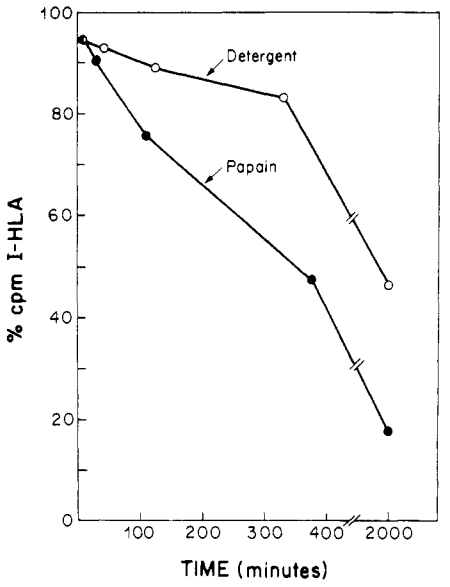

FIGURE 7: Comparison of dissociation rate of $HLA-B7_{det}$ with $HLA-B7_{pap}$. Time courses were carried out as described in Figure 4 at 33 °C with $HLA-B7_{pap}$ (●) or $HLA-B7_{det}$ (○). No corrections were made for P_0 or P_ω.

impact of the lower pH is to lower the activation energy of the chain dissociation process. Direct measurement of the equilibrium constant at pH 5.8 revealed that it had increased to approximately 50 nM, which is about 10-fold higher than the equilibrium constant in the equivalent buffer at neutral pH (data not shown).

Rate of Dissociation of Detergent-Solubilized HLA-B7. To determine whether removal of the carboxy terminus during papain digestion has a large effect on the rate of dissociation of intact HLA, iodinated β_2m was exchanged into $HLA-B7_{det}$. As shown in Figure 7, the rate of dissociation of $HLA-B7_{det}$ was much slower. Analysis of the data in Figure 7 yields values of 3.0×10^{-4} min^{-1} and 2.0×10^{-3} min^{-1} for $HLA-B7_{det}$ and $HLA-B7_{pap}$, respectively. I-β_2m was also much more slowly incorporated into $HLA-B7_{det}$ than into $HLA-B7_{pap}$ at an equivalent concentration. Similar time courses have been obtained by Church et al. (1982) for association of either human I-β_2m or bovine I-β_2m into human detergent-solubilized class I antigens, although no rate constants were reported.

Comparison of Different HLA Specificities. The rates of dissociation of $HLA-B7_{pap}$, $HLA-A2_{pap}$, $HLA-Bw40_{pap}$, and $HLA-A28_{pap}$ were determined at several different pHs (data not shown). In no case was the rate of dissociation of any one specificity different by much more than a factor of 2 from any other specificity. However, there was occasional evidence that $HLA-A2_{pap}$ and $HLA-A28_{pap}$ were somewhat slower to dissociate than $HLA-B7_{pap}$ and $HLA-Bw40_{pap}$. Preliminary data suggest that $HLA-A28_{pap}$ may be the slowest specificity to dissociate, while $HLA-Bw40_{pap}$ may be the fastest. This experiment needs to be repeated extensively before this result can be accepted, since the differences are not very large and minor changes in temperature were difficult to control. In addition, in these experiments P_ω was sometimes greater than 10%, causing the corresponding value for k_2 to become more uncertain.

Discussion

Class I histocompatibility antigens provide an interesting example of a heterodimeric system for the study of subunit/subunit interactions. HLA-B7 is thought to dissociate directly to free heavy chain and free β_2m (Hyafil & Strominger, 1979):

$$\text{I-HLA} \underset{k_1}{\overset{k_2}{\rightleftharpoons}} \text{I-}\beta_2\text{m} + \text{H} \qquad (12)$$

The free heavy chain is then able to recombine with any β_2m free in solution. The data obtained here support these concepts. The majority of iodinated HLA-B7 labeled only on tyrosine-67 of β_2m dissociates according to a single rate constant if a large excess of unlabeled β_2m is added to block observation of the reverse reaction. Moreover, the dissociation rate is independent of the initial I-HLA concentration, as expected for a first-order reaction (data not shown). There are several interpretations for the I-HLA population that dissociates more slowly, as designated by the variable P_ω. The iodination itself may cause some chemical heterogeneity, even though every effort was made to prepare a homogeneous population of labeled I-β_2m. The heterogeneity may also originate in the starting HLA-B7, although the endoglycosidase experiment (Figure 4) and the specificity comparison experiment (data not shown) demonstrate that neither carbohydrate polymorphism nor allelic variations significantly influence the rate of dissociation of complex. HLA-B7 heterogeneity due to proteolysis by residual papain, partial deamidation, or aggregation may also contribute to P_ω. The most interesting possibility, however, is that P_ω reflects HLA-B7 conformational heterogeneity and thus may be related to HLA function (see below).

When unlabeled β_2m is omitted from the system, the I-HLA approaches an apparent equilibrium that can be described by a single equilibrium constant and by the same rate constant obtained in the presence of excess unlabeled β_2m. The complications described above make the equilibrium constant difficult to determine, since it is necessary to subtract out contributions made by material that dissociates at a slower rate. Nevertheless, the data fit theoretical expectations well enough so that there can be no doubt that the system does approach a state of equilibrium.

It has recently been observed that when dinitrophenylated β_2m is incubated with $HLA-B7_{pap}$, the number of dinitrophenyl groups per β_2m is higher in the $HLA-B7_{pap}$ fraction than in the material that does not exchange. This result has been interpreted to mean that there exists a complex containing two light chains per heavy chain (Ward & Saunderson, 1983). If such an intermediate were obligatory in HLA-B7 dissociation, then one would expect that the dissociation rate should be a function of both the unlabeled β_2m concentration and the HLA complex concentration. Moreover, the rate of dissociation in the absence of β_2m should be much different than in the presence of unlabeled β_2m, since in the absence of unlabeled β_2m the intermediate with two light chains could be formed only after dissociation of another HLA complex, presumably by a second mechanism. The data presented here

are not consistent with either of these predictions.

The simplest way to examine the forward reaction would be to start with purified H and I-β_2m and follow association into I-HLA. Unfortunately, there have been no convincing reports in the literature of complete reconstitutiton of HLA following separation of chains [see Lancet & Strominger (1979) and Brodsky et al. (1979)]. The simplest feasible method of studying chain association is to follow incorporation of I-β_2m into HLA–B7. According to the free heavy-chain model, the rate of incorporation should be described by the equation

$$-\mathrm{d}[\text{I-}\beta_2\text{m}]/\mathrm{d}t = k_1[\text{I-}\beta_2\text{m}][\text{H}] - k_2[\text{I-HLA}] \quad (13)$$

where k_1 equals k_2/K as defined above. Clearly, uptake of I-β_2m into HLA should be a function of H where H is defined by the equilibrium constant according to

$$[\text{H}] = 0.5[-K + (K^2 + 4K[\text{HLA–B7}])^{0.5}] \quad (14)$$

Normally, incorporation of I-β_2m into I-HLA should be limited by the availability of H, whose formation requires prior dissociation of complex. However, when the complex has been preequilibrated at the initial conditions, the concentration of H should be constant. At higher HLA concentrations, incorporation should proceed more rapidly, due to a higher equilibrium concentration of H. Although a more rapid rate of incorporation of I-β_2m into I-HLA is observed at higher HLA concentrations, the extent of the increase is less than expected. This suggests that the chemical activity of either the HLA heavy chain or HLA complex itself is lower at high concentrations of HLA-B7, so that the active concentration is much lower than the theoretical concentration (Table I). This can be mathematically incorporated into the model by postulating dimerization or reversible aggregation of either the free heavy chain or complex. However, gel permeation chromatography of high concentrations of HLA–B7 does not reveal any dimers or other aggregates. The equivalent experiment cannot be done on the free heavy chain, since it cannot be isolated in active form. The HLA heavy chain appears to be very "sticky", so that it is lost on any matrix used for its purification, whenever the buffer used does not result in irreversible denaturation.

The data in Figure 2 can also be used to calculate the denaturation rate of H, since as H denatures, the percentage of isotope that migrates as HLA should decrease (see also Figure 6). Such a decrease in I-HLA is observed after prolonged incubation of I-β_2m and HLA–B7, but it is clearly slow compared to the association reaction. Such a denaturation reaction should also be manifested in the approach-to-equilibrium experiment in Figure 1. It theoretically should contribute to the value of P_ω; however, the data in Figure 2 show that it is so small that it can be neglected. Together, these results are consistent with the idea that the free heavy chain behaves as a metastable intermediate in vitro. The basis of the instability of the free heavy chain appears to be aggregation, which is normally prevented by association with β_2m.

HLA denaturation, as assayed by loss of antigenic activity, has universally been observed upon separation of chains by strong acid, guanidine, or SDS (Cresswell et al., 1973; Nakamuro et al., 1975; Parham, 1977; Lancet et al., 1979). The data in Figure 3 indicate that some exchange activity remains after exposure to guanidine and dialysis into physiological buffers. Similar results have been obtained for alloantigenic activity (Parham, 1977; Lancet et al., 1979). No exchange activity at all was observed following chain separation, whereas very low amounts of alloantigenic activity have been detected under these conditions (Lancet et al., 1979). The concordance between these results suggest that alloantigenic activity and ability to participate in the exchange reaction require the same native structure.

Investigation of the ionic strength dependence of the rate of subunit dissociation reveals that the complex dissociates more readily in high salt. This point has been a matter of controversy in the past. Peterson et al. (1974) found that complete subunit dissociation could be achieved in 3 M KCl, while Turner et al. (1975) did not observe substantial dissociation in 3 M KCl. The results obtained here do not directly address the situation at equilibrium, but it seems likely that the equilibrium constant should increase as the ionic strength increases due to the faster rate of dissociation.

Endoglycosidase F treatment of glycoproteins has been shown to cleave complex carbohydrates leaving only a single *N*-acetylglucosamine residue attached to the polypeptide (Elder & Alexander, 1982). When HLA–$B7_{pap}$ was cleaved with endoglycosidase F, there was no detectable impact on the rate of dissociation. This is not surprising, since the known location of the carbohydrate on asparagine-86 is far in primary sequence from amino acid residues 182–276, which have been shown to interact with β_2m (Yokohama et al., 1983). β_2m/HLA heavy-chain association takes place during biosynthesis prior to conversion of the high mannose oligosaccharide to the complex form (Krangel et al., 1979), so if the carbohydrate plays any role in chain association, the high mannose form of the heavy chain would be the most biologically relevant form to test. So far this has not been available for study. However, chain association still takes place in tunicamycin-treated cells, in which the heavy chain has no carbohydrate attached (Ploegh et al., 1981b). The rate of carbohydrate processing of the heavy chain has been found to be slower when chain association does not take place (Krangel et al., 1979), as in the cell line Daudi, which has a defective β_2m gene (Rosa et al., 1983). Presumably the presence of β_2m maintains the heavy chain in a conformation that allows access of much of the carbohydrate to enzymes. The lack of impact of carbohydrate removal on subunit dissociation argues that the role of the carbohydrate is not to stabilize that part of the HLA complex involved with β_2m association.

Study of the dependence of the rate of subunit dissociation on temperature allows calculation of an activation energy for the dissociation reaction by the Arrhenius equation

$$\ln k_2 = -\frac{E_a}{RT} + \text{constant}$$

where R is the gas constant and E_a is the activation energy. For HLA–$B7_{pap}$, E_a is about 20 kcal. The dependence of the equilibrium constant on temperature can be used to calculate the entropy and enthalpy of reaction on the basis of the van't Hoff equation

$$\frac{\mathrm{d} \ln K}{\mathrm{d}(1/T)} = \frac{-\Delta H}{R}$$

The value obtained is +30 kcal for ΔH and +70 cal/(deg K) for ΔS. Hence, the binding of β_2m is enthalpically favored and entropically disfavored. If one assumes that hydrogen bonds and salt bridges contribute largely to enthalpy, whereas hydrophobic interactions contribute mostly to entropy, then these data support the idea that salt bridges and hydrogen bonds play a larger role in stabilizing the complex than hydrophobic forces. Since β_2m has no tendency to dimerize by itself (unlike immunoglobulin light chains), it makes sense that hydrophobic forces should play a minor role in its association with other proteins. On the other hand, the data in Figure 2 and the failure to isolate HLA heavy chain under conditions

that promote reversible dissociation of HLA complex suggest that hydrophobic forces may be important for the stability of the heavy chain. In any case, the unfavorable entropy of chain association can be explained by a loss of conformational flexibility of the heavy chain upon association with β_2m.

The differences in dissociation rate between different HLA specificities was observed to be small and not always reproducible. In contrast, the dissociation rate of detergent-solubilized HLA is much slower than that of papain-solubilized HLA. This could be explained by a detergent micelle steric effect or by direct interaction between part of the carboxy terminus and β_2m. Alternatively, the presence of the carboxy terminus may merely limit the conformational flexibility of the heavy chain by contributing to the stability of the σ_3 domain. Nevertheless, these results suggest that papain-solubilized HLA is not a perfect model for HLA/β_2m interactions. If the dissociation reaction plays a physiological role in HLA function, then the carboxy terminus may be important for limiting complex dissociation, in addition to holding the HLA complex at the plasma membrane surface.

The exact mechanism of participation of class I antigens in recognition of viral antigen by CTLs is unknown. Some forms of the altered self-theory suggest that each antigen seen by T cells causes a unique alteration in the conformation of class I antigen, which is then recognized by the cytotoxic T cell as altered self. This altered conformation of class I antigen is supposed to mimic the conformation of an allelic class I antigen, thus explaining the ability of individual CTL clones to recognize virus plus self as well as allogeneic cells. Recently, it has been shown that different monoclonal antibodies directed against HLA-B7 stabilize different conformations of HLA-B7 (Parham, 1984). If several different conformations of HLA-B7 are slowly interconvertible over periods of hours, then they would be detected in dissociation experiments as deviations from ideal behavior if each conformation had a distinct β_2m dissociation rate. The conformation(s) with the slowest dissociation rate would contribute to the parameter P_ω described above. Some conformations could readily aggregate, thus explaining the difficulty in isolating the free heavy chain in an active form. It is tempting to speculate that the free heavy chain is the form of class I antigen that associates with viral antigen in CTL recognition because its tendency to aggregate might cause it to have higher affinity for viral antigen than the β_2m-associated complex.

It has recently been discovered that β_2m exchange occurs for certain specificities on the cell surface in vivo (Kimura et al., 1983) and in tissue culture (Bernabeu et al., 1984; Kefford et al., 1984). In addition, class I antigen internalization into acidic compartments has been demonstrated by immunofluorescence on T cell surfaces (Tse & Pernis, 1984). Since the dissociation and reassociation reactions are much faster at low pH, it seems likely that some dissociation occurs upon internalization and acidification in vivo. If HLA dissociation plays any role in T cell recognition, then class I antigen internalization by either the T cell or the target cell may be essential to this process.

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