The kinetic basis of peptide exchange catalysis by HLA-DM

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The mechanism by which the peptide exchange factor HLA-DM catalyzes peptide loading onto structurally homologous class II MHC proteins is an outstanding problem in antigen presentation. The peptide-loading reaction of class II MHC proteins is complex and includes conformational changes in both empty and peptide-bound forms in addition to a bimolecular binding step. By using a fluorescence energy transfer assay to follow the kinetics of peptide binding to the human class II MHC protein HLA-DR1, we find that HLA-DM catalyzes peptide exchange by facilitating a conformational change in the peptide-bound complex, and not by promoting the bimolecular MHC–peptide reaction or the conversion between peptide-receptive and -averse forms of the empty protein. Thus, HLA-DM serves essentially as a protein-folding or conformational catalyst.

MHC proteins are heterodimeric cell surface proteins that serve as antigen-presenting elements for the cell-mediated immune system. Class II MHC proteins bind peptide antigens produced by endosomal proteolysis and present them at the cell surface for recognition by $CD4^+$ T cells (1, 2). Newly synthesized class II MHC α and β glycoprotein subunits associate with the invariant chain protein that directs transport to an endosomal compartment (3). Endosomal proteins cleave the invariant chain, leaving a small peptide fragment (known as CLIP) bound in the peptide-binding site. CLIP remains in the binding site until it is exchanged for peptides generated from cell-surface or endocytosed proteins in a process facilitated by the peptide exchange factor HLA-DM (4). Peptide-loaded MHC proteins are transported to the surface for presentation to T cells.

HLA-DM is important for efficient endosomal cellular loading of most class II MHC allotypes, and in its absence MHC-CLIP complexes accumulate at the surface (5–8). Crystal structures for HLA-DM and its murine equivalent H2-DM reveal that the overall fold of the molecule is similar to other class II MHC proteins (9, 10), except that the usual MHC-peptide binding groove is largely closed by rearrangements of the flanking helices. DM is not believed to bind peptides but rather to interact with the MHC protein to facilitate peptide binding and release. DM interaction sites on a class II MHC protein have been mapped recently (11). The mechanism by which DM acts to facilitate peptide exchange is an outstanding problem in the field.

DM catalyzes both peptide release and binding reactions, and exhibits catalytic turnover such that more than one MHC-bound peptide can be exchanged per DM (12–14). Thus, DM can be considered an enzyme that catalyzes the peptide-binding reaction. Within this formalism, the kinetic parameters $K_M \approx 10^{-6}$ M and $k_{cat} \approx 10\ min^{-1}$ have been estimated (15). The presence of catalytic turnover indicates that DM cannot act simply by binding to empty or peptide-loaded class II molecules, which would lead to a stoichiometric but not catalytic process. It has been hypothesized that preferential reaction of DM with rapidly dissociating species would result in editing of the peptide repertoire presented by the cell, such that only stable MHC–peptide complexes are able to get to the surface (16). DM has also been shown to bind to the empty MHC protein and prevent its inactivation and aggregation (13, 17).

The mechanism of DM-mediated peptide exchange catalysis would be clarified by determination of the exact step or steps in the MHC antigen-loading mechanism that are affected. The MHC–peptide-binding mechanism is complex, and currently several kinetic models exist for peptide binding to class II MHCs. Rabinowitz *et al.* (18) demonstrated the production of a "peptide-receptive" form of the empty MHC protein after incubation with a fast-dissociating peptide. The peptide-receptive form was able to bind peptides with rate constants nearly 1,000-fold greater than previously measured (19–21). The peptide-receptive form reverted to a less-active form with a half time of ≈5–10 min (18). From these data, it was concluded that class II MHCs undergo a reversible isomerization between peptide-receptive ($MHC_{receptive}$) and peptide-averse (MHC_{averse}) states with different affinities for peptides. Natarajan *et al.* (22) also observed similar behavior and found that some fraction of reverted MHC molecules cannot bind peptide any further, suggesting a competing irreversible inactivation or denaturation step. Within this mechanistic framework, the same group in another study suggested that DM edits the peptide-MHC repertoire by specifically recognizing unstable conformations of MHC–peptide and actively converting them to peptide-receptive MHC (23). We have proposed an elaboration of this mechanism, in which the productive binding pathway includes a peptide-induced conformational change after the bimolecular step. This mechanism was based on our observations of saturation of the peptide-binding rate with increasing concentrations of peptide or protein (24). These data are consistent with a unimolecular conformational change between transient (MHCpep′) and stable (MHCpep) conformations after the initial binding complex, in addition to the conversion between peptide-receptive and -averse forms of empty MHCs (Scheme 1).

$$\begin{array}{ccccc} MHC_{receptive} & + \text{pep} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} & MHCpep' & \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} & MHCpep \\ {\scriptstyle k_{act}}\uparrow\downarrow{\scriptstyle k_{inact}} & & & & \\ MHC_{averse} & & & & \end{array}$$

The transient species MHCpep′ is similar to the transient peptide-bound intermediate forms Ci (25) and{$\alpha\beta$P*} (26), which have been described in kinetic studies of murine class II MHC proteins (25, 27–30). An alternate model consistent in principle with much of the kinetic data is that the transient intermediate MHCpep′ does not convert directly to MHCpep but passes through an empty MHC state (30). However, for HLA-DR1 binding to CLIP and hemagglutinin (HA) peptides, this model leads to an apparent k_{on} for the MHCpep state of $<100\ M^{-1}s^{-1}$, as compared with the corresponding $k_{on} > 10^4\ M^{-1}s^{-1}$ for the kinetic model in Scheme I (19, 24). The

Abbreviations: HA, hemagglutinin; AMCA, 7-amino-4-methylcoumarin-3-acetic acid; FRET, fluorescence resonance energy transfer.

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latter value is more reasonable for a bimolecular binding step, and we have used this mechanism to interpret the kinetic data for this system.

In this study, we have measured the effect of soluble HLA-DM on each of the individual reaction steps of Scheme I. The only step that seems to be directly affected by HLA-DM is the conversion of the intermediate MHCpep′ to the final peptide-bound MHCpep state, i.e., the unimolecular peptide-induced conformational change (31).

Experimental Procedures

Peptides. Peptides CLIP (Ac-VSRMRMATPL**δ**MQ, where δ is 2,4-diaminobutyric acid), derived from the class II-associated invariant chain, and HA (Ac-PRFV**K**QNTLRLAT), derived from influenza virus HAn (3, 20), were produced and labeled with 7-amino-4-methylcoumarin-3-acetic acid succinimide ester (AMCA-NHS, Pierce) as described (24). Side chains shown in bold indicate the position of side-chain amino groups used for introduction of the fluorescent label.

Production of HLA-DR1 and HLA-DM. The extracellular portion of DR1 was produced by expression of individual subunits in *Escherichia coli* inclusion bodies followed by refolding *in vitro* as described (32). Refolded empty HLA-DR1 protein was recovered by immunoaffinity chromatography by using the conformation-specific mAb LB3.1, and stored at 4°C. These preparations generally contained ≈90% active DR1, as judged by the ability to bind peptide after extended incubation, with 1–5% of the total active DR1 in the peptide-receptive form. For preparation of DR1–peptide complexes, purified empty protein was incubated with a 2–3-fold molar excess of AMCA–peptide at 37°C in the dark for 3 days, and DR1-peptide complexes were isolated in 30–70% yield by size-exclusion chromatography.

The extracellular portion of HLA-DM, carrying M2 and KT3 epitope tags, was expressed in *Drosophila melanogaster*-derived S2 cells and purified by M2 affinity chromatography as described (12, 33), followed by size-exclusion chromatography on a TSK G3000 (TosoHaas, Montgomeryville, PA) with pooled fractions concentrated by centrifugal ultrafiltration (nominal MW cutoff 30,000). Final yield, purity, and catalytic activity were similar to those reported (33).

Fluorescence Spectra and Standard Curves. MHC (Trp) to peptide (AMCA) fluorescence resonance energy transfer (FRET) was measured by using 285-nm excitation and 447-nm emission, which correspond to peaks in the protein fluorescence excitation and AMCA emission spectra, respectively, in a described assay (24). To convert fluorescence intensities to MHC–peptide complex concentrations, standard conversion factors were obtained from fluorescence spectra of purified DR1 (or mixtures of DR1 and DM), purified AMCA-labeled peptides, and purified MHC–peptide complexes at various concentrations as described (24).

Measurement of Initial Rates of Reaction. For measurement of association kinetics as initial rates of reaction, solutions of DR1 or peptide were prepared in CBST (20 mM citrate buffer, pH 5.0/150 mM NaCl/0.01% Tween 20/0.02% sodium azide) at twice the desired final concentration (25 nM–25 μM). Equal volumes of protein solution and peptide solution in CBST were mixed, and the increase in the FRET signal was measured at 15-sec intervals for 10 min. A constant concentration of DR1 (500 nM) was incubated with varying concentrations of peptide or a constant concentration of peptide (100 nM) was incubated with varying concentrations of DR1. Initial rates of association (k_{obs}) were determined from the slope of a linear fit to the early (linear) part of each association curve.

For investigation of the effect of DM on the concentration dependence of the initial rate of peptide binding, a fixed (excess) concentration of DM (1 to 7 μM) was included in the reactions. For investigation of the concentration dependence of DM's effect on the reaction, DM concentration was varied (500 nM–25 μM) relative to fixed total empty DR1 (12 μM) and peptide (100 nM) concentrations. The effect of DM concentration on the reaction was also tested for 0.5 μM DR1 and 800 nM peptide, with similar results. Control experiments showed that DM incubated with peptide alone showed no appreciable FRET signal.

For determination of kinetic parameters k_2 and $K_1 = k_{-1}/k_1$, the initial rates of association (k_{obs}) were plotted against the concentration of either DR1 or peptide. These plots were fit to a quadratic equation describing the initial rate of formation of MHC–peptide complex for a simplified linear two-step reaction, in which the bimolecular first step is in rapid equilibrium, the second unimolecular step does not back-react under initial rate conditions, and the peptide-averse form does not participate (24). The rate constant k_2 is determined by the initial rate at saturating reactant concentration, and the apparent equilibrium constant $K_1 = k_{-1}/k_1$ by the dependence of the initial rate on the reactant concentrations (24). The apparent peptide-receptive fraction $MHC_{receptive}/MHC_{total}$ was determined by comparison of the apparent rate constants k_2 measured in peptide and protein titrations, respectively, as described (24). This fraction is consistent with the active fraction estimated by comparison of the protein and peptide concentrations required for half-maximal initial rate in the presence of a limiting concentration of the other component (24).

Interrupted Initial Rate of Peptide Binding. The rate constant k_{-1}, describing the decay of the intermediate MHCpep′ backward into MHC and pep (Scheme I), was estimated by an interrupted initial rate experiment in which 800 nM HA and 500 nM DR1, in the absence or presence of 500 nM DM, were allowed to react for 5 min, at which time 100-fold excess unlabeled HA was added. A small decrease in fluorescence was observed after the addition. The initial rate of decay occurring over ≈300 sec was fit to an exponential function. No consistent fluorescence changes were observed at longer times.

Off-Rate Determination. For measurement of dissociation kinetics, purified MHC–peptide complexes (100 nM) in the absence or presence of DM (10–200 nM) were incubated with a 100-fold M excess of unlabeled peptide in CSBT at 37°C in the dark. At various times, fluorescence spectra were obtained by the FRET assay, and the decay in fluorescence was fit to an exponential function. A control experiment confirmed that photobleaching was not substantial in this protocol and did not account for the observed decrease in FRET as the reaction progressed.

Inactivation of Empty DR1. The inactivation of empty DR1 was measured as the decrease with time of the peptide-binding activity for $MHC_{receptive}$ freshly dissociated *in situ* from a fast-dissociating complex by following a described approach (18). Samples enriched in the peptide-receptive form were prepared by dissociation of the complex of DR1 with HA_{F308A} (Ac-PRAVKQNTLRLAT), which has a weak affinity (K_d ≈20 μM) and short lifetime ($1/k_{off}$ ≈ 6 min) at 37°C, but which is stable for several days at 4°C (J.A.Z., Z.Z.R., and L.J.S., unpublished observations). DR1–HA_{F308A} complexes were prepared by reacting empty DR1 with a 50-fold M excess of peptide at 37°C for several days, followed by size-exclusion chromatography in the cold to prevent dissociation once excess peptide was removed. The purified DR1–HA_{F308A} complex was diluted to 1 μM and warmed to 37°C to allow generation of empty peptide-receptive DR1. At various times, an equal volume of HA peptide solution (800 nM final) was added, and the initial rate of binding was plotted against the time of incubation. The decay of the initial rate to the value observed for untreated empty DR1 preparations was interpreted as the decay of the initial population of $MHC_{receptive}$ into the equilibrium mixture MHC_{averse} + $MHC_{receptive}$. For eval-

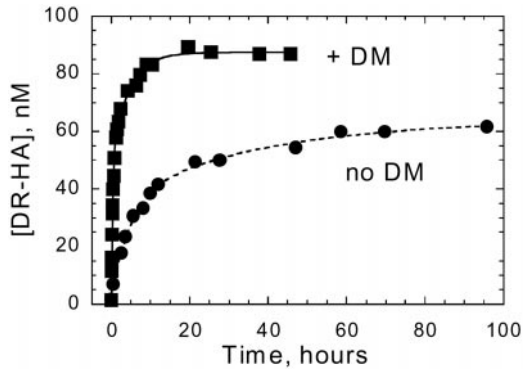

Fig. 1. DM catalyzes peptide binding. Overall reaction profiles for the reaction of DR1 and HA peptide in the presence or absence of DM. AMCA-labeled peptide (100 nM) was added to empty DR1 (100 nM) in the presence (squares) or absence (circles) of 100 nM DM, with peptide association measured by FRET. Values from the double exponential fits (lines) are given in the text. All experiments were performed at 37°C in CBST pH 5.0.

uation of the effect of DM on this reaction, equimolar DM (500 nM) was present with DR1 at all times after isolation of the HA_{F308A} complex.

Results

Overall Reaction Rates (k_{on}, k_{off}). By using a FRET assay to track the association of peptide with the class II MHC protein HLA-DR1 (24), we investigated the effect of DM on the various steps in the mechanism of the peptide-binding reaction. To examine the effect of DM on the overall reaction, we followed HA peptide binding by the FRET assay for several days in the presence and absence of DM (Fig. 1). Two kinetic phases were observed in each case. In the absence of DM, one phase constituting 55% of the reaction with τ = 4 h, and one much slower with τ = 24 h, were observed (Fig. 1). In the presence of DM, biphasic kinetics were also observed with a first phase of τ = 21 min constituting 58% of the reaction, and a second phase with τ = 24 h (Fig. 1). The first phase was accelerated by ≈12-fold, whereas the second phase was unaffected. A similar effect of DM has been reported (23). We have shown that the first kinetic phase corresponds to the conformational change step, and that the second phase corresponds to the conversion of MHC_{averse} to $MHC_{receptive}$ (Scheme I; ref. 24). The simplest explanation of this result is that DM affects the conformational change step but not the activation step, although other explanations are possible. We performed additional experiments to characterize the effect of DM on the individual steps of the overall reaction, as described below.

Peptide Binding and Conformational Change (k_{-1}/k_1, k_2). The overall binding reaction can be broken down into several separate rate processes. In previous work (24), we showed that the initial rate of reaction is concentration-dependent at low reactant concentration, indicating a bimolecular rate-determining reaction step, and concentration-independent at high reactant concentration, indicating a rate-limiting unimolecular step. Under initial rate conditions, where back-reaction is minimal, this behavior is diagnostic of a two-step mechanism with a first fast bimolecular step (described by a quasi-equilibrium constant $K_1 = k_{-1}/k_1$) and a second slower unimolecular step (characterized by the saturating rate constant k_2). We investigated the effect of DM on these processes. Fig. 2*A* shows the dependence of the initial rate of binding on the concentration of HA peptide, in the presence and absence of DM. The saturating rate, which corresponds to k_2[DR1], is enhanced 20-fold by the presence of DM. The peptide concentration required for half-maximal initial rate, which corresponds to k_{-1}/k_1, is unaffected by DM (Fig. 2*A*, right panel). Fig. 2*B* shows the concentration dependence of the initial rate of binding on the DR1 concentration, in the presence or absence of DM (7 μM). The same behavior is observed as in the peptide titration, with a 24-fold enhancement of the saturating rate in the presence of DM and no change in the value of the midpoint k_{-1}/k_1. In this experiment, a fixed concentration of DM was maintained, whereas the DR1 concentration was varied. Similar results were observed when a constant (1:1) DR:DM ratio was used, except that for [DM] <0.3 μM a smaller effect was observed. Extracted rate constants for these processes are given in Table 1. Similar results were obtained for the effect of

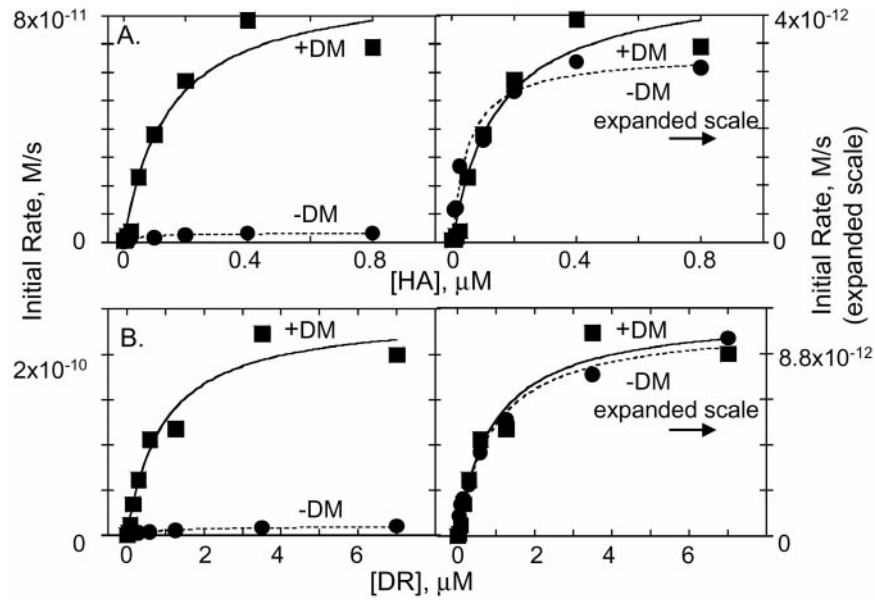

Fig. 2. Concentration dependence of initial rate of binding for DR1 and HA peptide. (*A*) HA peptide concentration dependence, using 500 nM DR1 and varying HA concentrations in the presence (squares) or absence (circles) of 1 μM DM. (*B*) DR1 concentration dependence by using 100 nM HA and varying DR1 concentration, in the presence (squares) or absence (circles) of 7 μM DM. Concentration dependence is shown on a normalized scale (*Right*). Initial rates were determined from linear fits to the early portion of binding traces (24). Kinetic parameters derived from quadratic fits to these data are given in Table 1.

Table 1. Effect of DM on kinetic parameters for the peptide-binding reaction

Experiment	Parameter	HA peptide		CLIP peptide	
		no DM	+ DM	no DM	+ DM
Inactivation of peptide-receptive MHC*	k_{inact}, $\times 10^{-3}$ s^{-1}	1.3	1.1	ND	ND
Peptide-receptive fraction†	$MHC_{receptive/total}$	0.046	0.038	0.035	0.030
Formation of intermediate MHCpep′‡	k_{-1}/k_1, nM	32	63	44	38
Peptide release from MHCpep′§	k_{-1}, $\times 10^{-2}$ s^{-1}	1.5	1.2	ND	ND
Conf. change MHCpep′ → MHCpep¶	k_2, $\times 10^{-3}$ s^{-1}	1.0	24	6.3	45
Peptide dissociation‖	k_{-2}, $\times 10^{-5}$ s^{-1}	1.3	14	4.9	31

ND, not determined.

*Determined by measurement of the rate of inactivation of empty DR1 freshly released from a fast-dissociating complex, as in Fig. 6. Values represent the average of three trials, with average SD of 0.6 $\times 10^{-3}$ s^{-1} (50%), and a range of observed DM effects from 0.8- to 2-fold.

†Calculated from the ratio of apparent rate constants extracted from the saturating initial rate observed in peptide and protein titrations as in Fig. 2. Values represent the average of two trials, with average SD of 0.013 (30%), and a range of observed DM effects from 0.3- to 1.0-fold.

‡Determined from the peptide concentration dependence of the initial rate as described in *Materials and Methods.* Values shown (from Fig. 2) are representative of two experiments with a range of observed DM effects from 0.8- to 3.5-fold.

§Estimated by measurement of the decay of MHCpep′ observed after interruption of a binding reaction with an excess of unlabeled peptide, as in Fig. 5. Values represent the average of three trials with average SD of 0.35 $\times 10^{-2}$ s^{-1} (35%) and a range of DM effects from 0.8- to 1.7-fold.

¶Determined from measurement of the saturating initial rate at high protein concentration. Values shown (from Fig. 2) are representative of two experiments with a range of observed DM effects from 17- to 24-fold for the HA peptide and 6- to 7-fold for the CLIP peptide.

‖Determined by measurement of the dissociation of DR1–peptide complex, as in Fig. 4. Values represent the average of three to five trials, with average deviation of 1.7 $\times 10^{-5}$ s^{-1} (22%) in the absence of DM, 5.8 $\times 10^{-5}$ s^{-1} (60%) in the presence of DM, and a range of DM effects from 9- to 17-fold for the HA peptide and 6- to 10-fold for the CLIP peptide. The overall dissociation reflects mostly k_{-2}.

DM on reaction of DR1 with a different peptide (CLIP), except that the rate enhancement by DM on the rate-determining step was 6.5-fold (Table 1). The concentration dependence for the effect of DM on the initial rates of peptide binding was similar for the two peptides (Fig. 3).

Peptide Release (k_{-2} and k_{-1}). To examine the effect of DM on the overall dissociation rate, we tracked the off-rate of HA and CLIP from the MHC–peptide complex in the absence and presence of DM at pH 5.0, near the pH optimum for DM action¶ (12). The dissociation of HA (100 nM) from DR1 proceeds with k_{off} = 1.3 $\times 10^{-5}$ s^{-1} (τ = 21 ± 3 h; Fig. 4*A*). In the presence of DM (100 nM), HA dissociation is substantially increased, with k_{off} = 1.4 $\times 10^{-4}$ s^{-1} (τ = 2.0 ± 0.2 h; Fig. 4*A*). The dissociation of CLIP from DR1 is faster than for HA, with $k_{off} = 4.9 \times 10^{-5}$ s^{-1} (τ = 5.6 ± 1.9 h) at pH 5.0. Again, in the presence of DM dissociation is faster, with $k_{off} = 3.1 \times 10^{-4}$ s^{-1} (τ = 0.9 ± 0.4 h; Fig. 4*A*). The overall off-rate k_{off} measured in these experiments essentially corresponds to the rate constant k_{-2}, as $k_{-1} >> k_{-2}$ (24). Thus, k_{-2} is substantially affected by DM for both peptides, as was k_2. The rate enhancement by DM increased with increasing concentration (Fig. 4*B*). We were not able to observe a saturating rate enhancement for peptide release. Dissociation rate enhancements of up to 80-fold have been reported in a similar system (14).

Both rate constants k_2 and k_{-2} are affected by DM, whereas the ratio k_{-1}/k_1 is not (Table 1). However, the individual steps k_{-1} and k_1 possibly could be affected in parallel, and we designed an experiment to measure k_{-1} in isolation to evaluate this possibility. According to Scheme I, interrupting the progress of the ongoing binding reaction by the addition of an excess of unlabeled peptide will prevent the formation of more fluorescent complex and should allow observation of the disproportionation of MHCpep′ into MHC + pep (backward) and into MHCpep (forward), with the back-reaction expected to predominate, as $k_{-1} >> k_2$. When a reaction between HA (800 nM) and DR1 (500 nM) was interrupted with a pulse of 100-fold M excess unlabeled HA, we observed a small fluorescence decay with τ = 66 sec (Fig. 5*A*). In the presence of 500 nM DM, the initial rate was increased as previously observed (Fig. 2), but the decay after the addition of unlabeled peptide had τ = 110 sec, similar to the value in the absence of DM (Fig. 5*B*). Thus, under conditions where DM is catalyzing the peptide-binding reaction,

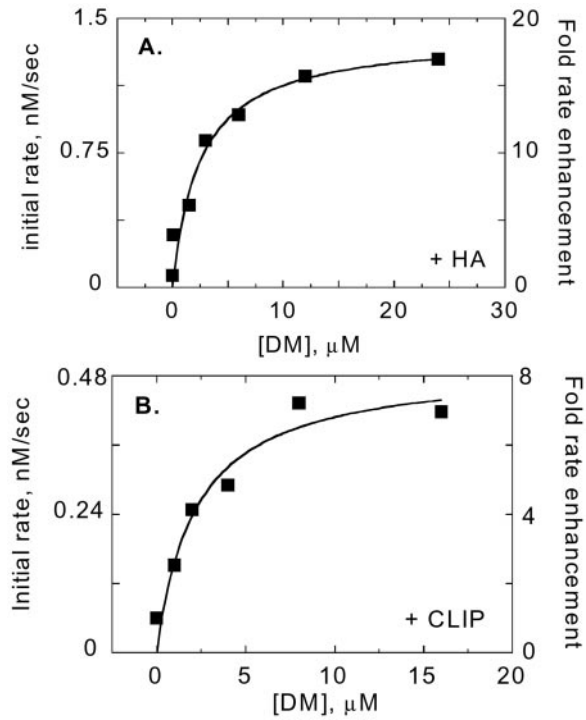

Fig. 3. Dependence of initial rates on DM concentration. (*A*) Initial rate of reaction between 12 μM total empty DR1 and 100 nM HA plotted against the DM concentration present in the reaction. Rate enhancement relative to the uncatalyzed rate is shown on the right axis. (*B*) Similar experiment for the CLIP peptide.

¶At pH 7.0, the dissociation of both peptides is significantly slower.

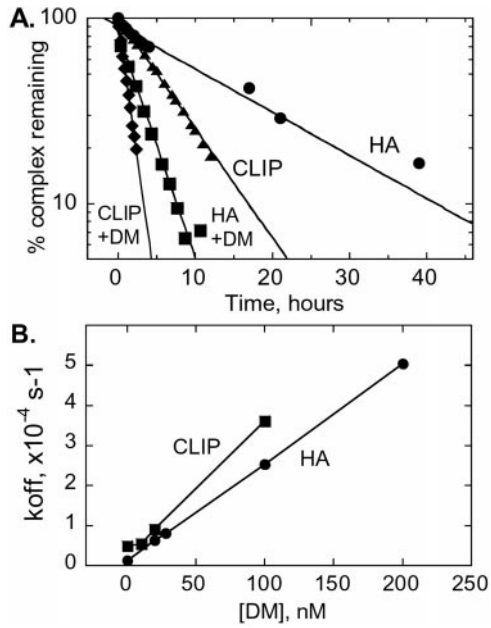

Fig. 4. (*A*) Dissociation assay for DR1–HA and DR1–CLIP complexes. MHC–peptide FRET was measured after addition of excess unlabeled peptide (10 μM) to purified complex (100 nM) in the presence (circles) or absence (squares) of 100 nM DM. Values from single exponential decay fits (lines) are given in Table 1. (*B*) Enhancement of peptide dissociation for DR1–HA (circles) and DR1–CLIP (squares) complexes induced by DM, plotted against the DM concentration.

the decay of MHCpep′ does not seem to be affected by DM. In this experiment, the relative amount of MHCpep′ and MHCpep may be different in the two instances, as in the presence of DM the overall reaction has proceeded further toward the stable MHCpep complex. To control for any effect of a varying MHCpep′:MHCpep ratio, we repeated the interrupted binding experiment, this time adding DM along with the unlabeled peptide. Again, the presence of DM had no effect on the observed fluorescence decay (not shown). Because the presence of DM did not affect the observed $K_1 = k_{-1}/k_1$ in the initial rates experiment, nor did it affect the k_{-1} estimated in this assay (Table 1), we conclude that DM does not substantially affect the rapid binding equilibrium between MHC + pep and MHCpep′.

Peptide-Receptive Fraction. Preparations of peptide-free class II MHC proteins contain both peptide-receptive and -averse forms, with the peptide-averse form usually predominating at equilibrium (≈95%; refs. 18 and 24). Although the peptide-averse protein can participate in the binding reaction after conversion to the peptide-receptive form (Fig. 1), the conversion is slow, and the initial rate experiment measures only the active form. The fraction of peptide-receptive DR1 relative to the total DR1 can be estimated by comparison of the concentrations of MHC and peptide required for half-maximal initial rate of binding, or alternately as the ratio of the saturating rates observed during MHC and peptide titrations (see ref. 24 and *Materials and Methods*). DM does not substantially affect the ratio of peptide-receptive to -averse DR1 (Table 1). Moreover, the presence of DM did not substantially change the relative amplitudes of the rapid (≈60%) and slow phases (≈40%) of the overall peptide-binding reaction (Fig. 1). Finally, preincubation of 500 nM DR1 with 500 nM DM for various times before performing the initial rate assay did not increase the fraction of peptide-receptive DR1 (J.A.Z. and L.J.S., unpublished results). Thus, under our assay conditions, DM does not seem to affect the $MHC_{receptive}$ to MHC_{averse} ratio (Table 1).

Inactivation of DR1 (k_{inact}). Although the peptide-receptive/-averse ratio is not altered by DM, it is possible that both k_{act} and

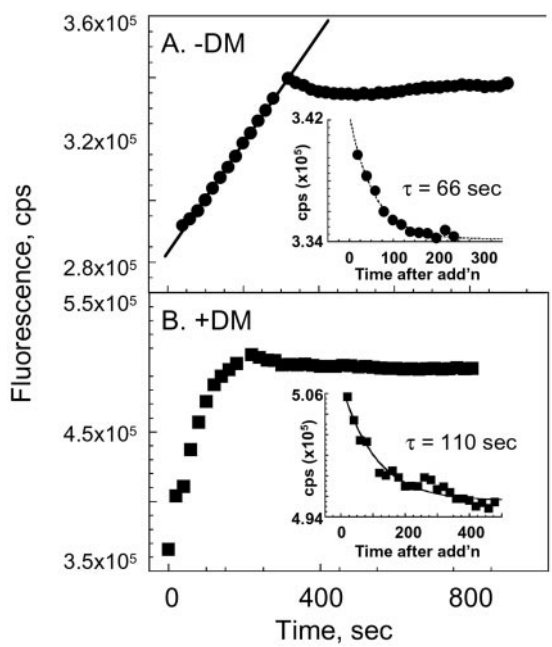

Fig. 5. Determination of k_{-1} by interrupted binding reaction. (*A*) The reaction of 800 nM HA-AMCA and 500 nM DR1 was interrupted by addition of 100-fold excess unlabeled HA. (*B*) Similar experiment performed in the presence of 500 nM DM. *Insets* show the decays fit to a single exponential function (line).

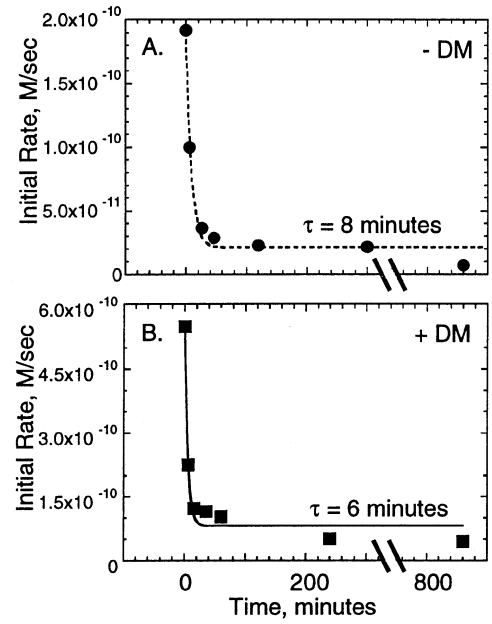

Fig. 6. Determination of inactivation rate constant k_{inact}. The initial rate of peptide binding was assayed at various times after dissociation of the weakly bound complex DR1–HA_{F308A}. (*A*) HA (800 nM) and freshly dissociated DR1 (500 nM) were reacted together at the indicated times after dissociation. The points were fit to a single-exponential decay. (*B*) Similar experiment, with 500 nM DM added to the DR1–HA_{F308A} complex immediately after it was isolated. The points were fit to a single-exponential decay (lines).

k_{inact} could be affected in parallel. To measure the inactivation rate in isolation, we prepared samples enriched in $MHC_{receptive}$ by release *in situ* of a weakly bound peptide, an approach previously used in this system (18). The peptide HA_{F308A} binds weakly to DR1, because key MHC–peptide interactions are abrogated by the alanine substitution (34). We isolated DR1-HA_{F308A}, allowed the peptide to dissociate to form $MHC_{receptive}$, and tracked the inactivation by the initial rate assay. Immediately after dissociation, the initial rate of reaction of HA (800 nM) with freshly dissociated DR1 (500 nM total) is 2×10^{-10} M/sec (Fig. 6*A*). This value is about 10-fold higher than normally observed for empty DR1 that had never bound peptide and consistent with an increased fraction of $MHC_{receptive}$ in the freshly dissociated preparation. With continued incubation, the initial rate decays with $\tau = 8$ min to the value typical of that observed at 37°C for untreated empty DR1, 2×10^{-11} M/s (Fig. 6*A*, Table 1). When freshly dissociated DR1 was incubated in the presence of 500 nM DM, similar behavior was observed, with the initial rate decaying from 5×10^{-10} M/sec to 5×10^{-11} M/sec with $\tau = 6$ min (Fig. 6*B*, Table 1). As before, the rate observed at long times was similar to that observed for untreated empty DR1. Because the ratio k_{act}/k_{inact} is not changed by the presence of DM, and because k_{act} is unaffected as well, we conclude that DM does not affect the conversion between peptide-receptive and -averse DR1 under our conditions.

Discussion

HLA-DM facilitates peptide loading to class II MHC molecules and is required for efficient loading in the physiological cellular context (6). In this report we have investigated the effect of DM on the kinetics of peptide binding for a human class II MHC, HLA-DR1. The data presented here indicate that the primary effect of DM is to catalyze the interconversion of the peptide-bound intermediate MHC-pep′ and the stable form MHCpep. We observed a large rate increase for both forward and reverse reactions in the presence of DM. By contrast, we observed that DM had little or no effect on the conversion between peptide-receptive and -averse DR or on the rapid bimolecular binding reaction between empty DR and free peptide. Thus, the principal effect of DM in facilitating peptide binding and release reactions is through interactions with peptide-bound forms.‖

DM has been reported to stabilize empty class II MHC proteins against inactivation (13, 17, 23). We did not observe a substantial effect of DM on the conversion between "peptide-receptive" and "peptide-averse" forms of the empty protein, although it should be noted that we did not investigate the irreversible inactivation that occurs within a longer time frame. Furthermore, any stabilizing interactions involving the transmembrane and cytoplasmic regions would not be apparent in our system, which includes only the extracellular domains. The stabilization that has been observed previously may be secondary to an effect on the peptide-binding reaction, with DM preventing inactivation indirectly by catalyzing productive binding which competes with inactivation. Whatever the reason for lack of DM chaperone function observed in our experiments, it is clear that such a chaperoning effect does not account for DM's catalysis of the peptide-binding reaction.

How could DM catalyze the transformation between the transient species MHCpep′ and the stable form MHCpep? One possibility is that DM acts as a conventional enzymatic catalyst, by stabilizing the transition state for the conversion between the MHCpep′ and MHCpep conformations. At present, the physical nature of such a transition state remains speculative, as the conformational change itself has not been characterized at high resolution (29, 31). In HLA-DR1, the peptide-induced conformational change is believed to include folding or rearrangement of a region including the prominent kink in the β1 domain helical region (β58–69), along with other changes (31), but no molecular details are available. Conversion between MHCpep′ and MHCpep is slow ($\approx 10^{-3}$–10^{-5} s^{-1}) and could involve formation of nonproductive species, for example complexes carrying peptides bound in an inappropriate register or conformation (27, 35). It is possible that DM modifies the reaction pathway to restrict sampling of such nonproductive species. In any case, DM would have to interact directly with one or more peptide-bound forms to effect the observed catalysis. Interestingly, many of the putative DR–DM interaction sites mapped by mutagenesis (11) cluster near the MHC P1 pocket, a region important in formation of the stable MHC–peptide complex (27, 34, 35). However, the role of particular residues in promoting conversion between the stable and transient MHC–peptide complexes remains to be elucidated.

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‖We cannot exclude the possibility that in a more elaborate kinetic scheme DM would function to promote a step different from the conversion between MHCpep′ and MHCpep, for example the formation of a hypothetical species MHCpep′′ between MHCpep′ and MHCpep.

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