Zinc Induces Dimerization of the Class II Major Histocompatibility Complex Molecule That leads to Cooperative Binding to a Superantigen*

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Dimerization of class II major histocompatibility complex (MHC) plays an important role in the MHC biological function. Mycoplasma arthritidis-derived mitogen (MAM) is a superantigen that can activate large fractions of T cells bearing specific T cell receptor Vβ elements. Here we have used structural, sedimentation, and surface plasmon resonance detection approaches to investigate the molecular interactions between MAM and the class II MHC molecule HLA-DR1 in the context of a hemagglutinin peptide-(306–318) (HA). Our results revealed that zinc ion can efficiently induce the dimerization of the HLA-DR1/HA complex. Because the crystal structure of the MAM/HLA-DR1/hemagglutinin complex in the presence of EDTA is nearly identical to the structure of the complex crystallized in the presence of zinc ion, Zn2+ is evidently not directly involved in the binding between MAM and HLA-DR1. Sedimentation and surface plasmon resonance studies further revealed that MAM binds the HLA-DR1/HA complex with high affinity in a 1:1 stoichiometry, in the absence of Zn2+. However, in the presence of Zn2+, a dimerized MAM/HLA-DR1/HA complex can arise through the Zn2+-induced DR1 dimer. In the presence of Zn2+, cooperative binding of MAM to the DR1 dimer was also observed.

Major histocompatibility complex (MHC)3 molecules can present a wide variety of antigens to T lymphocytes. Two classes, class I and class II MHC, recognized by CD8+ and CD4+ T lymphocytes, respectively, have been discovered. Class II MHC molecules are heterodimers, composed of an α-chain and a β-chain. These αβ heterodimers are highly polymorphic, type I integral membrane proteins, assembled in antigen-presenting cells (APCs) such as B cells and macrophages. Because the first crystal structure of a class II MHC molecule was reported in 1993 (1), crystal structures of more than 20 MHC class II molecules from both human and mouse have been determined. Some of these structures have revealed that the MHC class II heterodimer can self-associate to further form a (αβ)2, double dimer, termed the superdimer (2). In the crystals the superdimer is arranged in such a way that allows simultaneous binding of two T cell receptors (TCRs) and two CD4 molecules (2). Dimerization of class II MHC, either as preformed complexes on the APC membrane or as induced upon TCR engagement, appears consistent with many aspects of class II immunology, including signaling and T cell activation (2–4). However, it remains controversial as to whether the crystallographic superdimer constitutes evidence that the superdimer exists as a physiologically relevant conformation of MHC class II (5–8).

In addition to peptide antigens, class II MHC can present a number of proteins, known as superantigens (SAgs), to T lymphocytes (9, 10). Upon binding to the MHC class II molecules on APCs, SAgs are recognized by TCRs carrying specific Vβ subsets, leading to polyclonal activation of a large portion (up to 20%) of T lymphocytes. SAgs have been hypothesized to play important roles in a number of human diseases, including food poisoning, toxic shock syndrome, and autoimmune diseases such as multiple sclerosis and rheumatoid arthritis (10–14). SAgs are known to bind to the MHC molecules in diverse ways. Currently, two sites have been identified on the MHC, one with a low binding affinity for SAgs and the other with a high affinity (9, 15, 16). The low affinity site (Kd = 10−5 M) is on the MHC α1 domain, whereas the high affinity site (Kd = 10−7 M), which is Zn2+-coordinated, is on the MHC β-chain. Some SAgs have a single MHC-II-binding site; for instance, staphylococcal enterotoxin B and toxic shock syndrome toxin-1 bind only to the MHC α-chain (17, 18). Others, such as SEA, bind to both low and high affinity sites on MHC-II (15, 19). In addition, it has been proposed that dimerization or oligomerization of the MHC antigens by SAgs is critical for T cell activation (20–24).

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The atomic coordinates and structure factors (code 2OJE) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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3 The abbreviations used are: MHC, major histocompatibility complex; MAM, mycoplasma arthritidis-derived mitogen; SPR, surface plasmon resonance; HA, hemagglutinin peptide-(306–318); TCR, T cell receptor; APC, antigen-presenting cell; SAg, superantigen; SEA, staphylococcal enterotoxin A; AUC, analytical ultracentrifugation; SV, sedimentation velocity; HBS, Heps-buffered saline; RU, resonance unit; r.m.s., root-mean-square.
MAM is a potent T cell mitogen produced by *Mycoplasma arthritidis* (25), a natural pathogen of rodents. MAM can induce spontaneous chronic arthritis, which resembles human rheumatoid arthritis, in genetically susceptible strains of rodents. In vitro, administration of MAM can induce MHC class II-dependent T cell activation in a Vβ-restricted fashion, leading to B cell proliferation and differentiation and triggering cytokine expression (26). A structural study revealed that a MAM dimer can cross-link to two MHC molecules via interactions with the MHC α1 domains (27). It was also reported that the superantigenic activity of MAM via MHC class II molecules is Zn²⁺-dependent (28–30).

In this study, we have used structural, sedimentation, and surface plasmon resonance (SPR) detection approaches to investigate the molecular interactions between MAM and class II MHC molecule HLA-DR1, in the context of a hemagglutinin peptide (HA). Unexpectedly, we find that Zn²⁺ can induce the dimerization of a soluble class II MHC HLA-DR1/HA complex in solution. We also find that MAM binds the HLA-DR1/HA complex at a 1:1 stoichiometry, with a high affinity, and independent of zinc ion. Although Zn²⁺ is not directly involved in the binding between MAM and HLA-DR1, Zn²⁺-induced dimerization of the HLA-DR1/HA complex resulted in a dimerized MAM-MHC complex, in which cooperative binding of MAM to the HLA-DR1/HA complex dimer was observed.

**EXPERIMENTAL PROCEDURES**

**Protein Production**—Soluble MAM was expressed as a glutathione S-transferase fusion protein as described previously (27, 31, 32). The HLA-DR1/HA complex was prepared using a refolding protocol as described (27, 32, 33).

To generate biotinylated HLA-DR1 with covalently linked HA peptide for the BIAcore binding study, we made a new construct, using a two-step PCR protocol. Two sets of overlap linkers, the HA peptide-(306–318), and a BirA recognition sequence encoded a linker (QSTRGGASGG) covalently linked to the N terminus of DR1 pET26b vector (Novagen) using the NdeI and HindIII sites. The amplified DNA was then ligated to the pET26b vector (Novagen) using the NdeI and HindIII sites (underlined in the primer sequences). The HA peptide was covalently linked to the N terminus of DR1β through a flexible linker (GGGGSLVGGGSGGGS) similar to that used in our previous study (29). The engineered DR1β also contained a C-terminal amino acid tag encoding a linker (QSTRGGASGG) and a signal sequence (GLNDIFEAQKIEWHE) for attaching biotin with the BirA enzyme (34, 35). Modified HA-DR1β was expressed, solubilized, and refolded with DR1α, using a protocol as described (27, 32, 33), with a modification that no synthetic HA peptide was added. Correctly folded HA/HLA-DR1 complex was purified, using ion exchange chromatography, on a Mono Q column (GE HealthCare); it was biotinylated with the BirA enzyme (Avidity), and size-purified on a Superdex 200 FPLC column (GE HealthCare). The extent of biotinylation was estimated with the EZ biotin quantitation kit (Pierce). Typically, 99% of the protein was biotinylated.

**Crystallization**—Crystals of the MAM/HLA-DR1/HA complex were grown under the conditions described previously (27, 32), except that EDTA was substitute for Zn(OAc)₂. Crystals of the complex were also grown in the absence of both EDTA and Zn²⁺. To fully address whether zinc is involved in the complex formation, we soaked the complex crystals from the no-EDTA and no-Zn²⁺ condition in the mother liquor containing 10 mM CdCl₂. If a protein is known to bind zinc, cadmium ion is frequently used to substitute zinc for the determination of protein structure using the multiple isomorphous replacement method (36, 37). Microseeding was used to produce large crystals for x-ray diffraction data collection.

**X-ray Diffraction Data Collection, Structure Determination, and Refinement**—Crystals of the complex obtained in the absence of zinc ion or in the presence of EDTA are isomorphic with the crystals previously obtained in the absence of zinc ion (27, 32). Diffraction data for crystals of the MAM-DR1 (no-Zn²⁺), MAM-DR1 (EDTA), and MAM-DR1 (Cd²⁺) complexes were collected at 100 K at beamline X25 of the National Synchrotron Light Source at the Brookhaven National Laboratory (Table 1).

The crystal structure of the MAM/HLA-DR1/HA complex at 2.6 Å resolution (27) was served as a starting model for structural refinement, using CNS (38) with a protocol similar to that described previously (27). Noncrystallographic symmetry restraints defined by pairs of individual domain were used throughout the refinement. The refinement statistics are summarized in Table 1.

**Analytical Ultracentrifugation**—Sedimentation velocity (SV) experiments were conducted at 20 °C in a Beckman Optima XL-I analytical ultracentrifuge at a rotor speed of 50,000 rpm. Double-sector cells were loaded with 400 µl of proteins samples and with 410 µl of references solutions, respectively. Unless otherwise specified, the reference solution is Hepes-buffered saline (HBS) containing 10 mM Hepes buffer (pH 7.4), 150 mM NaCl. In certain experiments, 2 mM EDTA or ZnCl₂ was added to both protein and reference solutions. Data were recorded with absorbance detection at wavelengths of 280, 294, and 300 nm for low, moderate, and high concentrations of proteins, respectively. Absorbance profiles were analyzed with the software SEDFIT (39), using a model for continuous sedimentation coefficient distributions c(s) (40). Distributions were calculated with maximum entropy regularization at a predetermined confidence level of 1 S.D.

**AUC Sedimentation Equilibrium**—Sedimentation equilibrium studies were conducted at a temperature of 20 °C and at three or four rotor speeds for each protein or mixture. The individual protein or protein mixture (100 µl) at various concentrations in HBS, with or without EDTA/ZnCl₂, was loaded into an Epon double-sector centerpiece. Reference cells were loaded with 110 µl of reference solution. For the MAM/HLA-DR1/HA complex in the presence of 5 mM EDTA, mixtures of MAM and DR1 at various molar ratios and concentrations (5:2,
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TABLE 1
Data collection, refinement, and model details

| MAM/HLA-DR1/HA complex | No- Zn2+ | EDTA | Cd4+ |
|-------------------------|---------|------|------|
| Cell dimensions (Å)     | 137.3 × 179.2 × 179 | 137.7 × 179.6 × 180.0 | 138.2 × 178.8 × 180.0 |
| Resolution (Å)          | 41.4–2.8 | 40–3.0 | 44.6–2.6 |
| Redundancy (%)          | 4.1 (3.6) | 5.2 (4.5) | 3.8 (3.1) |
| Completeness (%)        | 91.4 (90.6) | 89.8 (91.7) | 96.7 (82.5) |
| Average I/0(n)          | 19.2 (2.9) | 17 (3.2) | 14.8 (1.7) |
| R sym (%)               | 8.6 (44.1) | 12.4 (56.3) | 9.8 (56.4) |

Refinement

| Resolution limits (Å)   | 41.4–2.8 | 40–3.0 | 44.6–2.6 |
| No. of reflections      | 50071 | 40345 | 66025 |
| Rwork (%)               | 27.2 | 21.1 | 23.4 |
| Rfree (%)               | 31.2 | 26.1 | 26.6 |

Non-H-atoms

| Protein                 | 9888 | 9888 | 9888 |
| PO4                    | 4 | 4 | 4 |
| Water                  | 106 | 118 | 126 |
| Average B (Å²)         | 45.1 | 37.5 | 48.0 |

Geometry

| r.m.s.d. bond length (Å) | 0.009 | 0.008 | 0.008 |
| r.m.s.d. bond angle (%)   | 1.5 | 1.4 | 1.4 |

5:3, 15:1, and 1:4, in a real micromolar ratio of concentrations for MAM:HLA-DR1 were used for sedimentation equilibrium analysis with three rotor speeds (20,000, 25,000, and 30,000 rpm). For the complex with 1 mM ZnCl2, MAM and DR1, at 16:1 and 80:5 in a real micromolar ratio of concentrations, were analyzed with four rotor speeds (15,000, 20,000, 25,000, and 30,000 rpm). For DR1 alone, sedimentation equilibrium of DR1 was analyzed at concentrations of 1 and 5 μM with or without 1 mM ZnCl2, with three rotor speeds (18,000, 21,000, and 24,000 rpm). In the Zn2+ titration experiment for DR1 alone, 2 μM DR1 was used with various Zn2+ concentrations (0, 0.001, 0.01, 0.1, 0.3, 0.5, 0.7, and 1 mM). For MAM alone, the equilibrium experiment was conducted at protein concentrations of 2 and 5 μM with or without 1 mM ZnCl2, and with three rotor speeds (20,000, 25,000, and 30,000 rpm).

All equilibrium absorbance profiles were acquired at a 280-nm wavelength. The equilibrium sedimentation data were analyzed using the software SEDPHAT (39). Data analysis was performed by global least squares analysis of data from multiple concentrations and multiple rotor speeds, using conservation of mass constraints (39).

Affinity Measurement Using SPR—Affinity and kinetic analyses of the interactions between MAM and HLA-DR1/HA were determined using a BIACore 3000 SPR instrument (BIACore) at 25 °C. Biotinylated HLA-DR1/HA complex was immobilized (~260 RU) onto a streptavidin (SA) sensor chip (BIACore). The concentrations used for the injected MAM samples ranged from 8 μM to 32 nM, with 2-fold dilutions. A blank surface blocked by biotin was used as the control surface. To minimize nonspecific binding, we carried out all of the binding experiments in 10 mM Hepes buffer containing 1 mM sodium chloride, 3.4 mM EDTA, 0.005% surfactant P20, at a flow rate of 30 μl/min. Pulses of 10 mM NaOH were used to regenerate both surfaces between injections. Association (k_on) and dissociation (k_off) rates, as well as the dissociation constant (K_d), were obtained by global fitting of the SPR data from multiple concentrations to a simple 1:1 Langmuir binding model, using BIAevaluation software version 4.1.

RESULTS

Crystal Structures of the MAM/HLA-DR1/HA Complex in the Presence of EDTA—Previously, we determined the crystal structure of a MAM/HLA-DR1/HA complex (27). No Zn2+ could be detected coordinated into the crystals, even though the crystallization buffer contained 1 mM Zn(OAc)2 (27, 32). However, zinc has been reported to be critical for the biological function of MAM (29–31). To better understand the role of zinc in the biological activity of MAM, we determined the crystal structures of the binary complex under several contrasting conditions. The MAM/HLA-DR1/HA complex was successfully crystallized under conditions, similar to those previously described (32), but in the absence of Zn(OAc)2 and/or in the presence of 2 mM EDTA (see “Experimental Procedures”). These new crystals are isomorphous with those obtained under the Zn2+ condition. We also soaked the complex crystals in the mother liquor with 10 mM CdCl2.

Diffraction data were collected to 2.8, 3.0, and 2.6 Å resolution for the crystals without Zn2+, with EDTA, and with Cd2+, respectively. The crystal structures of these complex crystals were refined to R_factor values of 27.2% (no Zn2+), 21.1% (EDTA), and 23.4% (Cd2+), with R_free values of 31.2, 26.1, and 26.6%, respectively (Table 1). The structures determined from these crystals can be readily superposed with the structure of the complex crystal obtained in the presence of Zn2+ (Fig. 1) (27). The root-mean-square (r.m.s.) deviations in the C-α positions of all amino acids among these structures are within 0.35 Å. For the Cd2+-soaked crystals, no Cd2+ ion could be located in the electron density map. These structures could suggest that the Zn2+ ion is not directly involved in the interaction between MAM and class II MHC molecules.

MAM Binds to the HLA-DR1/HA Complex with a 1:1 Stoichiometry, in the Absence of Zn2+—To further investigate the molecular interaction between MAM and class II MHC molecule, we performed sedimentation velocity analytical ultracentrifugation with individual proteins and mixtures (Fig. 2).
Previously, we demonstrated that MAM exists as a monomer at low protein concentration (≤11 μM) and that it can dimerize at high concentrations (≥25 μM) independent of Zn$^{2+}$ (41). The MAM monomer showed a single peak with a sedimentation coefficient (S) of about 2.1 (Fig. 2A), whereas the S value for a MAM dimer is 3.0 (41). In the absence of Zn$^{2+}$ ion, the HLA-DR1/HA complex showed a large peak, corresponding to a DR1 monomer, at 3.3 S (Fig. 2A). The sedimentation coefficient distribution of a mixture of 6 μM MAM and 6 μM HLA-DR1/HA showed two peaks at 2.1 and 4.0 S, respectively. The peak at 2.1 S corresponds to residue-unbound MAM monomer, whereas the peak at 4.0 S represents some species other than the unbound HLA-DR1/HA complex (at 3.3 S). This result demonstrates an interaction between the two molecules. Indeed, the value of the new peak at 4.0 S is very close to the S value (4.1 S) predicted by hydrodynamic modeling of a MAM/HLA-DR1/HA complex with a 1:1 stoichiometry. Increasing the protein concentrations of MAM (to 30 μM) and HLA-DR1/HA (to 10 μM) resulted in a peak for the sedimentation coefficient at 4.0 S, similar to that for the MAM-DR1 complex at low protein concentrations (Fig. 2). Addition of EDTA to the MAM/HLA-DR1/HA complex did not alter the 4.0 S peak position (Fig. 2B).

These experiments demonstrated that MAM forms a complex with the HLA-DR1/HA complex in a 1:1 stoichiometry. Because addition of EDTA to the MAM/HLA-DR1/HA complex mixture did not change the sedimentation profile, we conclude that formation of the MAM/HLA-DR1/HA complex is not dependent on divalent metal ions such as Zn$^{2+}$.

MAM Binds to the HLA-DR1/HA Complex with High Affinity, in the Absence of Zn$^{2+}$.—By the SPR technique, we measured the affinity and kinetics of MAM binding to the HLA-DR1/HA complex. We produced a C-terminal biotinylated DR1 molecule using a strategy similar to that described by Wu et al. (42) (Fig. 3A). A construct was made to express the β-chain of HLA-DR1 with an HA peptide covalently linked to its N terminus through a linker peptide similar to what we and others described previously (29, 43). In addition, an amino acid tag encoding a signal sequence for attaching biotin with the BirA enzyme was attached to the C terminus of the DR1 β-chain through a peptide linker as described (42).

The biotinylated HLA-DR1/HA complex was immobilized onto an SA sensor chip, for binding and kinetic studies with MAM. To obtain accurate kinetic data and minimize potential mass transport effects, we optimized experimental conditions by immobilizing biotinylated DR1 at a low ligand density (260 RU), and by introducing a high concentration of NaCl in the running buffer (see “Experimental Procedures”). As shown in Fig. 3B, a concentration-dependent binding to the immobilized HLA-DR1/HA complex was observed for MAM. The data were successfully fitted by a 1:1 model, with a low deviation ($\chi^2$) of 0.3. The fast association rate ($k_{a1}$) and slow dissociation rate ($k_{d1}$) were determined to be 2.18 $\times$ 10$^4$ M$^{-1}$ s$^{-1}$, and 1.35 $\times$ 10$^{-2}$ s$^{-1}$, respectively. Thus, a binding affinity (dissociation constant, $K_D$) of 0.6 μM was obtained for the interaction of MAM with HLA-DR1/HA.

The affinity $K_D$ of 0.6 μM is lower than the value (25 nM) that we previously measured using a $^{125}$I-labeled cellular competition assay (29). The discrepancy could result from several factors. One obvious factor that may contribute to this discrepancy is the difference in method. In the SPR method, what we measure is the binding affinity between MAM and the HLA-DR1/HA complex; in the cell-based method, however, the class II MHC molecules are associated with additional co-receptors that could increase the overall binding affinity of MAM to the receptor complexes. In vivo, HLA-DR molecules expressed on human B cells are loaded with a mixture of peptides. We have demonstrated that a significant portion of the interface between MAM and the HLA-
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DR1/HA complex in the co-crystal structure is contributed by the HA peptide at the MHC peptide-binding groove (27). Therefore, differences in the MHC-loaded peptides could result in differences in the binding affinity of HLA-DR for MAM. Our previous functional studies also suggested that the MHC-loaded peptides influence the presentation of MAM to T cells (29). In the SPR analysis, there could be other factors that give rise to a weaker binding affinity. For instance, the high salt BIAcore running buffer could weaken the binding of MAM to the immobilized HLA-DR1 molecule. Analysis of the crystal structure of the MAM/HLA-DR1/HA complex indicated that as many as 19 hydrogen bonds, including several salt bridges, are present at the interface of the complex (27). Although mutagenesis studies have not been performed on the interacting residues, it is a reasonable assumption that electrostatic forces play an important role in the binding between the two molecules. Therefore, high salt concentration could decrease the extent of electrostatic-dependent binding and give rise to a weaker binding affinity. As the salt concentration was increased, various folds of decrease in binding affinities have been observed in an SAg-TCR-Vβ system (44). In the SPR analysis, another factor that could contribute to a weaker binding affinity is ligand immobilization. Although we immobilized the HLA-DR1/HA complex under a very gentle condition via the DR1β C-terminal biotin that is at the opposite end of the MAM-binding site of DR1, partial inactivation of the HLA-DR1/HA complex or steric hindrance to block the access of MAM to the binding site cannot be completely ruled out. It has been reported that immobilization can result in complete loss of binding activity (45, 46).

To address the discrepancy in values obtained for the binding affinity between MAM and HLA-DR1, between our earlier study and the present one, we used an alternative technique, the AUC sedimentation equilibrium method, to provide another determination of the binding affinity. The sedimentation equilibrium is an efficient AUC method for measuring the $K_D$ values for reversible protein-protein interactions (39). Because both interacting species are in solution, any possible artifacts arising from chemical modifications such as ligand immobilization in the SPR analysis could be avoided. When 3 μM HLA-DR1/HA was mixed with 5 μM MAM molecule in the presence of 5 mM EDTA, we were able to model the sedimentation equilibrium data using a noninteracting single species model, with a reasonable r.m.s. deviation of $A_{	ext{exp}}$ but a relatively large global $\chi^2$ of 2.3 (data not shown). By using SETPHAT (39), we obtained an apparent molecular mass of 62.8 kDa, with the molecular mass being the only fitting parameter. This value is significantly larger than the calculated molecular mass for MAM (25.7 kDa) and that for the HLA-DR1/HA complex (44.5 kDa), but it is smaller than the mass for a MAM/HLA-DR1/HA complex (70.2 kDa). This result is in good agreement with the fact that the apparent molecular mass estimated from analysis of sedimentation equilibrium data represents an average molecular mass for the equilibrium mixture, in which MAM (the smaller species) is in slight excess. Therefore, the resulting mass for such a mixture is expected to be smaller than the theoretically calculated mass for the complex. Nevertheless, although it was not a perfect fit, this result indicated an interaction between MAM and the HLA-DR1/HA complex. Indeed, when we analyzed the equilibrium data from multiple concentrations and multiple speeds with a $(A + B \rightleftharpoons AB)$ hetero-association model using SEDPHAT (40), a much better fitting was obtained. Fig. 3C showed a representative fitting of the experimental equilibrium data. A $K_D$ of 0.1 μM was obtained, with a much improved global $\chi^2$ of 1.4 and an r.m.s. deviation of 0.005 OD.

The affinity (0.1 μM) determined using sedimentation equilibrium is higher than that (0.6 μM) determined by BIAcore and is much closer to that (25 nM) determined using the cell-based assay that likely involved other factors for binding with MAM (29). It has been reported that the $K_D$ values obtained by sed-
mentation equilibrium are consistently higher than those from BIAcore (46). It was suggested that the sedimentation equil-
rium values could more accurately reflect the true binding con-
stants, given that the sedimentation method does not involve
chemical coupling of ligands to a solid support (46). In our case,
the $K_d$ value determined by sedimentation is more trustworthy,
because we used near physiological condition (low salt concen-
tration) in the sedimentation, in contrast to the high salt con-
centration used in the BIAcore analysis.

Nevertheless, the affinity determined by both methods could
be regarded as high for the interactions between TCR, SAg, and
MHC (9). Because the running buffers for our SPR and equilib-
rium analyses contained excessive amounts of EDTA, our result
clearly indicated that Zn$^{2+}$ is not directly involved in the high
affinity binding of MAM with class II MHC, the HLA-DR1/HA
complex.

**Zn$^{2+}$ Promotes Dimerization of the MAM/HLA-DR1/HA
Complex**—Although Zn$^{2+}$ does not directly participate in the
binding between MAM and class II MHC, it is possible that
Zn$^{2+}$ may be involved in modulation of the MAM-MHC com-
plex through other mechanisms. For instance, Zn$^{2+}$ has been
found to be important in dimerization of the MAM/HLA-
DR1/HA complex (30); such dimerization of the complex is
critical for the biological function of SAg (29, 31).

To investigate the role of zinc in the MAM-MHC interaction,
we repeated the SV experiment for the MAM/HLA-DR1/HA
complex but in the presence of Zn$^{2+}$. As shown in Fig. 2B,
a mixture of 6 μM MAM and 4 μM HLA-DR1/HA led to a new S
value (6.0 S) for the complex. The S value at 6.0 corresponds to
an MAM/HLA-DR1/HA complex in a 1:1 stoichiometry (Fig. 2
B). This supported our previous conclusion that the highly
saturated under our experimental conditions.

To obtain a completely dimerized complex, we increased the
micromolar ratio to 16:1 for MAM (16 μM) and the HLA-
DR1/HA complex (1 μM). In the presence of Zn$^{2+}$, a new broad
peak emerged at 6.6 S in the velocity sedimentation profile (Fig.
2B). Hydrodynamic modeling of a 2:2 MAM-MHC complex
leads to a predicted S value of 6.5. Our data thus demonstrate
that Zn$^{2+}$ promotes the dimerization of the MAM/HLA-
DR1/HA complex. Indeed, addition of 10 mM EDTA to MAM/
HLA-DR1/HA complex suppressed the effect caused by addition
of 1 mM Zn$^{2+}$, resulting in a peak at 4.0 S that represents a
MAM/HLA-DR1/HA complex in a 1:1 stoichiometry (Fig. 2B).
These results indicated that dimerization of the MAM/HLA-
DR1/HA complex is Zn$^{2+}$-dependent.

**Dimerization of the MAM/HLA-DR1/HA Complex Is Mediated
by a Zn$^{2+}$-dependent HLA-DR1/HA Dimer**—The dimer-
ization of the MAM-MHC complex could be mediated by a
dimer of either MAM or the HLA-DR1 molecule. We have
shown previously that MAM only dimerize at a high protein
concentration and that MAM dimerization is independent of
Zn$^{2+}$ (41). This implies that the dimerized MAM-MHC com-
plex is mediated by a Zn$^{2+}$-dependent HLA-DR1/HA dimer.
However, whether Zn$^{2+}$ can induce dimerization of the HLA-
DR1/HA complex requires experimental confirmation. There-
fore, we performed an SV experiment for the HLA-DR1/HA
complex with Zn$^{2+}$ or both EDTA and Zn$^{2+}$. Addition of 1 mM
Zn$^{2+}$ to the HLA-DR1 solution (15 μM) resulted in a sedimen-
tation coefficient peak at 5.2 S (Fig. 2B), corresponding to a
HLA-DR1 dimer. The addition of 10 mM EDTA to the Zn$^{2+}$-
incubated HLA-DR1 solution resulted in a monomer peak for
the DR1 molecule at 3.3 S (Fig. 2B). To investigate the effective
concentration for dimerization of the HLA-DR1/HA complex
in the presence of Zn$^{2+}$, we lowered the concentration of the
MHC molecule. Our experiment demonstrated that the HLA-
DR1/HA complex forms a dimer in the presence of 1 mM Zn$^{2+}$
at a concentration as low as 0.28 μM (data not shown). Because
of experimental limitations, we could not use a HLA-DR1 con-
centration lower than 0.28 μM. Nevertheless, our data demon-
strate that Zn$^{2+}$ triggers the dimer formation of the HLA-
DR1/HA complex. This implies that the dimerized MAM/
HLA-DR1/HA complex is formed via a Zn$^{2+}$-mediated HLA-
DR1 dimer.

**Determination of the Dissociation Constant of the DR1 Dimer**—To verify our SV results and to determine the dissoci-
association constant of the DR1 dimer, we performed sedimentation
equilibrium studies, either in the presence or in the absence
of Zn$^{2+}$, for the HLA-DR1/HA complex and for MAM by itself
(Fig. 4).

At equivalent protein concentrations (15 μM), the sedimentation
equilibrium profile for MAM with 1 mM Zn$^{2+}$ was very similar
to that for MAM without Zn$^{2+}$ (Fig. 4A). The subtle differences
between the two equilibrium profiles could result from small differences in the effective concentration of MAM
loading in two different cells. Indeed, with a single species
model, modeling of the equilibrium data of MAM with or with-
out Zn$^{2+}$ resulted in similar molecular mass values, with excel-
lent global $\chi^2$ and r.m.s. deviation (Fig. 4A). This supported our
previous conclusion that Zn$^{2+}$ is not involved in the dimeriza-
tion of MAM. In contrast, at a protein concentration of 5 μM, it
is obvious that the sedimentation profile for DR1 in HBS buffer
supplemented with 1 mM ZnCl$_2$ is very different from that for
DR1 in HBS buffer only (Fig. 4B). By using SETPHAT (39), we
could readily model the sedimentation equilibrium data of DR1
in the absence of ZnCl$_2$, using a single species model with a
r.m.s. deviation of 0.004 OD and a global $\chi^2$ of 1.2. An apparent
molecular mass of 46.4 kDa was obtained, with molecular mass
being the only fitting parameter. This value is in good agree-
ment with the theoretically calculated mass of 44.5 kDa for a
HLA-DR1/HA complex, suggesting that the HLA-DR1/HA
complex exists as a monomer in the absence of Zn$^{2+}$. In con-
trast, global modeling of equilibrium data of DR1 in the pres-
ence of 1 mM ZnCl$_2$ resulted in an apparent mass of 79.3 kDa,
with an r.m.s. deviation lower than 0.28 μM. Nevertheless, we
could not use a HLA-DR1 concentration lower than 0.28 μM. Despite
our experimental limitations, we could not use a HLA-DR1 con-
centration lower than 0.28 μM. Nevertheless, our data demon-
strate that Zn$^{2+}$ triggers the dimer formation of the HLA-
DR1/HA complex. This implies that the dimerized MAM/
HLA-DR1/HA complex is formed via a Zn$^{2+}$-mediated HLA-
DR1 dimer.
model was used in SEDPHATE (40), a much better fit was obtained, with an r.m.s. deviation of 0.003 OD and a global χ² of 1.1. A \( K_D \) of 0.33 μM for the DR1 monomer-dimer equilibrium was obtained.

Zinc Titration on the DR1 Dimerization—After determination of the dissociation constant for the DR1 dimerization, it is important to determine the effective Zn²⁺ concentration for DR1 dimerization. Accordingly, we performed a Zn²⁺ titration experiment using the AUC sedimentation equilibrium method (Fig. 4C). The HLA-DR1/HA complex at 2 μM concentration was incubated with 0, 0.001, 0.01, 0.1, 0.3, 0.5, 0.7, or 1.0 mM ZnCl₂, with HBS buffer and was then subjected to the equilibrium experiment. A representative fitting of the experimental data for DR1 in the presence of ZnCl₂, at concentrations of 0.1 and 0.3 mM, is shown in Fig. 4C. Single species modeling of the equilibrium data of DR1 with Zn²⁺ concentrations between 0 and 0.1 mM resulted in molecular masses ranging from 44.8 to 45.7 kDa (Fig. 4C). These results indicate that the HLA-DR1/HA complex exists as a monomer in the absence of or at low concentrations of Zn²⁺. At Zn²⁺ concentrations higher than 0.1 mM, fitting of the sedimentation equilibrium data for DR1 led to molecular masses ranging from 57 to 74 kDa, with the largest mass obtained for DR1 in the presence of 1 mM Zn²⁺. With 2 μM DR1 and 1 mM Zn²⁺, the molecular mass value that we determined here is slightly smaller than the one modeled with a 1:1 interaction between HLA-DR1/HA and MAM. If only 1:1 complex was assumed, global fitting of experimental data using SEDPHATE (39) generated high global reduced χ² (4.0) and high local r.m.s. error of 0.042 values. In contrast, if we used a reversible \( A + B \rightleftharpoons AB \) complex model, with \( A \) referring to DR1, \( A \) representing DR1 dimer, and \( B \) representing MAM, a general analysis of the equilibrium data gave an excellent fit, with a global reduced χ² of 0.8 and local r.m.s. error of 0.004 OD (Fig. 4D). Because of the very weak dimerization of MAM (KD = 1.4 mM) compared with that of DR1 (KD = 0.33 μM), we omitted a MAM dimer from the above model, to reduce the fitting complexity. Nevertheless, inclusion of the MAM dimer in the model equation generated similar results (data not shown).

Under the current model, several dissociation constants could be estimated. For instance, we estimated a \( K_D(AB) \) of 0.33 μM for DR1 homo-dimerization, from the equilibrium data of the DR1-MAM complex in the presence of Zn²⁺. This value is identical with the \( K_D \) value that we determined using DR1 alone, thereby providing a good validation for our modeling method. From this analysis, we estimated a slightly lower binding affinity (\( K_D(AB) \)) for DR1 binding to MAM in the presence of Zn²⁺ (0.14 μM) than the \( K_D \) for the complex in the absence of Zn²⁺ (0.1 μM). In addition, a \( K_D(AB) \) of 47 nM was estimated for MAM (B) binding to the DR1 dimer (AA). The affinity for
MAM binding to a DR1 dimer (47 nM) is about 3-fold higher than that for MAM binding to a DR1 monomer (140 nM). The relatively small magnitude of this difference could imply that DR1 dimerization slightly enhances the binding affinity for the first MAM molecule. In contrast, a \( K_d = (A40B) \) of 5.0 \( \mu \text{M} \) was estimated for the binding of a second MAM molecule to the MAM-(DR1)\(_2\) complex in the presence of Zn\(^{2+}\). This value of 5.0 \( \mu \text{M} \) is >100-fold higher than that (47 nM) for the binding of the first MAM molecule to the DR1 dimer. The 100-fold decrease in affinity strongly suggests that cooperative binding of MAM to the HLA-DR1/HA complex occurs upon addition of Zn\(^{2+}\). Thus, in the presence of Zn\(^{2+}\), binding of the first MAM molecule to the DR1 dimer may reduce the binding affinity of the second MAM molecule to the pre-formed MAM-(DR1)\(_2\) complex.

**DISCUSSION**

Receptor oligomerization is frequently observed in biological systems, as a mechanism by which the avidity of receptor-ligand interactions can be increased, while at the same time active intracellular signal transduction complexes are formed (47–49). In the immune system, clusters of macromolecules, termed the immunological synapse, have been observed during T cell activation, indicating that multimerization of receptors could be critical for the biological functions of these molecules (50, 51). X-ray crystallographic analyses have indicated that class II MHC can further form a (\( \alpha \beta \))\(_2\) superdimer of the MHC heterodimer (2). However, dimerization of soluble class II MHC molecules has not been observed in solution, even under the extreme acidic conditions used for crystallization (1). Although several cell-based studies have suggested that the class II MHC superdimer exists on the surface of living cells (5–7), some of the evidence to support this assertion could contribute to biochemical artifacts (8). In addition, several mutagenesis studies indicated that most of the substitutions made at the MHC superdimer interface did not affect T cell activation (49, 52–55). Although a few of the mutations seemed to modulate T cell activation, it was not clear whether those latter mutations abolish the actual formation of the MHC superdimer or whether they induce other effects, such as reduction of cell surface expression of class II MHC and/or abrogation of binding of accessory molecules (49).

In this study, we have investigated the molecular interaction between MAM and class II MHC. Our results indicated that Zn\(^{2+}\) neither participates directly in the binding between MAM and class II MHC molecules nor induces the dimerization of MAM in solution. Velocity sedimentation experiments revealed that formation of the complex between MAM and HLA-DR1/HA does not require the Zn\(^{2+}\) ion; after addition of chelator (EDTA), the same peak, representing the complex, was seen. Consistently, no Zn\(^{2+}\) ion was detected in the crystal structures of the MAM/HLA-DR1/HA complexes under various conditions, with EDTA, Cd\(^{2+}\), or Zn\(^{2+}\) (this study and see Refs. 27 and 32). Binding studies using the AUC and SPR techniques further confirmed that the Zn\(^{2+}\) ion is not directly involved in the binding between MAM and the HLA-DR1/HA complex, given that MAM binds the HLA-DR1/HA complex with high affinity in the absence of the Zn\(^{2+}\) ion.

Although Zn\(^{2+}\) does not directly participate in the interaction between MAM and class II MHC, we unexpectedly found that the Zn\(^{2+}\) ion triggers dimerization of the HLA-DR1/HA complex. In the presence of Zn\(^{2+}\), the HLA-DR1/HA complex forms a dimer of the heterodimer, with a dissociation constant of 0.33 \( \mu \text{M} \). The dimerized HLA-DR1 binds the first MAM molecule with a slightly higher affinity (3-fold) than does the monomer HLA-DR1. Furthermore, our results showed that binding of the first MAM molecule to the dimerized MHC complex greatly reduced the binding affinity of the DR1 dimer for the second MAM molecule. Our data strongly suggest that the addition of Zn\(^{2+}\) induces cooperative binding of MAM to the HLA-DR1/HA complex. Evidently, Zn\(^{2+}\) triggers the dimerization of the MAM/HLA-DR1/HA complex via a Zn\(^{2+}\)-mediated HLA-DR1/HA dimer. It is currently not clear whether the HLA-DR superdimer described by Brown et al. (1) represents the structure of the Zn\(^{2+}\)-induced DR1 dimer, because no Zn\(^{2+}\) was found in those structures. Structure determination of the DR1 molecule in the presence of Zn\(^{2+}\), and structure-based mutagenesis studies, will be required to investigate whether the Zn\(^{2+}\)-induced dimerization of HLA-DR1 plays any roles in the biological function of the class II MHC molecule. Nonetheless, several studies, including ours, clearly demonstrated that dimerization or even oligomerization of MHC class II molecules at the cell surface is absolutely required for inflammatory cytokine gene expression in human monocytes. What makes our observation unique is the ability of the HLA-DR dimer to bind two different MAM molecules. Such recognition of MAM-bound HLA-DR by T cells will significantly increase the ability of these complexes to trigger TCR microclustering at the immunological synapse where signaling is initiated and sustained leading to optimal T cell activation (56).

Previously, we failed to find Zn\(^{2+}\) in the crystal structure of the MAM/HLA-DR1/HA complex when the crystals were grown in the presence of 1 mM Zn\(^{2+}\) (27), although our new studies have shown that the HLA-DR1/HA complex forms homodimer in the presence of Zn\(^{2+}\). It is not uncommon that Zn\(^{2+}\) ion was not detected in the solved structures for protein crystals grown in or soaked with Zn\(^{2+}\). We searched the protein data base and found that more than 20 structures involving HLA-DR molecules have been determined. Only four of these crystals, namely the SEA\(_{p227A}\)-DR1 complex (Protein Data Bank 1I05 (57)), the SpecC-DR2a complex (Protein Data Bank 1I05 1HQR (58)), the SEH-DR1 complex (Protein Data Bank 1HXY (59)), and the MAM-DR1 complex (Protein Data Bank 1R5I (27)), were grown or soaked in various concentrations of Zn\(^{2+}\). SpecC and SEH are known to require Zn\(^{2+}\) for binding to class II MHC. Indeed, one Zn\(^{2+}\) ion was found at the interface between the SAg and the DR molecule in each of the complexes. In contrast, although the crystal of the SEA\(_{p227A}\)/HLA-DR1/HA complex was grown in the presence of 1 mM ZnCl\(_2\), no zinc was found in that crystal structure (57).

We have demonstrated that a dimer of the MAM/HLA-DR1/HA complex can form via a Zn\(^{2+}\)-induced HLA-DR1 dimer. It is intriguing that the MAM-DR1 complex forms a dimerized complex through a MAM homodimer in the crystal structures (27). In fact, all other three complexes mentioned above (SpecC-DR2a, SEH-DR1, and SEA\(_{p227A}\)-DR1 complexes)
are monomeric in the crystal structures, even though Zn²⁺ was included in the crystallization buffer (57–59). The absence of a Zn²⁺-coordinated HLA-DR dimer in the crystal structures could indicate that interactions of class II MHC with the SAg suppress the Zn²⁺-induced dimerization of the HLA-DR molecule. Alternatively, crystal-packing forces could play a role in elimination of the DR1 dimer in the crystal.

Nevertheless, our results clearly indicate that a dimer of the class II MHC heterodimer is formed in the presence of zinc. In fact, zinc is known to be essential for the highly proliferating cells in the immune system (60). Notably, it has been reported that zinc can induce polyclonal activation of human B lymphocytes from blood, spleen, and lymph nodes in vitro (61). In serum, zinc occurs at concentrations in the range of 10–120 μM (62–65). A much higher concentration of Zn²⁺ (0.3 mM) was required in this study to allow detection of the effect of Zn²⁺ on the oligomeric state of HLA-DR1. The possible functional significance of dimers of class II MHC heterodimers has been extensively discussed (1, 2, 5, 6, 49, 53, 54, 66). In addition, it was recently reported that zinc homeostasis plays significant roles in the maturation of dendritic cells and regulation of surface expression of MHC class II (67). Addition of a zinc chelator was found to increase the surface expression of MHC class II on dendritic cells, leading to enhanced CD4+ T cell activation (67). Although speculative at this point, zinc-induced dimerization of class II MHC could be involved in the regulation of surface expression of MHC II. By analogy to the zinc-induced dimerization of the inhibitory receptors of human natural killer cells that is critical for their inhibitory function (68), it is possible that Zn²⁺-induced dimerization of MHC II could provide inhibitory signals for resting APCs. Thus, our results suggest that Zn²⁺-induced class II MHC dimerization is important for the biological function of the MHC molecules.

Our results are also consistent with the immunological finding that metal ions, Zn²⁺ in particular, play an important role in the biological function of certain SAgs (9, 15, 19, 29, 30, 58). Zinc may regulate the SAg activity in several ways (41). In addition to its direct participation in the interactions between SAgs and their host receptors (9, 58, 59), zinc, as our results have indicated, induces dimerization of host receptors such as class II MHC molecules, thereby modulating the activity of SAgs.

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