Reports

Energy Transfer Between Two Peptides Bound to One MHC Class II Molecule

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The 17–amino acid peptide from chicken ovalbumin, Ova(323-339), was labeled at the amino terminus with fluorescein [FOva(323-339)] and near the carboxyl terminus with Texas Red [AcOva(323-338)KTR]. Fluorescence spectroscopy was carried out on resolved electrophoretic bands on nonreducing polyacrylamide gels derived from incubation mixtures containing major histocompatibility complex (MHC) class II molecules IA^d and the FOva(323-339)- and AcOva(323-338)KTR-labeled peptides. Energy transfer between fluorescein and Texas Red was observed in the "floppy" αβ heterodimer band, but not in the "compact" αβ heterodimer band. Energy transfer was detected between the truncated peptides FOva(323-328)$CONH_2$ and AcOva(331-338)KTR in both the compact αβ and floppy αβ gel bands. The energy-transfer data suggest that the two binding sites of floppy αβ arise from splitting apart a putative large, single binding site region in compact αβ.

CLASS II MOLECULES OF THE MAJOR histocompatibility complex (MHC) are αβ heterodimeric membrane-bound glycoproteins (*1*). These molecules bind antigenic peptides and "present" them to T cell receptors on $CD4^+$ cells. It is widely assumed that the stoichiometry of peptide:MHC binding is 1:1 (*2*). We report that two fluorescently labeled peptides can bind to a single class II MHC molecule. This result is significant in that the observed pattern of two-peptide energy transfer fluorescent spectra provides a qualitative view of the structures of two previously described forms of class II MHC molecules, "floppy" and "compact" (*3*).

A simple line of reasoning led us to consider two-peptide binding to class II MHC molecules. Each of the αβ heterodimers has two long-lived conformations as shown on electrophoresis gels. The more mobile band is called compact, and the less mobile band is called floppy. Floppy αβ is an intermediate on the pathway for the dissociation of compact αβ into separate α and β chains under the conditions of the experiments (*3*). The floppy structure might then be one in which the peptide-binding regions are split apart. Since separated α and β chains from the class II MHC molecules IA^d and IE^k are known to bind the same peptides as do the αβ heterodimers, floppy αβ might then be able to bind two peptides (*4*). This view is consistent with a study of the pattern of single peptide binding to compact αβ, floppy αβ, and to separate chains (α and β) (*5*). This pattern is similar for α, β, and floppy αβ, but is distinct for compact αβ. The present study of energy transfer between two fluorescent peptides bound to a single class II MHC molecule provides strong additional evidence that the floppy αβ structure is derived from the compact αβ structure by splitting apart the binding site region.

We report investigations of truncated peptides in addition to the full-length 17 amino acid peptides based on the chicken ovalbumin peptide Ova(323-339) that are known to bind to IA^d. In the peptide sequence of Ova(323-339), ISQAVHAA-HAEINEAGKR (*6*), the pair of alanines were cut out so as to retain the histidines. In order to avoid possible charge effects due to the remaining amino and carboxyl termini, Ova(323-328)$CONH_2$ and AcOva(323-339) were synthesized (*7*). These truncated peptides bind to IA^d, but the amount of bound truncated peptide is five times less than the amount of bound full-length peptide (*5*). The binding and dissociation of full-length and truncated peptides are independent of the fluorophore and its position (amino or carboxyl terminus) (*8*).

In Fig. 1 a densitometer scan of the silver-stained nonreducing SDS-polyacrylamide gel (12.5%) shows the positions of the four protein bands. Binding of fluorophore-labeled peptides was monitored by a fluorescence scan (520 nm). The fluorescent lane of the gel was obtained by electrophoresis of incubation mixtures containing IA^d and equimolar concentrations of fluorescein (F)- and Texas Red (TR)–labeled Ova(323-339) peptide (solid line). In comparison, a fluorescence scan of a lane containing the same components as above, except that FOva(323-339) was replaced by a fluoresceinated fragment of herpes simplex virus (HSV) glycoprotein D [FHSV(8-23)], shows no binding of FHSV(8-23) to IA^d (dashed line). Emission spectra taken at the positions corresponding to the two heterodimers, floppy and compact, serve as background spectra.

Energy transfer fluorescence spectroscopy

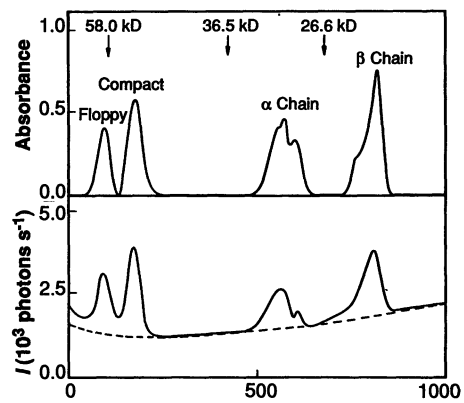

Fig. 1. Densitometer and fluorescence scans of an SDS-polyacrylamide gel (12.5%). Detergent-solubilized IA^d (*16*) was incubated for 1 day with an excess of fluorophore-labeled peptide at 37°C. The samples (20 μl) containing 1 μM IA^d, 0.1 mM fluorescent Ova peptide, 1 mM DM, 10 mM phosphate buffer, pH 7, and 150 mM NaCl, were applied to a SDS–polyacrylamide gel electrophoresis (*17*) under nonreducing and nonboiling conditions. The lanes were analyzed for peptide binding to resolved protein bands with a scanning laser microscope as described previously (*4*). The fluoresceinated peptides were excited by an argon ion laser (488 nm) in epi-illumination. The emission was recorded by a photomultiplier at 520 nm, and the lanes were scanned on a micromover translation stage. Fluorescence emission spectra (480 to 700 nm) were collected at each position in the lane. After silver staining (*18*) the protein bands were monitored with an LKB UltroScan XL laser scanner. (**A**) The background-corrected scan. The four bands correspond to the floppy and compact conformations of the heterodimer, as well as to the α and β chains, which migrate with an apparent molecular weight of 64, 55, 33, and 27.5 kD, respectively. All IA^d samples show the same protein pattern. In this "native" gel the molecular weight markers (58.0, 36.5, and 26.5 kD) run at positions that are different than in a denaturing gel. (**B**) The corresponding fluorescence scan of a lane containing IA^d, FOva(323-339), and Ova(323-338)KTR. The fluorescence scan (dashed line) of the lane containing IA^d, FHSV(8-23), and Ova(323-338)KTR was taken as background for the difference emission spectra shown in Fig. 2. The data in (B) are direct, and not corrected or scaled.

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on the αβ heterodimer bands was carried out on various gel lanes as shown in Fig. 2. All spectra were compared with a spectrum on a lane containing IA^d and only fluorescein-labeled peptide. An increase of fluorescence intensity in the region of the emission maximum of Texas Red (605 nm) corresponds to energy transfer between the fluorescein and Texas Red fluorophores. It is known that energy transfer efficiency is strongly dependent on the distance between the two dyes in the range from 1 to 8 nm (*9*). Two modes of two-peptide binding were found—two full-length peptides can bind to floppy αβ (Fig. 2A), and two truncated peptides can bind to compact αβ (Fig. 2D). Two truncated peptides can also bind to floppy αβ (Fig. 2C). No simultaneous binding of the two full-length peptides to the compact αβ conformation was detected (Fig. 2B).

At present it is not possible to deconvolute the observed energy transfer into the intrinsic efficiency of transfer, the interfluorophore distances, and the populations of the various molecular species. The transfer must arise from pairs of fluorophores on the same MHC molecule, otherwise the transfer probability is much too small. Also, it is likely that a relatively large fraction of the fluorescent MHC molecules has two fluorophores bound. Thus, the transfer emission intensities seen in Fig. 2 are of the order of 10% of the fluorescein emission intensity. If the intrinsic transfer efficiency were 100%, this would mean that of the order of 10% of the molecules with a bound fluorescein peptide also contain a bound Texas Red peptide. Since the transfer efficiency is doubtlessly less than 100%, more than 10% of the molecules with a bound fluorescein peptide also have a bound Texas Red peptide. The fluorescein fluorescence intensity itself corresponds to ~1% of the IA^d molecules, (*7*) and is typical of such preparations (*2, 8*). It is highly unlikely that the strong observed transfers can be due to minute contaminations of α_2 and β_2 homodimers. Also, no bands at the previously described positions of the α_2 and β_2 homodimers were seen (*10*).

We note that in Fig. 2 the Texas Red emission in the case of the truncated peptides bound to floppy shows a substantial blue shift, as well as possible multiple emission wavelength maxima. The blue shift may arise from Texas Red molecules in hydrophobic sites, since blue shifts of the order of 10 nm are found for similar fluorophores in going from water to methanol solvents. Multiple wavelength maxima can arise from Texas Red–labeled peptides in more than one site.

Bands on electrophoresis gels corresponding to floppy and compact have been found for IA^d, IE^k (*3*) and IA^k, IA^s. Thus these two conformations appear to be a general feature of class II MHC molecules. These two conformations cannot be purely gel or detergent artifacts, since the transition from compact to floppy can be induced in lipid bilayers, as well as in detergents (*3*). The floppy conformation is an intermediate in the dissociation of compact into separate α and β chains. Floppy is probably also a folding intermediate in the reassembly of α and β chains to form compact. Based on the results given in the present work, the floppy conformation should provide a mechanism for peptide exchange in αβ heterodimers, since the floppy conformation can bind two full-length peptides. This possibility is particularly significant since peptide-free αβ heterodimers and α and β chains may be unstable in the absence of bound peptide. A possible role of two-peptide intermediates in the reactions of αβ heterodimers and antigenic peptides has been discussed recently (*8, 11*).

The terms compact and floppy were introduced to characterize the mobilities of these long-lived protein conformations on electrophoresis gels, and not necessarily to describe any dynamic properties the αβ heterodimers may have (*3*). The terms may be compared to R ("relaxed") and T ("taut") that are used to describe two conformational states of hemoglobin (*12*). Based on the structure of class II molecules proposed by Brown *et al.* (*13*) and our observations of two-peptide binding, we believe that the binding site of the floppy αβ heterodimer is one in which the two α-helical domains of the binding site are moved apart, one carrying one-half of the β sheet, and the other carrying the other half of the β sheet. The remaining stem of the molecule presumably remains intact.

It is premature to speculate on the biological relevance of two-peptide binding to the two conformations of the αβ heterodimer. However, we note that with respect to the compact structure, and the binding of trun-

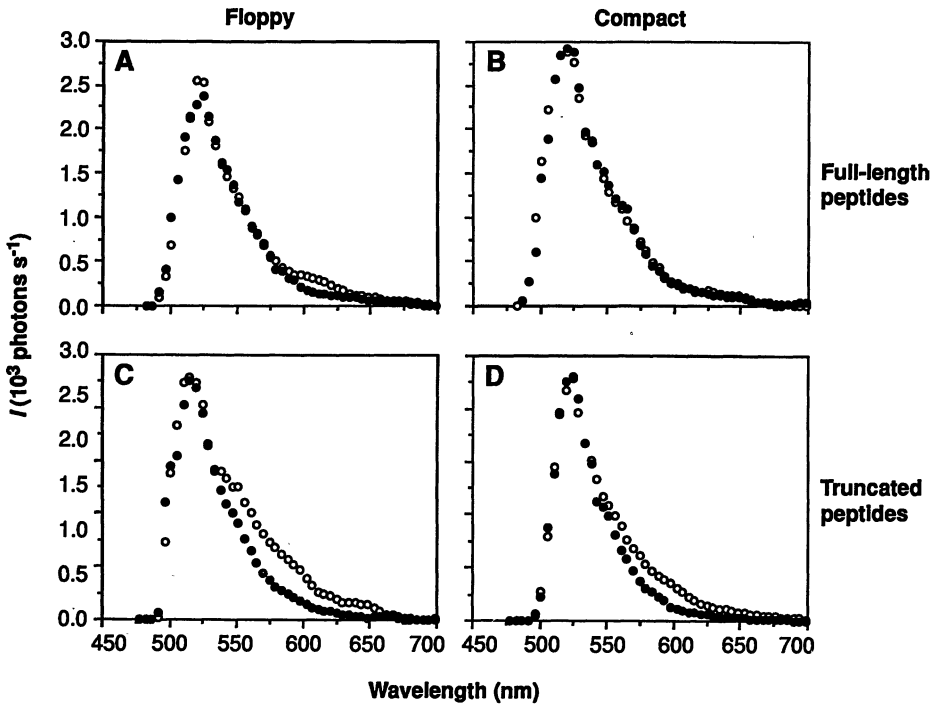

Fig. 2. Fluorescence spectra of resolved floppy and compact αβ heterodimer bands in lanes containing fluorescent peptides. Emission spectra of the full-length peptides, [FOva(323-339) and AcOva(323-338)KTR], are shown in (**A**) and (**B**), and those of the truncated peptides, [FOva(323-328)$CONH_2$ and AcOva(331-338)KTR], in (**C**) and (**D**). The emission spectra obtained with 488-nm excitation were corrected with the use of background spectra recorded from a position between the floppy and compact bands in order to exclude emission due to nonspecific association. Alternatively, background spectra of the two αβ heterodimer bands in a lane containing IA^d, FHSV(8-23), and AcOva(323-338)KTR were recorded (dashed line in Fig. 1). Typical resulting difference spectra of the floppy and compact heterodimer bands are shown in (A) and (C) and (B) and (D), respectively. Spectra on lanes containing peptides labeled with fluorescein and Texas Red are shown in open circles. Parallel spectra on lanes containing only fluorescein-labeled peptide are superposed and scaled to have the same intensity at 520 nm (closed circles). Only ~1% of the MHC molecules have a bound fluorescent peptide, and where energy transfer is observed, it represents ~0.1% of all the IA^d molecules. All of the spectra shown were obtained from single scans on a single gel. Scans at other positions on the protein band in the same lane yielded similar results, as did also scans on five additional gels.

cated peptides, two-peptide binding should give rise to combinatorial antigens. That is, although short peptides are probably not immunogenic because of homology with self short peptides (*14*), two short peptides bound to a single class II MHC molecule should be fully immunogenic from the point of view of potential T cell–specific recognition. With respect to the binding of full-length peptides, Bhayani and Paterson have reported the augmentation of an IE^k-restricted cytochrome c peptide–specific response of T cells by a bystander nonstimulatory peptide (*15*). On the basis of these results these authors suggested that two peptides might bind to a single IE^k molecule.

Irrespective of the immunological significance of two-peptide binding to class II MHC αβ heterodimers, this possible stoichiometry must be recognized in any kinetic analysis of the reactions of MHC molecules with peptides.

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5. K. Dornmair, B. Clark, H. M. McConnell, unpublished results. Our data on the binding of peptides to IA^d for different truncated peptides are only roughly similar to those of A. Sette *et al.* [*Nature* **328**, 395 (1987)]. This difference is not unexpected since different experiments are involved and none of the results correspond to thermodynamic equilibrium. Also, all of the truncated peptides used in the present work are different since the carboxyl and amino termini are blocked.
6. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K. Lys; K, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y. Tyr.
7. Peptide synthesis: The solid-phase technique was used to synthesize Ova(323-339)COOH, Ova(323-328)$CONH_2$, AcOva(323-338)K, AcOva(331-338)K and HSV(8-23) on a Milligen 9050 peptide synthesizer. After purification by high-performance liquid chromatography (HPLC) with a preparative Vydac C_{18} reversed-phase column (Separation Group, Hesperia, CA), peptides were labeled with fluorescein isothiocyanate or Texas Red sulfonyl chloride (Molecular Probes) at the amino terminus or at the ε-amino group of the carboxyl-terminal lysine, respectively. The reaction was carried out in dimethylsulfoxide or *N,N*-dimethylformanide in the presence of diisopropylethylamine or 2,6-collidine and monitored by HPLC with an analytical Vydac C_{18} reversed-phase column. After completion, the labeled product was purified with a preparative Vydac C_{18} reversed-phase column. Peptides were characterized by mass spectroscopy.
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16. Protein isolation: IA^d was isolated and purified as previously described (*4*). The cell line A20.1.11 was used as a source for IA^d. The non-ionic detergent-solubilized membrane proteins were purified by lentil lectin and MKD6-MAb affinity chromatography. After dialysis the final buffer contained 1 mM dodecyl-β-D-maltoside (DM, Calbiochem), 10 mM phosphate buffer, pH 7.0, 150 mM NaCl, and 0.02% NaN_3. The preparation was characterized by protein determination and SDS–polyacrylamide gel electrophoresis (12.5% acrylamide).
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Periodic Hot-Spot Distribution on Io

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The Jovian satellite Io has active volcanic hot spots. The point-to-point correlation of the hot-spot distribution indicates that the hot spots preferentially make chains. The arrangement of the chains is periodic with a typical spacing of 120 kilometers. The chains exhibit concordant trends with stresses imposed by the tidal deflection of the lithosphere, suggesting that the hot spots are formed along fissures in the lithosphere. The typical spacing may be controlled by lithosphere thickness.

THERE HAS BEEN A RAPID ADVANCE during the past two decades in our understanding of planetary volcanism (*1*), one of the key phenomena needed to develop models of the internal structure and evolution of terrestrial planets. In this report, I discuss a way to investigate the internal structure by looking into the spatial distribution of volcanoes.

Since the Voyager missions discovered active volcanism on Io, many tens of hot spots have been identified from imaging data (*2, 3*) (Fig. 1). Io is about the size of Earth's moon ($R_{Io} \cong 1820$ km). However, infrared observations show that the satellite discharges as much energy as Earth, and most of the energy emanates from the hot spots (*3, 4*). Jupiter's powerful tides bend and flex the interior of Io as it moves in an eccentric orbit, resulting in frictional heating in the interior and active volcanism (*5*). The tides tend to deform the satellite into a triaxial ellipsoid. The tides with the ellipsoidal symmetry are the engine of Io's tectonics; therefore, the symmetry of surface tectonic features would suggest the dynamic processes going on at depth.

I examined Io's hot-spot distribution using the self-correlation function of discrete points. Self-correlation is defined as

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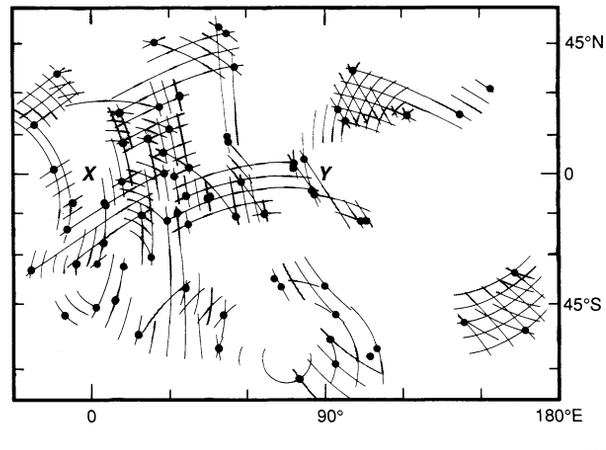

Fig. 1. Mercator projection of hot spots in the eastern hemisphere of Io where Voyager images have the best resolution (*3*). Closed circle, hot spot; thin line, arc on a small or great circle; thick line, stress trajectory induced by the migration of the tidal bulges (*16*). The *X* and *Y* axes point to Jupiter and the trailing direction of the orbital motion, respectively. The *Z* axis points to Iographic north. Three groups of small circles are drawn with the poles 10°N, 133°E; 57°S, 74°E; and 33°S, 7°E. The angular distance between the small circles is constant for each group.