Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 4134–4138, June 1990
Chemistry

Refolding and reassembly of separate α and β chains of class II molecules of the major histocompatibility complex leads to increased peptide-binding capacity

(protein folding/protein structure/antigen presentation/protein dynamics)

KLAUS DORNMAIR AND HARDEN M. MCCONNELL

Stauffer Laboratory of Physical Chemistry, Stanford University, Stanford, CA 94305

Contributed by Harden M. McConnell, March 12, 1990

ABSTRACT **Class II molecules of the major histocompatibility complex present antigenic peptides to helper T cells. These are heterodimeric glycoproteins consisting of one α and one β chain. Two different α/β heterodimeric conformations as well as the separate α and β chains bind specific peptides. The α chain is thought to have one and the β chain two intramolecular disulfide bonds. In the present study we have reduced these disulfide bonds in the murine major histocompatibility complex molecule I-A^d, which led to the release of bound peptides from all conformations and to unfolding of the separate chains. The separate α and β chains could be refolded to their native structure by reoxidation of the cysteines. Refolding was accompanied by reassembly of the separated chains to the α/β heterodimer. Both the separated α and β chains and the α/β heterodimer bound significantly higher amounts of antigenic peptide after reduction and reoxidation, as compared to the untreated protein.**

Class II molecules of the major histocompatibility complex (MHC) are heterodimeric glycoproteins consisting of one α and one β chain. Their function is to bind antigenic peptides and to present them to helper T cells (1–3). The peptide binding site is thought to be located in a groove between two α helices that lie on top of a platform formed by β strands (4). Two distinct α/β heterodimeric conformations ("compact" and "floppy") have been identified (5). It has also been found that both the α and the β chains independently bind peptides specifically (6). The on and off rates of peptides are remarkably small (2, 7), and only a small fraction of isolated MHC molecules are able to bind added peptides, even when the peptides are present in a great molar excess and are incubated for long periods of time (3, 8, 9). This is presumably due to the fact that many MHC molecules still have peptide bound after isolation (10, 11) and that these peptides dissociate very slowly.

In the present study we removed bound peptides by unfolding the MHC molecules. Refolding of the secondary structure and reassembly of the separated α and β chains then provides a higher binding capacity for newly added peptides.

Moreover, studying the folding of MHC molecules allows one to investigate the folding pathway and the targeting of a highly complex molecule. Many proteins have been unfolded and refolded successfully (for a review, see ref. 12), the thermodynamic and kinetic mechanisms have been studied in detail, and folding intermediates have been characterized (13–17). The role of disulfide bond formation has been investigated (18–20). Fab fragments of antibodies have been unfolded and refolded successfully (21).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

MATERIALS AND METHODS

Sample Preparation. I-A^d was affinity purified from A20.1.11 cells by using the antibody MKD6 as described (5). In the present study we replaced 30 mM octyl β-D-glucopyranoside by 2 mM dodecyl β-D-maltoside (DM) (Calbiochem), which has a lower critical micellar concentration. The I-A^d-containing fractions were pooled and dialyzed in 10 mM Tris·HCl, pH 8.3/150 mM NaCl/0.02% NaN_3/2 mM DM overnight. This preparation, which is referred to as I-A^d, contains the compact and floppy conformations and separate α and β chains. To isolate the α and β chains separately, we ran the purified I-A^d on preparative SDS/polyacrylamide gels; 2-mercaptoethanol was omitted and the samples were not boiled. The α and β chains were electroeluted (22) from these gels in 25 mM Tris/0.2 M glycine, pH 8.2/0.1% SDS.

Reduction and Reoxidation Reactions. I-A^d (30 μg/ml) was reduced with 1 mM and 10 mM dithiothreitol (DTT) in 10 mM Tris·HCl, pH 8.3/150 mM NaCl/0.02% NaN_3/2 mM DM by incubation for 1 hr at 37°C. The α and β chains (20 μg/ml each) were incubated for 1 hr at 37°C in 1 mM or 10 mM DTT in 25 mM Tris/0.2 M glycine, pH 8.2/0.1% SDS either separately or after mixing equal amounts of them. For reoxidation, the reduced samples were dialyzed in at least a 1000-fold volume of 10 mM Tris·HCl, pH 8.3/150 mM NaCl/0.02% NaN_3/2 mM DM for 18 hr. We used 9DC DiaCell dialysis capsules (BioDesign, New York) equipped with Spectrapor dialysis tubing with a molecular size cutoff of 12 kDa. The sample volume was typically 50 μl.

Peptide Incubation and Analytical Gel Electrophoresis. I-A^d or the α and β chains were incubated with a fluorescein-labeled peptide fragment of chicken ovalbumin [FOva-(323–339); 100 μM; Peninsula Laboratories] for 1 hr at 37°C before reduction or after reoxidation. Reduction was performed after incubation with 100 μM FOva-(323–339). After the incubations, the samples (typically a total of 1.5 μg of protein) were loaded on SDS/12.5% polyacrylamide gels. Prior to fixation and silver staining, the gels were scanned for fluorescent peptides on a fluorescence microscope as described (5, 6). The gels were silver stained (23), and relative protein concentrations were determined by scanning the silver-stained gels on a LKB UltroScan XL laser scanner. The scans for fluorescent peptides and for silver-stained proteins were corrected for background.

Western Blotting. Proteins were transferred from SDS/polyacrylamide gels to nitrocellulose membranes overnight at 30 V in 20 mM Tris/150 mM glycine, pH 8.0. MHC heterodimers were detected with the monoclonal antibody 28-16-8S (24). The concentration of 28-16-8S was 1 μg/ml, and the nitrocellulose membranes were incubated for 6 hr.

Abbreviations: MHC, major histocompatibility complex; FOva-(323–339), synthetic peptide representing amino acids 323–339 of chicken ovalbumin labeled at its N terminus with fluorescein isothiocyanate; DTT, DL-dithiothreitol; DM, dodecyl β-D-maltoside.

We used an alkaline phosphatase-conjugated goat anti-mouse antibody (Sigma) for detection of 28-16-8S and nitro blue tetrazolium (0.1 mg/ml) and 5-bromo-4-chloro-indolylphosphate (0.05 mg/ml) in 50 mM sodium carbonate buffer, pH 9.5/2 mM $MgSO_4$ for staining. We used 5 μg of total protein per lane. The refolded samples were therefore concentrated by putting the dialysis capsules on solid polyethylene glycol 8000, which is hygroscopic, but chemically inert.

RESULTS

Fig. 1 shows the effect of reduction with 1 mM and 10 mM DTT and reoxidation on I-A^d. Fig. 1*A* shows the silver-stained SDS/polyacrylamide gels, and Fig. 1*B* shows the corresponding scans for fluorescent peptides. The relative distribution of the two α/β heterodimeric conformations compact and floppy and the separated α and β chains as revealed by scanning the silver-stained gel on a gel scanner as well as the relative amount of peptide bound to each of these conformations are given in Table 1. I-A^d was incubated with 100 μM FOva-(323–339) in DM buffer for 1 hr at 37°C (Fig. 1 *A* and *B*, lane 1). Scanning of the silver-stained gel on a gel scanner revealed a relative distribution of floppy:compact:α chain:β chain of 1.23:2.19:0.15:0.13 (Table 1). As shown in Fig. 1*B*, lane 1 and reported earlier (6), all of these conformations bind specific peptides. However, the relative concentration of separate chains in the present preparation was particularly low. Reduction of the disulfide bonds with 1 mM and 10 mM DTT after prior incubation with FOva-(323–339) led in both experiments to dissociation of peptides bound to I-A^d (Fig. 1*B*, lanes 2 and 4, respectively, and Table 1). Only a small amount of peptide remained bound to the compact conformation after reduction with 1 mM DTT, which completely disappeared upon reduction with 10 mM DTT. This indicates that the compact conformation is the most stable conformation. The effect of reduction on the secondary structure can be seen in the silver-stained gel: whereas the compact conformation was unaltered, the floppy conformation disassembled almost completely (Fig. 1*A*, lanes 2 and 4) to separate chains (Table 1). Both the separated α and β chains migrated at positions corresponding to higher apparent molecular masses, indicating unfolding of the proteins (see below for details). Reoxidation by dialysis in air-saturated buffer restored not only the floppy conformation from the separated chains (Table 1) but also the folded conformation of both the α and the β chains (Fig. 1*A*, lanes 3 and 5). In addition, peptide binding was restored (Fig. 1*B*, lanes 3 and 5, and Table 1). Importantly, the amount of peptide bound to each of these conformations was significantly higher (Table 1). The fluorescence (obtained by multiplying the peak height by the half-width of the peak) associated with the floppy conformation rose from 4900 cps × channels for the untreated sample to 19,800 and 16,000 cps × channels for the reduced and reoxidized samples, respectively. Although less pronounced, a significant increase in peptide binding to the compact conformation and to the separated chains was observed. These results are interpreted as being related to the release of previously bound peptides by reduction. These peptides are removed by dialysis. Reoxidation and refolding yields MHC molecules lacking bound peptides. Therefore more binding sites are accessible for FOva-(323–339). With the particular preparation used here, we found the peptide binding of all conformations to be increased by a factor of >2.5 after reduction with 10 mM DTT and reoxidation (Table 1). However, other preparations showed peptide binding capacities to be increased by a factor of 10 or more.

To investigate the conformational changes and enhanced peptide binding capacity of I-A^d after reduction and reoxidation in more detail, we isolated the separated α and β chains by electroelution from preparative SDS/polyacrylamide gels. Fig. 2 shows the β chain migrating with an apparent molecular mass of 27.5 kDa (lane 1). After reduction with 1 mM DTT (Fig. 2, lane 2), 35% of this chain migrated at an apparent molecular mass of 28.5 kDa and 25% migrated with an apparent molecular mass of 30 kDa. After reduction with 10 mM DTT, 100% of the β chain migrated with an apparent molecular mass of 30 kDa (Fig. 2, lane 3). Boiling for 30 min in 2% SDS in the presence of 10 mM DTT did not alter the apparent molecular mass (Fig. 2, lane 4). The α chain migrated with an apparent molecular mass of 33 kDa (Fig. 2, lane 6). After reduction with 1 mM DTT for 1 hr at 37°C, 75%

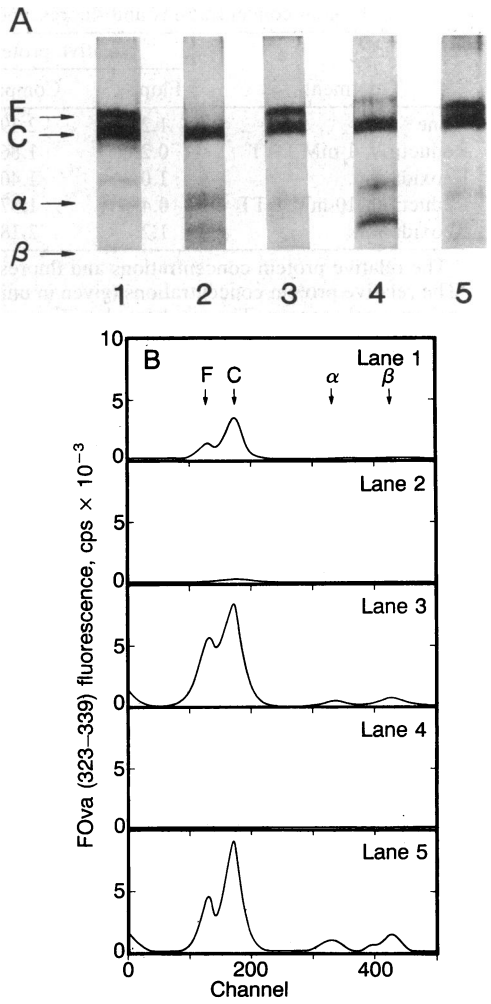

FIG. 1. Reduction and reoxidation of I-A^d. (*A*) Silver-stained SDS/polyacrylamide gel. Lane 1, I-A^d was incubated for 1 hr at 37°C in the presence of 100 μM FOva-(323–339). The α/β heterodimeric conformations floppy (F) and compact (C) migrate with apparent molecular masses of 64 and 55 kDa, and the separate α and β chains migrate with apparent molecular masses of 33 and 27.5 kDa, respectively. Lane 2, I-A^d was reduced with 1 mM DTT for 1 hr at 37°C in the presence of 100 μM FOva-(323–339). Lane 3, the sample of lane 2 was reoxidized by dialysis in air-saturated buffer and subsequently incubated for 1 hr at 37°C with 100 μM FOva-(323–339). Lane 4, identical to lane 2 except that 10 mM DTT was used for reduction instead of 1 mM DTT. Lane 5, the sample of lane 4 was reoxidized by air and subsequently incubated for 1 hr at 37°C with 100 μM FOva-(323–339). (*B*) Scans for fluorescent peptides bound to the different conformations of I-A^d. The samples in *A* (lanes 1–5) were scanned for fluorescent peptides on a fluorescence microscope prior to fixation of the gels. It is evident that reduction (lanes 2 and 4) leads to almost complete loss of peptide binding. Reoxidation (lanes 3 and 5) restores peptide binding. The binding capacity of all conformations of I-A^d was significantly higher after reduction and reoxidation, compared to the untreated material (lane 1).

Table 1. Protein concentrations and fluorescence intensities

Treatment	Relative protein concentration*				Relative fluorescence intensity†			
	Floppy	Compact	α	β	Floppy	Compact	α	β
None	1.23	2.19	0.15	0.13	4.9	12.2	<1.5	<2.0
Reduction, 1 mM DTT	0.25	1.86	0.41	0.62	<0.5	0.9	<1.5	<2.0
Reoxidation	1.07	1.40	0.10	0.13	19.8	29.0	2.0	3.0
Reduction, 10 mM DTT	0.43	1.87	0.76	1.05	<0.5	<0.5	<1.5	<2.0
Reoxidation	1.26	2.18	0.25	0.28	16.0	32.0	4.6	6.6

The relative protein concentrations and fluorescence intensities of the samples in Fig. 1 are given.

*The relative protein concentrations (given in units of absorbance × mm) were determined by scanning the silver-stained gel on a gel scanner. The numbers shown were obtained by integrating the area under the peaks.

†The relative fluorescence intensities (given in units of cps × 10^{-3} × channels) were determined by scanning the gel on a fluorescence microscope prior to fixation. The numbers shown were obtained by multiplying the peak height by the half-width of the peak. FOva-(323–339) was smeared over the gel. Thus the fluorescence background increased with the length of the gel. Therefore the detection limit for the β chain is higher than that for the α chain, which again is higher than that for the compact and floppy conformations.

of this chain migrated with an apparent molecular mass of 34.5 kDa (Fig. 2, lane 7). After reduction with 10 mM DTT, 100% of the α chain migrated at an apparent molecular mass of 34.5 kDa (Fig. 2, lane 8). Again, boiling did not alter the apparent molecular mass (Fig. 2, lane 9). Thus both chains migrate with higher apparent molecular masses after reduction. This is interpreted as being due to an increased effective volume of the proteins (i.e., to a partial or complete unfolding of their secondary structure). Because the unfolding of the β chain occurs in a two-step reaction, we presume that one of the two disulfide bonds of the β chain has a higher redox potential than the other bond.

Boiling in SDS in the presence of high concentrations of DTT doubtless leads to unfolding of the secondary structure. Therefore we assume that I-A^d, upon incubation in 10 mM DTT for 1 hr at 37°C, not only disassembles into separated chains but that these chains also unfold. This interpretation easily explains why reduced I-A^d does not bind peptides (Fig. 1). The compact conformation was found more resistant to reduction than the floppy conformation. This might be explained by assuming a higher redox potential of one or more

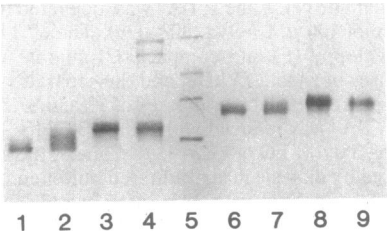

FIG. 2. Unfolding of separate chains. Lane 1, the β chain of I-A^d after electroelution from preparative SDS/polyacrylamide gels. The apparent molecular mass is 27.5 kDa. Lane 2, the β chain was incubated for 1 hr at 37°C with 1 mM DTT. Three bands are visible with apparent molecular masses of 27.5, 28.5, and 30 kDa. Lane 3, the β chain was incubated for 1 hr at 37°C with 10 mM DTT. Only one band is seen; it migrates with an apparent molecular mass of 30 kDa. Lane 4, the β chain was incubated for 30 min at 95°C in the presence of 10 mM DTT. The apparent molecular mass of the band is identical to that in lane 3. However, aggregates migrating with apparent molecular masses of 60 and 70 kDa are detected. Lane 5, molecular size standards. The proteins are bovine serum albumin, hen egg albumin, carbonic anhydrase, and glyceraldehyde-3-phosphate dehydrogenase. Trypsin inhibitor and lysozyme are not shown. Lane 6, the α chain of I-A^d after electroelution from preparative SDS/polyacrylamide gels. It migrates with an apparent molecular mass of 33 kDa. Lane 7, the α chain was incubated for 1 hr at 37°C with 1 mM DTT. Two bands are visible with apparent molecular masses of 33 and 34.5 kDa. Lane 8, the α chain was incubated for 1 hr at 37°C with 10 mM DTT. Only one band is left; it migrates with an apparent molecular mass of 34.5 kDa. Lane 9, the α chain was incubated for 30 min at 95°C in the presence of 10 mM DTT. The apparent molecular mass is identical to that in lane 8.

of the disulfide bonds in this α/β heterodimeric conformation than in the floppy conformation or in the separated chains. Alternatively, the disulfide bonds might be reduced, but the noncovalent interaction between the chains is stronger than in the floppy conformation, thus preventing disassembly and unfolding. We have not yet distinguished between these two possibilities. Boiling in SDS in the presence of DTT or mercaptoethanol does cause the compact conformation to disassemble and unfold (data not shown).

The separate chains can be reassembled to the α/β heterodimer. Fig. 3*A* shows the electroeluted α and β chains in lanes 2 and 3, respectively. A small fraction of both the α and β chains dimerized, migrating with apparent molecular masses of 68 kDa and 56.5 kDa, respectively. This is analogous to the dimerization of heavy and light chains of immunoglobulins (21). The chains were mixed and incubated at 37°C with and without peptide for 1 hr and 18 hr (Fig. 3*A*, lanes 4–7). Under these conditions, no reassembly could be achieved. The mixed, separated chains were reduced with 1 mM and 10 mM DTT for 1 hr at 37°C (Fig. 3*A*, lanes 8 and 9, respectively), thus unfolding the proteins (see above). Reoxidation by dialysis in air-saturated buffer (Fig. 3*A*, lanes 10–13) not only refolded the separated chains as evident from their lower apparent molecular masses but also yielded considerable amounts of α/β heterodimers migrating with an apparent molecular mass of 64 kDa. This corresponds to the floppy conformation and is distinctly different from the α- and β-chain homodimers discussed above. The yields of reassembled α/β heterodimer were 8% after reduction with 1 mM DTT and reoxidation (Fig. 3*A*, lane 10), 18% after reduction with 10 mM DTT and reoxidation (Fig. 3*A*, lane 11), 5% after reduction with 1 mM DTT and incubation with 100 μM FOva-(323–339) following the reoxidation (Fig. 3*A*, lane 12), and 17% after reduction with 10 mM DTT and incubation with 100 μM FOva-(323–339) following the reoxidation (Fig. 3*A*, lane 13). Thus, the yield is significantly higher when the proteins are first unfolded completely, indicating that reassembly occurs simultaneously with refolding. The incubation with peptides after refolding had no pronounced influence on the reassembly efficiency.

Fig. 3 *B* and *C* shows scans of lanes 6, 12, and 13 of Fig. 3*A* for proteins and fluorescent peptides, respectively. The positions of separated chains, homodimers, and the heterodimers compact and floppy are indicated. Fig. 3*B* provides clear evidence that reassembly occurs only if the separated chains have previously been unfolded. In Fig. 3*B*, lane 6, folded separated chains have been incubated. In lanes 12 and 13 of Fig. 3, they were first unfolded and reassembled during refolding to yield predominantly the floppy conformation and to some extent the compact conformation. At molecular masses higher than that of the floppy conformation, aggregated chains can be seen. However, comparison with Fig. 3*C*

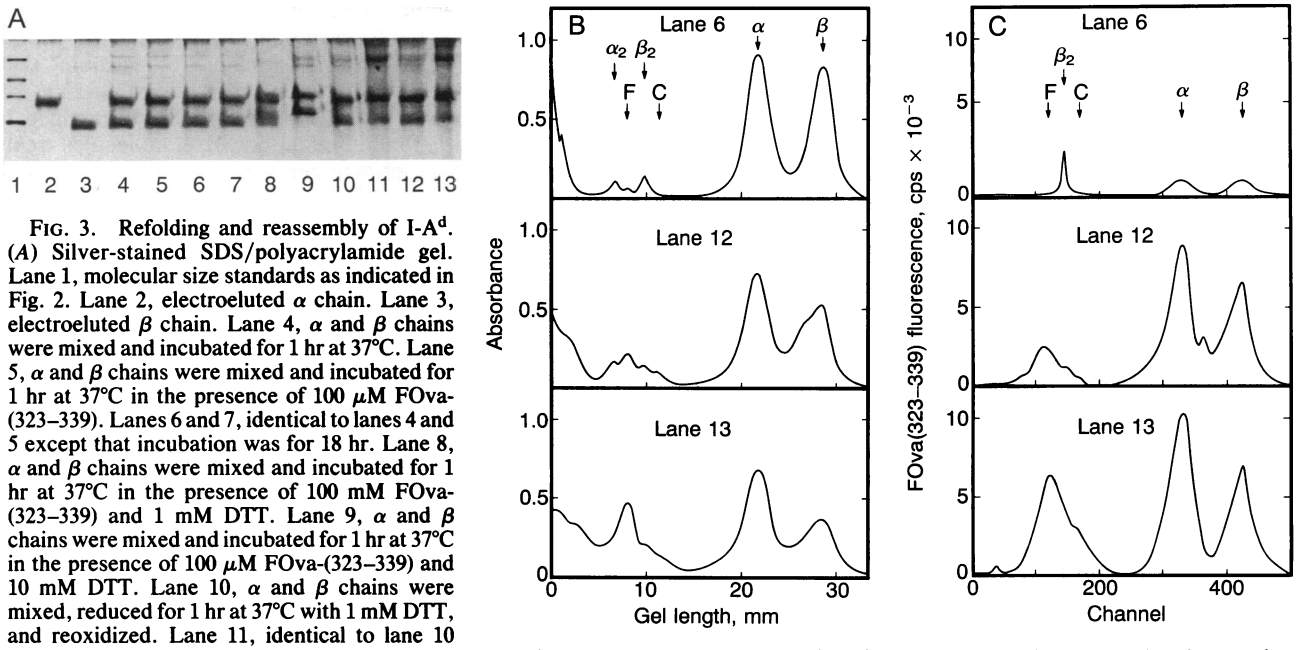

FIG. 3. Refolding and reassembly of I-A^d. (*A*) Silver-stained SDS/polyacrylamide gel. Lane 1, molecular size standards as indicated in Fig. 2. Lane 2, electroeluted α chain. Lane 3, electroeluted β chain. Lane 4, α and β chains were mixed and incubated for 1 hr at 37°C. Lane 5, α and β chains were mixed and incubated for 1 hr at 37°C in the presence of 100 μM FOva-(323–339). Lanes 6 and 7, identical to lanes 4 and 5 except that incubation was for 18 hr. Lane 8, α and β chains were mixed and incubated for 1 hr at 37°C in the presence of 100 mM FOva-(323–339) and 1 mM DTT. Lane 9, α and β chains were mixed and incubated for 1 hr at 37°C in the presence of 100 μM FOva-(323–339) and 10 mM DTT. Lane 10, α and β chains were mixed, reduced for 1 hr at 37°C with 1 mM DTT, and reoxidized. Lane 11, identical to lane 10 except that 10 mM DTT was used for reduction instead of 1 mM DTT. Lanes 12 and 13, identical to lanes 10 and 11 except that the samples were incubated for 1 hr at 37°C in the presence of 100 μM FOva-(323–339) after reoxidation. (*B*) Scans of the silver-stained SDS/polyacrylamide gel. To quantify the relative protein concentrations, the gel shown in *A* was scanned on a gel scanner. The positions of the separated chains, the α/β heterodimeric conformations compact (C) and floppy (F), and the homodimeric α and β chains (α_2 and β_2) are given. When the separated chains were isolated and incubated together without previous reduction and reoxidation, they did not reassemble to α/β heterodimers (lane 6). Reassembly to the floppy conformation was observed after reduction with 1 mM or 10 mM DTT and reoxidation (lanes 12 and 13, respectively). A shoulder at the position corresponding to the compact conformation is seen. (*C*) Scans of the gel shown in *A* for fluorescent peptides. The sample not subjected to reduction and reoxidation (lane 6) bound small amounts of FOva-(323–339). After reduction with 1 mM or 10 mM DTT and reoxidation (lanes 12 and 13, respectively), the peptide-binding capacity of the separated α and β chains was increased by factors of 11 and 9, compared to lane 6. The reassembled floppy conformation was associated with high amounts of peptide. Although β_2 bound peptide (lane 6), the β chain oligomers did not (lanes 12 and 13).

shows that those aggregates do not bind peptides and thus may be aggregated in an unfolded state. Since the concentration of free β chain is decreased compared to the α chain, we assume that these high molecular mass aggregates are β-chain oligomers. The samples of lanes 6, 12, and 13 (Fig. 3) were incubated with FOva-(323–339) under identical conditions directly before applying them to the gel. After normalizing the protein concentrations by using the scans for proteins shown in Fig. 3*B* (this is necessary as the overall recovery is not quantitative, and β chains oligomerize), the peptide binding capacity of the α and β chain increased by factors of 11 and 9, respectively, in the particular preparation used here.

To obtain additional evidence that the products of our reassembly experiment indeed are the heterodimeric floppy and compact conformations, we used the monoclonal antibody 28-16-8S in Western blotting experiments. 28-16-8S recognizes an epitope only expressed by α/β heterodimers. Fig. 4 shows a silver-stained gel of an untreated I-A^d preparation (lane 1). In Fig. 4, lane 2, the same preparation was blotted and stained by using 28-16-8S. Comparison of the silver-stained gel and the blot of the untreated sample provides evidence that 28-16-8S recognizes the compact and floppy conformations but not the separated chains, even though the relative concentrations of β chain:α chain:compact:floppy were 1:1.12:1.25:1.37. A blot of refolded and reassembled sample is shown in Fig. 4, lane 3. Two bands migrating at apparent molecular masses corresponding to floppy and compact are stained with 28-16-8S. This result provides evidence that our experiments yield the heterodimeric floppy and compact conformations from separated, unfolded chains.

DISCUSSION

Here we have shown that (*i*) reduction of disulfide bonds of I-A^d releases previously bound peptides, (*ii*) the separated chains may be unfolded and then refolded and reassembled to the floppy and compact conformations, and (*iii*) refolding and reoxidation of disulfide bonds of both the α/β heterodimers and the separate α and β chains lead to an increased peptide-binding capacity.

As evident from Fig. 1 and Table 1, it is not necessary to completely unfold and refold I-A^d in order to increase its peptide-binding capacity. Although the floppy conformation disassembled upon reduction and was restored from the separate chains upon reoxidation, the compact conformation remained unchanged under the same conditions. However, the compact conformation lost all of its peptides upon reduction and bound 2.5 times the amount of FOva-(323–339) after reoxidation (Table 1). We therefore surmise that the disulfide bonds of the compact conformation are reduced by DTT, but the protein does not unfold. It is known that the formation of disulfide bonds contributes considerably to the free energy stabilizing secondary structures (20), but on the other hand much of free energy is determined by noncovalent interactions. The observation that the compact conformation releases its bound peptides without disassembly is interpreted as being due to an increased flexibility despite an unaltered mean structure. The increased flexibility due to reduced disulfide bonds may thus allow the peptide to dissociate with a fast off rate, whereas it dissociates extraordinarily slowly when the protein is rigid (i.e., with oxidized disulfide bonds).

The flexibility of the chains also plays a major role in the reassembly to the α/β heterodimer. The chains did not reassemble when they were in the folded state. It is known that several proteins do not reassemble to homo- or heterodimeric conformations when the subunits are folded but may be reassembled when they are refolded simultaneously (25). Similar to the reassembly of heavy and light chains of

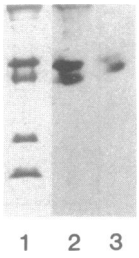

FIG. 4. Western blots provide further evidence for the reassembly of separate chains to the α/β heterodimeric floppy and compact conformations. Lane 1, silver-stained gel of a preparation of I-A^d. Lane 2, Western blot of the sample shown in lane 1. Lane 3, Western blot of a refolded and reassembled sample. Monoclonal antibody 28-16-8S was used for blotting. Although they are present in high concentrations, the separate chains are not recognized by 28-16-8S (compare lanes 1 and 2). Lane 3 shows that the reassembly experiments yielded the floppy and compact conformations. Because the sample was concentrated before loading on the gel, it contained higher amounts of DM and polyethylene glycol. This caused the bands to smear.

immunoglobulin Fab fragments (21), we observed refolding and concomitant reassembly only when we used unfolded chains. It is known from *in vivo* studies on the role of disulfide bond formation during assembly of heavy and light chains of Fab fragments (26) and of the human chorionic gonadotropin molecule (27) that the sequence of disulfide bond formation in the lumen of the endoplasmatic reticulum is crucial for successful assembly of the subunits. However, we cannot exclude that chaperones (see ref. 28 for a review) are involved, which might catalyze or delay the assembly in the cell. The results presented here show that high flexibility of the chains seems to be a requirement for successful reassembly, at least for *in vitro* reassembly. Figuratively, the two chains may refold and synchronously reassemble like a zipper. Once the heterodimer is formed, it is very stable because the chains are hooked into each other. This picture is consistent with the high stability of MHC molecules observed experimentally (5).

Rigid chains hooked into each other may act as a trap for bound peptides and thus may explain the slow off rates. This must be important for the function of MHC molecules in the cell. It is known that peptides are bound in an intracellular compartment, then the MHC–peptide complex is transported to the cell surface, where it "waits" for a specific T cell. If the peptide were not trapped or bound with a low off rate, it would dissociate during export or when in the plasma membrane. The biological task of MHC molecules is to bind a variety of antigenic peptides and to retain each of them for extended periods of time. When the mechanism of peptide–MHC assembly is ultimately understood, the term "trapping site" may be more appropriate than "binding site."

The reduction and reoxidation experiments described here quantitatively release peptides known to copurify with MHC molecules (10, 11). As shown in Fig. 1, reassembly is not essential for this purpose. Table 1 shows that the binding capacity for peptides is greatly increased after reduction and reoxidation. It has been reported that typically only 5–30% of MHC binding sites are accessible to freshly added peptides (3, 8, 9). Moreover, different preparations of the same haplotype are known to vary considerably in their peptide-binding capacity. This may be explained by assuming that a variety of peptides with different affinities or off rates preoccupy the binding site. We have not yet determined the stoichiometry of peptide binding to MHC molecules treated by the method described here. However, we found the binding capacity increased by factors ranging from 2.5 to >10; thus, it is possible that we might yield an almost 1:1 stoichiometry. Although slight modifications of experimental details may have to be introduced for haplotypes other than I-A^d, the present method provides a means to overcome the well-known problems of self-peptides occupying the binding site (3, 8, 9) and therefore allows for the preparation of chemical reagents in which one defined peptide occupies the binding site of one MHC molecule.

This work was supported by the European Molecular Biology Organization (K.D.) through Grant ALTF 113-1988 and by National Institutes of Health Grant 5R01 Al 13587-13.

1. Babbitt, B., Allen, P. M., Matseuda, G., Haber, E. & Unanue, E. R. (1985) *Nature (London)* **317**, 359–361.
2. Buus, S., Colon, S., Smith, C., Freed, J. H., Miles, C. & Grey, H. M. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 3968–3971.
3. Watts, T. H. & McConnell, H. M. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 9660–9664.
4. Brown, J. H., Jardetzki, T., Saper, M. A., Samraoui, B., Bjorkman, P. & Wiley, D. C. (1988) *Nature (London)* **332**, 845–850.
5. Dornmair, K., Rothenhäusler, B. & McConnell, H. M. (1990) *Cold Spring Harbor Symp. Quant. Biol.* **54**, in press.
6. Rothenhäusler, B., Dornmair, K. & McConnell, H. M. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 352–354.
7. Sadegh-Nasseri, S. & McConnell, H. M. (1989) *Nature (London)* **337**, 274–276.
8. Buus, S., Sette, A., Colon, S. M., Miles, C. P. & Grey, H. M. (1987) *Science* **235**, 1353–1358.
9. Adorini, L., Muller, S., Cardinaux, F., Lehmann, P. V., Falcioni, F. & Nagy, Z. A. (1988) *Nature (London)* **334**, 623–625.
10. Demotz, S., Grey, H. M., Appella, E. & Sette, A. (1989) *Nature (London)* **342**, 682–684.
11. Wallny, H. J. & Rammensee, H. G. (1990) *Nature (London)* **343**, 275–278.
12. Jaenicke, R. (1987) *Prog. Biophys. Mol. Biol.* **49**, 117–237.
13. Harrison, S. C. & Durbin, R. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 4028–4030.
14. Creighton, T. E. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 5082–5086.
15. Udgaonkar, J. B. & Baldwin, R. L. (1988) *Nature (London)* **335**, 694–699.
16. Roder, H., Elöve, G. A. & Englander, S. W. (1988) *Nature (London)* **335**, 700–704.
17. Matouschek, A., Kellis, J. T., Serrano, L. & Fersht, A. R. (1989) *Nature (London)* **340**, 122–126.
18. Maher, P. A. & Singer, S. J. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 9001–9005.
19. Goldenberg, D. P., Frieden, R. W., Haak, J. A. & Morrison, T. B. (1989) *Nature (London)* **338**, 127–132.
20. Matsumura, M., Signor, G. & Matthews, B. W. (1989) *Nature (London)* **342**, 291–293.
21. Björk, I. & Tanford, C. (1971) *Biochemistry* **10**, 1289–1295.
22. Hunkapiller, M. W., Lujan, E., Ostrander, F. & Hood, L. E. (1983) *Methods Enzymol.* **91**, 227–236.
23. Heukeshoven, J. & Dernick, R. (1985) *Electrophoresis* **6**, 103–112.
24. Ozato, K. & Sachs, D. H. (1979) *J. Immunol.* **126**, 317–321.
25. Huber, R. (1988) *Angew. Chem. Int. Ed.* **27**, 79–88.
26. Bergman, L. W. & Kuehl, W. M. (1979) *J. Biol. Chem.* **254**, 5690–5694.
27. Beebe, J. S., Mountjoy, K., Krzesicki, R. F., Perini, F. & Rudden, R. W. (1990) *J. Biol. Chem.* **265**, 312–317.
28. Rothman, J. E. (1989) *Cell* **59**, 591–601.