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Empty and Peptide-Loaded Class II Major Histocompatibility Complex Proteins Produced by Expression in *Escherichia coli* and Folding *in Vitro*

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The human class II major histocompatibility complex protein HLA–DR1 has been expressed in *Escherichia coli* as denatured α and β subunits and folded *in vitro* to form the native structure. DR1 folding yields are 30–50% in the presence or absence of tight-binding antigenic peptides. The protein produced in this manner is soluble and monomeric with the expected apparent molecular weight. It reacts with conformation-sensitive anti-DR antibodies and exhibits peptide-dependent resistance to SDS-induced chain dissociation and to proteolysis as does the native protein. The observed peptide specificity and dissociation kinetics are similar to those of native DR produced in B-cells and finally the protein exhibits circular dichroism spectra and cooperative thermal denaturation as expected for a folded protein. We conclude that the recombinant DR1 has adopted the native fold. We have folded DR1 in the absence of peptide and isolated a soluble, peptide-free αβ-heterodimer. The empty DR1 can bind antigenic peptide but exhibits altered far UV-circular dichroism and thermal denaturation relative to the peptide-bound form. © 1999 Academic Press

Major histocompatibility complex (MHC)[2] proteins are heterodimeric membrane glycoproteins found predominately on the surface of B-lymphocytes that are critical in stimulating a T-cell-mediated immune response. For class II MHC proteins, the α and β subunits carry extracellular peptide-binding and immunoglobulin-like domains and one transmembrane span and short cytoplasmic tail (<20 residues) per subunit (1). In a cell, class II MHC proteins tightly bind peptides derived from exogenous and endogenous antigens and present them to receptors on T-cells as part of the mechanism for initiating an immune response (2,3). Heterogeneous mixtures of endogenous peptides are found associated with class II MHC proteins isolated from their native source (4–7). These tightly bound peptides interfere with biochemical analysis of the peptide-binding reaction and complicate structural and other biophysical characterizations of class II MHC proteins.

Previous efforts to prepare recombinant complexes of class II MHC proteins with single, defined peptides (8–12) or empty, peptide-free molecules (9,10,13,14) have met with limited success. Although these methods were crucial to producing material for structure determinations (15,16) and other studies (10,13,15,17–19), they all suffer from chemical and physical heterogeneity and/or low yield. In particular, native protein produced in recombinant insect cells (8,9,13,16) or mammalian cells (14) is heterogeneously glycosylated and often has uncertain peptide occupancy, and refolded protein from *Escherichia coli* is obtained in very low yield and can be substantially aggregated or misfolded (10–12). Currently, structural and biophysical studies of class II MHC proteins are limited by the amount and homogeneity of the protein available for study. We are interested in producing milligram quantities of class II MHC proteins and their peptide complexes in homogenous form.

In this paper we describe a system for high-efficiency *in vitro* folding of a soluble version of the human class II MHC protein DR1 from subunits produced in *E. coli.* Empty and peptide-loaded DR1 complexes exhibited

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[2] Abbreviations used: MHC, major histocompatibility complex; DTT, 1,4-dithiothreitol; Ha, peptide 306–318 from influenza hemaglutinin (PKYVKQNTLKLAT); Ii, peptide 90–104 from human invariant chain (KMRMATPLLMQALPM).

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distinct physical properties suggesting that a conformational change may accompany peptide binding.

MATERIALS AND METHODS

Plasmids

The HLA–DR1 α chain (*DRA*0101*) and β chain (*DRB1*0101*) genes were reconstructed for T7 polymerase-directed expression in *E. coli* of their extracellular domains as follows: oligonucleotide primers for polymerase chain reaction were designed to incorporate an *Eco*RI restriction enzyme site, ribosome binding site (AGGAGG), spacer sequence (AATTTAAA), and initiation codon at the 5′-end and a stop codon (TAA) and *Hin*dIII restriction enzyme site at the 3′-end of the genes. The resulting PCR products encode protein sequence from Ile 1 to Ala 181 for DRα and Gly 1 to Ala 190 for DRβ. These sequences correspond to the mature extracellular region of the protein not including the membrane-proximal "connecting-peptide" region. The PCR products were cloned immediately after the T7 promoter of the expression vector pLM1 (20) and the correct constructions were confirmed by dideoxy sequencing.

Expression and Purification of Polypeptides

For protein expression, plasmids encoding the DRα and DRβ polypeptides were separately transformed into *E. coli* BL21(DE3)pLysS cells (21) which carry a *lac*-driven T7 polymerase gene. Individual colonies were grown to 1 liter in Luria broth with 50 μg/ml ampicillin, 35 μg/ml chloramphenicol, and 2 mg/ml glucose and induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside at an $OD_{600\ nm}$ of 0.6–0.8. After 1 h the cells were harvested by centrifugation (3000*g*, 20 min) and lysed, and inclusion bodies were isolated by a modified differential centrifugation–detergent wash procedure (22) as described below. The cell pellet was homogenized in $\frac{1}{10}$ the culture volume in 50 mM Tris–Cl, 735 mM sucrose, 1 mM EDTA, 15 mM sodium azide, 10 mM 1,4-dithiothreitol (DTT), pH 8.0. The cell suspension was lysed by the addition of 2.5 vol 20 mM Tris–Cl, 1% deoxycholate, 1% Triton X-100, 0.3 mg/ml lysozyme, 100 mM NaCl, 15 mM sodium azide, 10 mM DTT, pH 7.5. The suspension was stirred approximately 15 min at room temperature, 0.5 mM $MgCl_2$ and 20 μg/ml DNase I were added, and the solution was stirred again until the viscosity decreased. Lysates were then frozen at −20°C, thawed, and collected by centrifugation at 4°C, 10,000*g* for 20 min. Pellets were resuspended with a polytron or dounce homogenizer in 50 mM Tris–Cl, 0.5% Triton X-100, 100 mM NaCl, 1 mM EDTA, 15 mM sodium azide, 10 mM DTT, pH 8.0, and collected as above. This procedure was repeated twice and then once using the same solution without Triton X-100 or NaCl. The washed inclusion body pellets were finally taken up in 20 mM Tris–Cl, 8 M urea, 10 mM DTT, 0.5 mM EDTA, pH 8.0, and centrifuged at 30,000*g* for 1 h at room temperature. Supernatants containing solubilized inclusion bodies were stored at −80°C or immediately purified by denaturing anion-exchange high-pressure liquid chromatography. The subunits were purified on Poros HQ20 resin (Perseptives Biosystems) in 20 mM Tris–Cl, 8 M urea, 1 mM DTT, pH 8.0 (DRα) or pH 9.0 (DRβ), with a linear gradient over 10 column vol of 0 to 300 mM NaCl in the same buffer. Fractions containing DRα or DRβ by SDS–PAGE were pooled, 0.5 mM EDTA was added, the pH was adjusted to 8.0 in the case of DRβ, and purified subunits were stored at −80°C in small aliquots. Subunit preparations were >70% pure as judged by scanning densitometry of SDS–PAGE experiments (72 ± 7%, n = 7). Purified subunits were quantitated by UV absorbance in urea solution using a calculated ϵ_{280} = 29,800 M^{-1} cm^{-1} (DRα) and 37,500 M^{-1} cm^{-1} (DRβ). For analysis by electrospray mass spectrometry, samples were further purified by reverse-phase chromatography (Poros R2 matrix, Perseptives Biosystems) with a linear acetonitrile gradient in 1% CH_3COOH.

Peptides

HLA–DR1-binding peptides Ha (PKYVKQNTLKLAT, from influenza hemagglutinin 306–318 (23,24)), A2 (VGSDWRFLRGYHQYA, from human HLA–A2 103–117 (5)), Cs (EKKIAKMEKASSVFNVV, from malaria circumsporozoite protein 380–396 (25)), Ii (KMRMATPLLMQALPM, from human invariant chain 90–104 (5)), and nonbinding control peptide β_2m (SDLSFSKDWSFYL, from human β_2 microglobulin 52–64) were synthesized using solid-phase FMOC chemistry, deprotected, and purified by reverse-phase chromatography on Vydac C4 or C18 using acetonitrile gradients in 0.1% CF_3COOH. The identity of the purified peptides and the lack of incomplete deprotection products were confirmed by fast-atom bombardment mass spectrometry.

Folding of Subunits

Purified subunits were thawed and diluted dropwise at least 100-fold with constant stirring to a final concentration of 0.08 μM each subunit into standard folding solution: 20 mM Tris–Cl, 25% (w/v) glycerol, 0.5 mM EDTA, 3 mM reduced glutathione, 0.3 mM oxidized glutathione, pH 8.5. Folding mixtures were kept for 3 days at 4°C with or without 0.5 μM peptide. These conditions were varied as noted for some experiments. Folding mixtures containing peptide were concentrated by a batchwise ion-exchange step. DEAE–Sephadex A-50 was added to the folding mixture at 1 g dry resin per liter, swollen overnight at 4°C, col-

lected in a column, washed with 10 vol 20 mM Tris–Cl, pH 8.0, and eluted with $\frac{1}{40}$ of the original folding volume 0.5 M NaCl, 20 mM Tris–Cl, pH 8.0. The eluate was exchanged into 20 mM Tris–Cl, pH 8.0, using ultrafiltration (Amicon YM10 membrane, 10,000 MWCO). In addition to concentrating the folding mixture, the batchwise ion-exchange and filtration steps partially enriched the DR$\alpha\beta$–peptide complex from unfolded subunits and removed components of the folding mixture that interfered with subsequent steps. The folded heterodimer was then purified by native anion-exchange chromatography on PorosHQ in 20 mM Tris–Cl, pH 8.0, with a linear gradient over 15 column vol of 0 to 400 mM NaCl. Heterodimer folded in the absence of peptide was recovered only in low yield using the ion-exchange procedure and was instead concentrated using ultrafiltration (Amicon YM10 membrane, 10,000 MWCO) and purified by immunoaffinity chromatography using monoclonal antibodies specific for the folded conformation L243 (26) or LB3.1 (27) as previously described (4,9). Empty and peptide-loaded DR1 were exchanged into phosphate-buffered saline (PBS):7 mM Na/K phosphate, 135 mM NaCl, pH 7.0, and stored at 4°C. Folded DR1 was quantitated by UV absorbance using the experimentally determined (9) ϵ_{280} of 52,000 M^{-1} cm^{-1}, corrected if necessary for peptide aromatic groups.

Folding Time Course

For determination of folding time course, a standard 100-ml folding mixture containing 0.05 μM Ha peptide was incubated at 4°C and 10-ml samples were taken at different times. Iodoacetamide (100 mM) was added to the samples to trap free cysteines, and then the samples were frozen at −80°C to stop the folding process. After the last time point the samples were thawed, analyzed by ELISA, and concentrated by ultrafiltration for nonreducing SDS–PAGE analysis as described below.

ELISA

The amount of folded DR1 in folding mixtures or column eluates was measured with a sandwich ELISA using monoclonal antibodies specific for the folded conformation L243 (26) or LB3.1 (27) as previously described (9). Immulon IV 96-well plates were coated with 2 μg/ml monoclonal antibody, washed with 0.05% Triton X-100 in PBS (T-PBS), and blocked with 3% bovine serum albumin (BSA) in PBS. Samples, purified DR1, and negative controls were applied to the plates as 100 μl of solution in 0.3% BSA, 0.1% Triton X-100 in PBS (BT-PBS) and incubated at room temperature at least 1 h. Plates were washed with T-PBS, incubated with rabbit anti-DR1 serum diluted 1:25,000 in BT-PBS, washed again, incubated with 0.2 μg/ml horseradish peroxidase-labeled goat anti-mouse IgG (Boehringer Mannheim) BT-PBS, washed again, and developed with 1 mg/ml 2,2-azino-di-[3-ethylbenzthiazoline sulfonate] (Boehringer Mannheim) and sodium perborate in citrate–phosphate buffer according to the manufacturer's recommendations. Sample concentration was determined by comparison to a four-parameter standard curve (9) using DR1 standards purified after expression in Sf9 cells or folding *in vitro* using material expressed in *E. coli.* The useful range of the assay was 0.5–50 ng DR1 in a 100-μl sample.

Peptide Dissociation Rate

Purified Ha peptide was radioactively labeled on tyrosine using [^{125}I]NaI and chloramine-T as described(28) to a specific activity of at least 2.5 × 10^4 cpm/pmol. Purified Ii peptide was fluorescently labeled at its amino terminus using fluorescein isothiocyanate (FITC). Labeled peptides were isolated by gel filtration on Sephadex G-10 (Pharmacia) and were stored in the dark at 4°C in PBS. For off-rate determinations, DRα and DRβ subunits were folded in the presence of labeled peptides, and labeled peptide complexes were isolated as described above. The purified DR1-labeled peptide complexes were incubated at 0.1 μM with 10 μM unlabeled peptide at 37°C (Ii) or at room temperature (Ha) and samples were taken at different time points. Samples were analyzed by HPLC gel filtration (Biosep SEC-3000, Phenomenex) and the amount of DR1-labeled peptide complex quantitated by gamma counting for ^{125}I-labeled Ha or by an in-line fluorescence detector equipped with a 436-nm band pass excitation filter and a 495 nm long pass emission filter for FITC-labeled Ii measurements.

Papain Cleavage

For papain cleavage experiments, papain (2× crystallized, Sigma) was activated at 2.8 mg/ml in 10 mM sodium phosphate buffer, 1 mM DTT, 1 mM EDTA, pH 6.5, at room temperature for 10 min. Papain activity was standardized between experiments using the chromogenic substrate *N*α-benzoyl-L-arginine *p*-nitroanilide diluted into standard folding solution. DR1 samples were digested at a protein concentration of 2 μM for 2 h at room temperature, and the reaction mixtures were stopped by the addition of 10 μg/ml *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E64) and analyzed by SDS–PAGE.

SDS–PAGE

Samples were prepared for SDS–PAGE analysis (29) by either boiling for 5 min in sample buffer or incubating at room temperature before loading onto the gel. Nonreduced sample buffer has final concentrations of

60 mM Tris–Cl, pH 6.8, 1% SDS, 10% glycerol, and 0.001% bromophenol blue. Reduced sample buffer also includes 100 mM DTT. Polyacrylamide gels (10%) were run at a constant voltage of 150 V/10 cm length and then stained with Coomassie brilliant blue R-250. For SDS–PAGE analysis, folding mixtures were concentrated approximately 20-fold in Centricon 10 (Amicon) ultrafiltration devices.

For quantitation of folding yield, concentrated folding mixtures and purified DR1 were analyzed by SDS–PAGE. The stained gels were digitized and the integrated density of the bands measured using the NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.gov/nih-image/). Differences in staining efficiency among the α (boiled), β (boiled), and $\alpha\beta$ (nonboiled) bands were determined using purified DR1–Ha. The relative staining of the bands was 1.0 (α boiled) to 0.7 (β boiled) to 1.4 ($\alpha\beta$ nonboiled). These values were used to calculate the fraction of the $\alpha\beta$–peptide band for folding mixtures.

Circular Dichroism

For circular dichroism (CD) analysis, folded DR1 was purified as described above, exchanged into 20 mM phosphate buffer, pH 7.0, and concentrated to approximately 20 μM. Measurements were made using a 1 mm path length cuvette in an AVIV 60DS CD spectrophotometer. Parameters for wavelength scans were 1.5-nm bandwidth, constant 10°C temperature, and 1-nm sampling with 5 s dwell time per point. Parameters for thermal denaturation experiments were 2-nm bandwidth and 204-nm detection wavelength, with 2°C intervals, a 1-min equilibration time, and 1-min dwell time at each temperature. Although the overall denaturation reaction is irreversible, test experiments showed that the denaturation traces were independent of scan speed in this regime (0.6–2°C/min), allowing the use of an equilibrium thermodynamic analysis (30). For determination of midpoint denaturation temperature (T_m) thermal denaturation data were fit to a seven-parameter function that describes a reversible two-state transition (31,32):

$$\theta = (\theta_u + m_u T) + \left[\frac{(\theta_f - \theta_u) + T(m_f - m_u)}{1 + \exp\left[\frac{\Delta H}{RT} + \frac{\Delta Cp}{R} \left(\frac{T_m}{T} - 1 + \ln \frac{T}{T_m} \right) \right]} \right],$$

where θ_u and m_u describe the y intercept and slope of the unfolded state baseline, θ_f and m_f describe the y intercept and slope of the folded state baseline, T_m is the midpoint of the transition (where $\Delta G = 0$), ΔCp is the apparent heat capacity change upon unfolding, and ΔH is the apparent enthalpy of unfolding at the T_m. In Fig. 8, the DR1–Ha curve uses ΔH and ΔCp values of 90 kcal/mol and 2.0 kcal/mol · *K*, respectively, while for DR1–no peptide the same parameters were 35 kcal/mol and 0.8 kcal/mol · *K*.

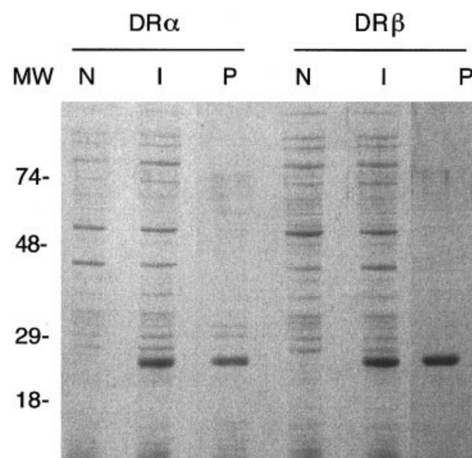

FIG. 1. Expression and purification of DR1 subunits. SDS–PAGE analysis of lysates of *E. coli* cells carrying genes for DRα and DRβ subunits and anion-exchange purified protein from inclusion bodies (N, not induced; I, induced; P, purified; MW, molecular weight markers, with masses indicated in kDa).

RESULTS

Expression and Purification of DR1 α and β Polypeptide

Genes coding for the extracellular domains of the human class II MHC protein HLA–DR1 α and β subunits were used for expression in *E. coli.* The DRα and DRβ subunits were expressed in good yields by T7-driven expression (Fig. 1). DR subunits comprised 10–20% of the total cellular protein and were found exclusively in the insoluble fraction. The insoluble inclusion bodies were washed extensively, solubilized in 8 M urea, and then further purified by denaturing anion-exchange chromatography. Subunit yields after purification were approximately 10 and 7 mg per liter of *E. coli* culture for the α and β chains, respectively. The isolated subunits were substantially pure, as judged by SDS–PAGE (Fig. 1). Electrospray mass spectrometry analysis of the purified α subunit indicated a mass of 21,262.2, corresponding to a deformylated N-terminal methionine residue (calculated mass 21,264.0), and of the purified β subunit indicated a major species of mass 22,052.8, corresponding to removal of the N-terminal methionine (calculated mass 22,053.6), and a minor species of mass 22,188.7, corresponding to a deformylated N-terminal methionine (calculated mass 22,184.8). The denatured, solubilized subunits appeared homodisperse and monomeric by gel filtration in 8 M urea (not shown).

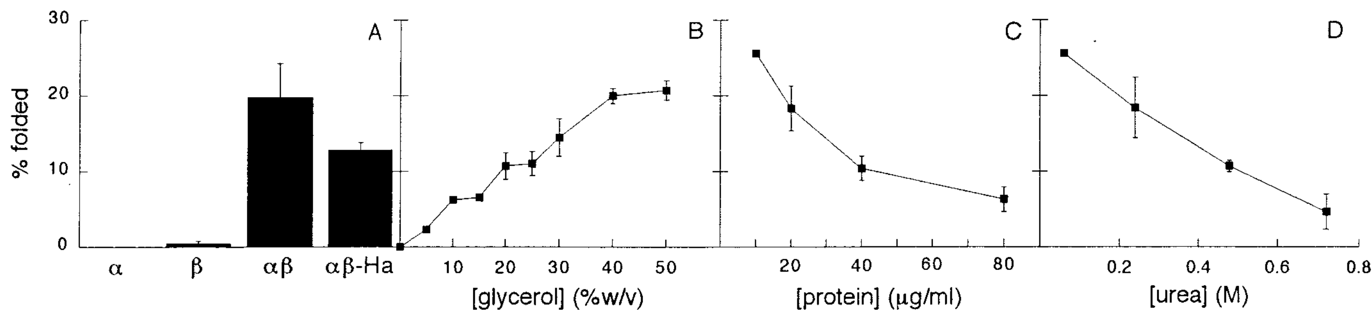

FIG. 2. Folding of DR1. (A) Folding yields of mixtures containing only the α subunit (α), only the β subunit (β), both α and β ($\alpha\beta$), or both α and β in the presence of excess Ha peptide ($\alpha\beta$-Ha). (B–D) Effect of final glycerol, protein, and urea concentration on folding yield. Folding yields determined by ELISA.

Folding in Vitro

We investigated several folding protocols and found that dilution of the urea-solubilized subunits into denaturant-free buffer solution promoted folding to the native structure, as indicated by reactivity with antibodies specific for the folded DR $\alpha\beta$-heterodimer. Folding was strongly inhibited by a component of the inclusion body preparation that was removed during the subunit purification, and attempts to fold the urea-solubilized but unpurified subunits were unsuccessful. Folding proceeded in the presence or absence of peptide, but required both α and β subunits (Fig. 2A). Folding was strongly favored by increased glycerol concentration (Fig. 2B), decreased protein concentration (Fig. 2C), and decreased temperature (not shown). This suggested that an aggregation process was competing with the productive folding pathway. Other compounds shown previously to promote folding of proteins containing immunoglobulin-like domains (ammonium sulfate, arginine, urea, ethylene glycol, and detergents (10,33–35)) had no effect or were detrimental to DR1 folding. In fact, residual urea from the denatured subunits inhibited folding (Fig. 2D). As expected by the need to form three disulfide bonds during the folding reaction, folding was dependent on the pH and the redox potential of the reaction solution. Optimal folding occurred at pH 8.5 with solution redox potential maintained by a 10:1 molar ratio of reduced:oxidized glutathione (not shown).

To confirm the identity of the ELISA-reactive material as folded DR1 we performed other characterizations. Many DR$\alpha\beta$–peptide complexes are resistant to SDS-induced chain dissociation (4,5,9), and we used this property to measure peptide binding in the folding mixture. Figure 3 shows that folded DR1 exhibits a SDS-resistant $\alpha\beta$–peptide complex band when folded in the presence of Cs, A2, and Ha peptides, all known to promote SDS resistance in the native protein, but not in the presence of the control β_2m peptide or in the absence of peptide. The difference in mobility of the SDS-resistant $\alpha\beta$–peptide with different peptides confirms the presence of peptides in these bands and the ability of the folded DR1 to bind peptide.

The time course of folding was quite slow, with a half-time for folding of approximately 12 h under standard conditions (Fig. 4A). Both folding as measured by ELISA and peptide binding as measured by SDS–PAGE analysis proceeded at the same rate. DR1 has one disulfide bond within the α subunit and two disulfide bonds and one free cysteine within the β subunit. Formation of the intramolecular disulfide bonds in the DR α and β chains can be observed by nonreducing SDS–PAGE as increased mobility upon disulfide-bond formation (Fig. 4B). Formation of intramolecular disulfide bonds characteristic of the native structure proceeded with approximately the same slow kinetics as observed for peptide binding and ELISA reactivity. Neither increased peptide concentration nor addition of protein disulfide isomerase substantially increased the rate of folding.

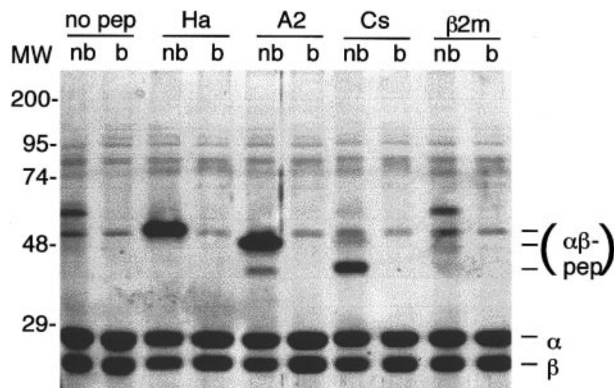

FIG. 3. Folding with different peptides. SDS–PAGE analysis of folding mixtures without peptide (no pep) or containing a fivefold molar excess of DR1-binding peptides Ha, A2, and Cs; control peptide β_2m; with samples boiled (b) or not (nb) before gel analysis. The additional faster migrating band in lane A2, nb, is due to a contaminant in the peptide preparation.

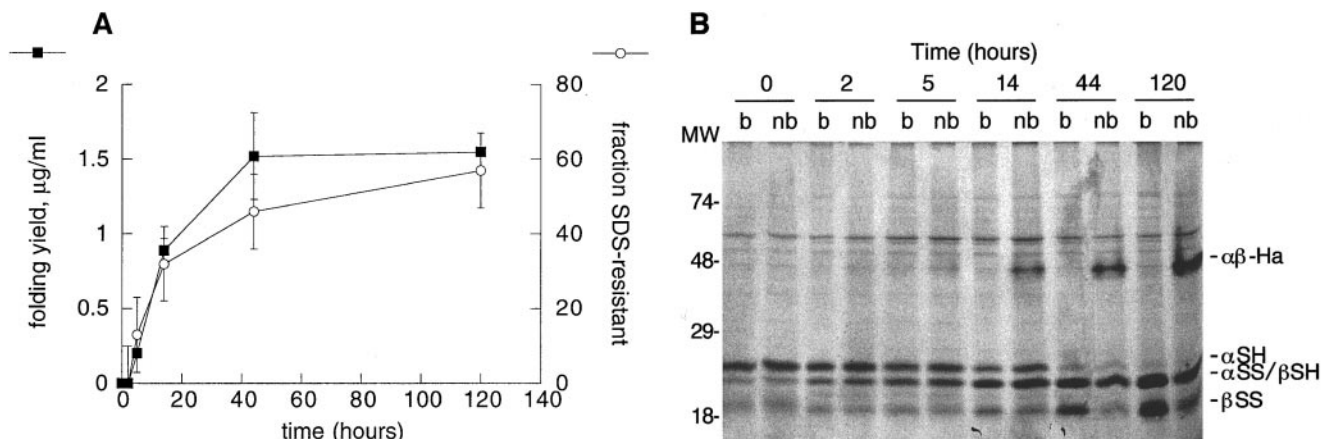

FIG. 4. Folding time course. (A) Samples were removed from a folding mixture containing a fivefold molar excess of Ha peptide at the indicated times and folding yield measured by ELISA (filled squares) or nonreducing SDS–PAGE (open circles). (B) SDS–PAGE, with samples boiled (b) or not (nb) before loading. The α subunit has one and the β subunit has two intramolecular disulfides. Mobilities of subunits containing intramolecular disulfides (αSS, βSS) or with only free cysteines (αSH, βSH) determined by reducing and nonreducing SDS–PAGE of purified DR1 from *E. coli* and Sf9 insect cells.

Characterization of Folded DR1 αβ–Peptide Complex

For the folding experiment in Fig. 5, a folding yield of 45% was determined as the fraction of DR1 resistant to SDS-induced chain dissociation, calculated using the relative staining efficiencies for α (boiled), β (boiled), and $\alpha\beta$–peptide (nonboiled) bands using purified DR1 in Fig. 5 as a standard (see Materials and Methods). Folding yields in other experiments ranged from 15 to 50%. This correlates well with yields determined by ELISA.

Isolation of folded $\alpha\beta$–peptide complexes from the folding reaction mixture was hampered by the low protein concentration and significant viscosity. For large-scale foldings (typically 2 liters or more), $\alpha\beta$–peptide complexes were first collected by a batch ion-exchange absorption step and then eluted and further purified by anion-exchange chromatography. The purified, folded DR1 was >95% $\alpha\beta$–peptide complex, exhibiting essentially no free subunits by nonboiled SDS–PAGE analysis (Fig. 5A). The $\alpha\beta$–peptide complex exhibited a single species by gel filtration with MW ~45,000 kDa (Fig. 5B). Recovery from the batch absorption step was typically 70–90%, and from the anion-exchange chromatography 50–80%. Overall recovery for folding and isolation of $\alpha\beta$–peptide complexes from purified subunits was 12–25%.

DR1–peptide complexes folded and purified from *E. coli* exhibit similar peptide dissociation kinetics to those reported for native DR1. Figure 6 shows the dissociation of ^{125}I-labeled Ha peptide and FITC-labeled Ii peptide from folded *E. coli* DR1. Measured τ values ($1/k_{\mathrm{off}}$) for peptide release of ~222 and ~13 h were found for Ha and Ii peptides, respectively. Others have also observed a relatively slow release of Ha peptide (9,24) and a fast release of Ii peptides (36) from

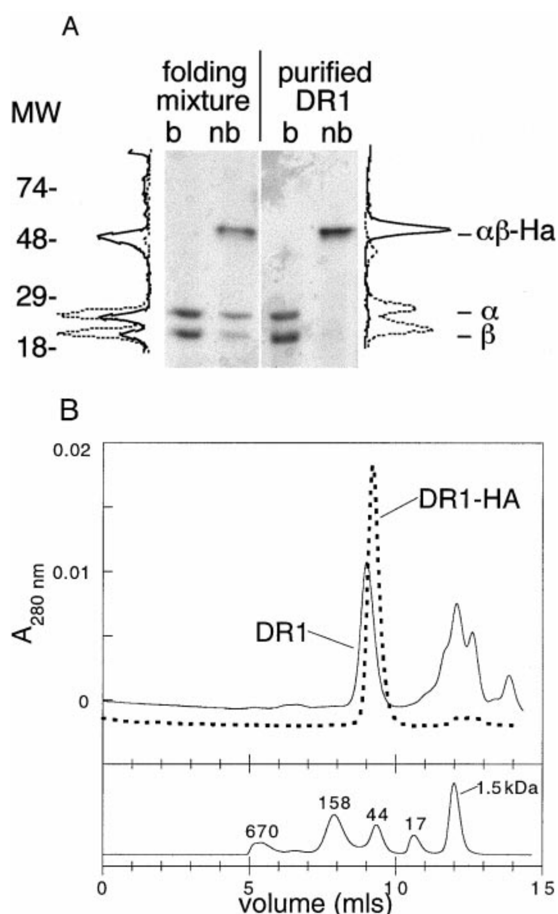

FIG. 5. Folding yield. (A) SDS–PAGE of folding mixture and purified DR1$\alpha\beta$–peptide, with samples boiled (b) or not (nb) before gel analysis. Adjacent densitometric scans correspond to boiled (dashed lines) or not boiled (solid lines) lanes. (B) Gel filtration traces of empty DR1 (solid line) and DR1–Ha (dashed line) are shown above calibration trace of molecular weight standards (Bio-Rad).

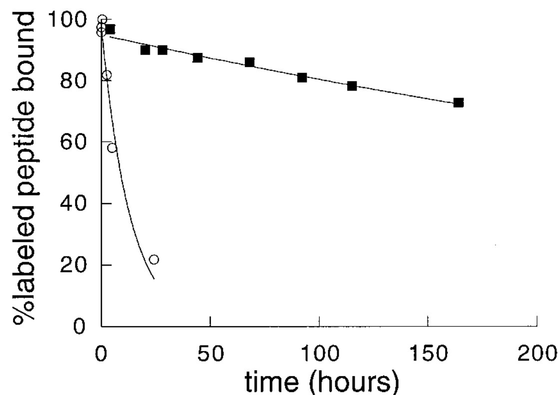

FIG. 6. Peptide release rates. DR1 folded in the presence of ^{125}I-labeled Ha (filled squares) or FITC-labeled Ii (open circles) was isolated and incubated with a large excess of unlabeled peptide. Labeled peptide remaining associated with DR1 was measured by gel filtration at the indicated times. Curves represent single exponential decays with τ = 222 h for DR1–Ha and τ = 13 h for DR1–Ii. Corresponding values for DR1 protein produced in Sf9 cells (9) were 200 h (DR1–Ha) and 16 h (DR1–Ii).

DR1 or other DR alleles. These data further confirm that the DR1–peptide complex we have isolated is folded correctly.

CD analysis of DR1–Ha complex from *E. coli* gave a spectrum (Fig. 7A, thick line) very similar to CD spectra of native DR1 from human LG2 B-cells (37) and of recombinant DR1–Ha from Sf9 insect cells (not shown) with a broad minimum near 220 nm. The DR1–Ha sample was heated to 95°C in 2-degree intervals and the CD signal was monitored at 204 nm. A cooperative denaturation transition with a T_m of 354K (81°C) was observed (Fig. 7B, top). After melting, the sample was scanned again at 4°C, giving the spectrum of heat-denatured DR1–Ha (Fig. 7A, dotted line). These CD data strongly suggest that the *E. coli* expression/*in vitro* folding procedure produces properly folded, thermally stable DR1-Ha.

DR1–αβ Complex Folded in vitro in the Absence of Peptide

ELISA analysis (Fig. 1A) indicated that folding proceeded to high yield in the absence of peptide. However, the purification method used for DRαβ–peptide complex gave extremely low yield for DR1 folded in the absence of peptide, and a milder procedure was developed. Folding mixtures were concentrated and exchanged into glycerol-free buffer by ultrafiltration and then purified by immunoaffinity chromatography using monoclonal antibodies that specifically recognize the folded species (26,27). A major species of approximately 45,000 kDa molecular weight was isolated that contained both α and β subunits when analyzed by SDS–PAGE. This material was essentially free of aggregated protein and exhibited apparent molecular weight similar to but slightly larger than the $\alpha\beta$–peptide complex (Fig. 5B). Overall recovery for folding and isolation of empty $\alpha\beta$ complexes from purified subunits was 5–8%. The empty DR1 isolated in this manner is SDS sensitive as expected for peptide-free $\alpha\beta$-heterodimer (9), but can bind the Ha peptide to give a SDS-stable complex (Fig. 8). In the experiment shown in Fig. 8, 88% of the folded empty DR1 was competent to bind peptide. The activity in other experiments ranged from 60 to 95%.

DR1–peptide complexes isolated from their native B-cell source are extremely resistant to protease digestion (4). We expected that DRαβ complexes folded in the absence of peptide would also be resistant if they indeed had adopted the native fold. To test this, we examined the protease sensitivity of DR1 folded in the presence or absence of peptide. DRαβ or DRαβ–peptide complexes were folded under standard conditions and

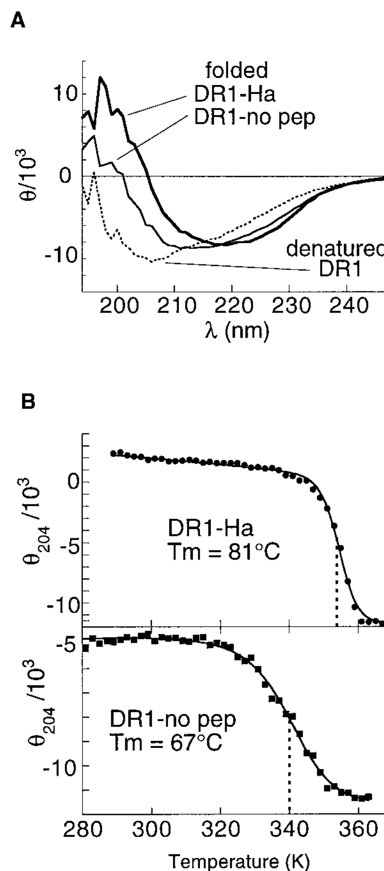

FIG. 7. Circular dichroism of empty and peptide loaded DR1. (A) CD spectra of DR1–Ha (thick line) or DR1 folded without peptide (thin line). Both proteins showed similar CD spectra after heat denaturation (dashed line). All spectra obtained at 4°C. (B) Thermal denaturation of DR1–Ha (top) and DR1 without peptide (bottom). CD signal measured at 204 nm. Curves indicate a least-squares fit to a seven-parameter equation describing a two-state transition with T_m as shown.

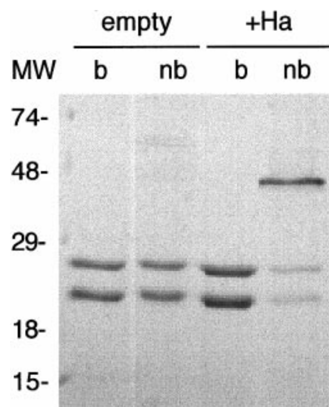

FIG. 8. Peptide binding by folded empty DR1. DR1 (18 μM) folded and isolated in the absence of peptide (empty) was incubated with a fivefold molar excess of Ha peptide (+Ha) for 36 h at 37°C before SDS–PAGE with samples boiled (b) or not (nb) before loading.

digested with papain. As a control, DRα and DRβ subunits that had been diluted separately into folding solution were combined immediately before digestion. Figure 9 shows that DR$\alpha\beta$ and $\alpha\beta$–peptide in folding mixture are both resistant to papain digestion under conditions where individual subunits are completely digested. In both cases approximately 30% of the total protein in the folding mixture was resistant to papain digestion, consistent with folding yields estimated by ELISA.

CD analysis of DR1 from *E. coli* folded without peptide gave a spectrum (Fig. 7A, thin line) with a broad minimum at 214 nm, shifted somewhat relative to that of DR1–Ha (Fig. 7A, thick line) but substantially different from denatured DR1–Ha (Fig. 7A, dotted line). The DR1 folded without peptide was heated to 95°C in 2-degree intervals and the CD signal was monitored at 204 nm as described previously (Fig. 7B, bottom). A cooperative denaturation with a T_m of 340K (74°C) was observed. After melting, the sample was scanned again at 4°C, giving the spectrum of heat-denatured DR1–no pep (figure 7A, thin line) which is indistinguishable from the heat-denatured DR1–Ha. These CD data suggest a folded structure for the DR1 that we have refolded and isolated in the absence of peptide.

DISCUSSION

Under appropriate conditions, refolding *in vitro* of HLA–DR1 subunits produced in *E. coli* inclusion bodies proceeds in relatively high yield in the presence or absence of peptide. The isolated protein is free of aggregates and monomeric α or β subunits and has adopted the native fold as judged by circular dichroism, peptide-binding specificity, resistance to protease digestion, reactivity with conformationally sensitive antibodies, and peptide-dependent resistance to SDS-induced chain dissociation. The absence of glycosylation and proteolytic degradation in the refolded material provides a homogenous protein ideal for structural and biophysical studies. We have used the folded DR1–Ha peptide complex to prepare crystals that diffract to <2.4 Å (not shown). The *in vitro* folding method reported here may be useful for isolation of DR1–peptide complexes that are difficult to obtain using other sources of DR1. For example, we have used a modified version of this procedure to prepare DR1 complexes with fast-dissociating peptides such as A_9KA_3 ((38) and S. Sadegh-Nasseri, personal communication). Although in principle the refolding procedure presented here should be applicable to other class II proteins, we have observed difficulties in expression and/or refolding of certain other human allotypes (unpublished observations).

Of the conditions examined during the optimization of folding conditions, the ion-exchange purification of the denatured subunits appears to be crucial, as has been previously observed (12). We suspect that the anion-exchange purification increased refolding efficiency by removing nonprotein contaminants such as lipids or nucleic acids. Because increased glycerol and decreased protein concentration promote folding of denatured class II subunits (Fig. 1 (11)) we speculate that an aggregation pathway may be competing with the correct folding mechanism. The time course of folding in this system is very slow (Fig. 4), suggesting a slow folding step or a slow recovery from a nonnative species. However, no folding intermediates distinguish-

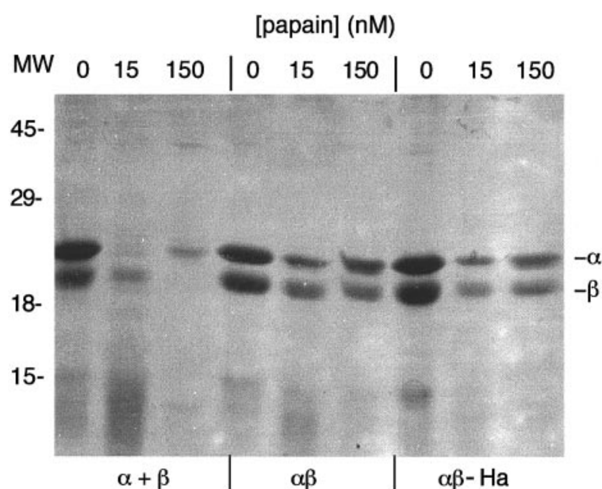

FIG. 9. Papain resistance of DR1 in folding mixture. Papain at the indicated concentrations was used to digest samples of α- and β-denatured subunits combined in folding mixture immediately before digestion ($\alpha + \beta$), DR1 folded in the absence of peptide ($\alpha\beta$), or DR1 folded with a fivefold molar excess of Ha peptide ($\alpha\beta$-Ha). Total DR1 concentrations were 2 μM. All samples were boiled before reducing SDS–PAGE analysis. Papain comigrates with the α subunit and is barely visible in the 150 nM lanes.

able by antibody reactivity or by native gel electrophoresis could be observed to accumulate during the folding reaction, and inclusion of protein disulfide isomerase in the folding mixtures did not increase the rate of folding.

Empty class II MHC proteins produced by other methods (9,10) or as produced *in vivo* (39) tend to aggregate in the absence of peptide. This aggregation has been suggested to play a role in preventing empty class II molecules from reaching the cell surface (40). Aggregation may be alleviated in our refolding procedure by the presence of glycerol and by maintaining the protein at low temperature. The empty HLA–DR1 is significantly more stable than empty class I MHC proteins (41,42) or class I MHC complexes with weakly binding peptides (43), which exhibit cooperative thermal denaturation at ~45°C and have been reported to denature at 37°C *in vivo* (44). Recently, an empty form of I-E^k, the murine homologue of HLA–DR, also has been shown to be relatively stable (42), with T_m ~62°C and ΔH ~45 kcal/mol at pH 7, similar to the values reported here for HLA–DR1 (74°C and 35 kcal/mol). Peptide binding stabilized I-E^k against thermal denaturation (ΔT_m = 8°C (42)) as observed for Ha peptide binding to DR1 (ΔT_m = 7°C).

The conformation of the empty DR1 appears to be somewhat different from that of DR1–Ha, as indicated by alterations in the CD spectrum in the region λ ~225 nm (increase) and λ ~200–215 nm (decrease) (Fig. 7A). The spectrum is distinct from that for either DR1–HA or denatured DR1 (or any combination thereof) as shown by the lack of an isodichroic point. The differences in the CD spectrum cannot be accounted for by the spectrum of the bound Ha peptide, which would be expected to contribute a negative feature centered at λ ~204 nm with θ ~−1600 (45) for 13 residues of polyproline type II-like helix. Conformational alterations associated with peptide binding by class II MHC molecules have been proposed previously (9,17,42,46–48) but the exact nature of any conformational difference between the empty and peptide-loaded class II MHC proteins remains unclear. Study of the empty refolded DR1 may prove useful in investigation of this structure and in understanding the mechanism of peptide loading and antigen presentation by class II MHC proteins.

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