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Heavy chain of HLA-A and HLA-B antigens is conformationally labile: A possible role for β_2-microglobulin

(histocompatibility antigens/circular dichroism/β-pleated sheet/chain association)

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ABSTRACT The three-dimensional organization of HLA antigens has been investigated by spectroscopic and immunochemical techniques. Measurement of the circular dichroism shows that in papain-solubilized HLA the heavy chain as well as the previously studied light chain (β_2-microglobulin) consists predominantly of β-pleated sheet structures. When heavy chain is separated from the light chain under denaturing conditions and is allowed to renature, about 50% of the β structure is lost, concomitantly with most of the alloantigenic activity. Analysis of the two acid-cleaved fragments of HLA-B7 heavy chain shows that β structure is preferentially lost from the COOH-terminal region of the heavy chain. Exposure to denaturants per se does not inevitably result in irreversible loss of antigenic activity. However, recovery of antigenic properties does seem to depend on reassociation of the two chains. The results reported here provide further evidence for (*i*) the similarity of HLA antigens and immunoglobulins at the three-dimensional level and (*ii*) two distinct and physiologically important conformations of the HLA heavy chain, depending upon whether it is associated with the light chain.

HLA, the major human histocompatibility antigens,* are integral membrane proteins present on most cells of the body (1). They are composed of a 42,000-dalton† glycoprotein (heavy chain) noncovalently associated with an extracellular light chain of 12,000 daltons (2). The light chain is identical to β_2-microglobulin (β_2m), a protein originally isolated from the urine of patients with kidney dysfunction (3). Multiple differences exist among the amino acid sequences of the HLA heavy chains of different alloantigenic specificities (4), which presumably relate to their role in graft rejection and the restriction of T cell-mediated lymphocytotoxicity. These differences seem, however, to be limited to about 20% of the residues of the heavy chain, whereas the remaining 80% of its residues plus the entire sequence of the light chain are common to all HLA specificities examined. Thus, different HLA molecules have certain common features, including overall chain structure and association, mode of membrane insertion, susceptibility to limited papain cleavage, glycosylation pattern, and reactivity with certain nonallospecific monoclonal antibodies. Most of these features also appear in the homologous antigens of the mouse (H-2D,K) and of other mammalian and avian species (5). The preservation of these common properties during evolution suggest they may be important functionally.

The complete amino acid sequence of HLA-B7 has been determined (4) and from limited comparisons with other HLA and H-2 antigens is believed to provide a representative structure for all major histocompatibility antigens (2). The extracellular portion of this molecule, which can be isolated by papain solubilization (6), may be divided into four regions on the basis of chain structure, sequence homology data, and disulfide loop arrangement (4). These are the 100-residue-long light chain (β_2m) and three consecutive 90-residue segments of the heavy chain (α) denoted α1, α2, and α3. The α1 region (residues 1–90) has no cysteines and contains the molecules's single asparagine-linked carbohydrate side chain (7). The α2 (residues 91–180) and α3 (residues 181–270) regions each contain a disulfide loop.

Similarities between HLA antigens and immunoglobulins that have been cited (8–11) include: (*i*) both are centrally involved in immune responses; (*ii*) they have a basic two-chain structure with extensive noncovalent interactions; (*iii*) the heavy and light chains of both Ig and HLA contain linearly arranged disulfide loops of similar size; (*iv*) the light chain (β_2m) (12) and α3 region of the HLA heavy chain (11) show amino acid sequence homology to Ig constant domains; (*v*) as in Ig, there is some indication of internal homology within the HLA heavy chain (4); (*vi*) β_2m binds the first component of complement, similarly to monomeric Ig or its Fc fragment (13).

Comparison of the secondary and tertiary structures of Ig and HLA has been limited so far to the finding that β_2m, like Ig and its fragment, contains a high percentage of β-pleated sheet (13, 14). A preliminary report of circular dichroism (CD) studies on HLA indicated that the heavy chain is also rich in this structure (15). In this paper these results are confirmed and extended.

MATERIALS AND METHODS

HLA Antigens. Papain-solubilized HLA antigens from B lymphoblastoid cell lines JY (A2,2; B7,7) and LB (A28,28; B40,40) were purified as described (7). Further purification was obtained by lentil lectin affinity chromatography (7, 16). Final antigen solutions were in 0.15 M NaCl/0.02 M Tris·HCl, pH 8.0 (Tris/NaCl) and when necessary were concentrated by using an Amicon PM 10 membrane.

β_2m was purified from the urine of patients with kidney dysfunction (17). HLA heavy chain was prepared by two different methods. (*i*) HLA (1 mg/ml) was incubated in 6 M Gdn·HCl for 2–4 hr at 25°C, chains were separated on a Bio-Gel P 300 column equilibrated with 6 M Gdn·HCl, and fractions were dialyzed extensively against Tris/NaCl. (*ii*) HLA was incubated at 25°C for 3 hr in 3 M NaSCN and then separated on a G-100 column equilibrated in Tris/NaCl and preloaded with 3 times the sample volume of 3 M NaSCN (see ref. 18). The emerging heavy chain was in Tris/NaCl.

Abbreviations: CD, circular dichroism; β_2m, β_2-microglobulin (the light chain of HLA); α, the HLA heavy chain; α1, α2, α3, regions of the α chain.

* In this paper, HLA will collectively refer to papain-solubilized HLA-A and -B antigens, consisting of a 34,000-dalton heavy chain (α) and an 11,600-dalton light chain (β_2m).

† This molecular weight is based upon the amino acid sequence of the detergent solubilized HLA-B7 heavy chain (4).

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Acid cleavage of HLA heavy chain was performed as described (19) in the presence of 0.5% 2-mercaptoethanol and under nitrogen to prevent oxidation of tryptophan and disulfide bridges. Fragments were separated on a column of Sephadex G-75 superfine in 1 M propionic acid and then dialyzed against Tris/NaCl. HLA antigen activity was measured by inhibition of antibody-mediated immune lysis as described (20).

Protein Quantitation. UV absorption spectra were measured on a Cary 219 spectrophotometer. Extinction coefficients ($\epsilon_{280}^{1\%}$ in dl g^{-1} cm^{-1}) of 16.7 for β_2m, 19.1 for the HLA-B7 heavy chain, and 21.4 and 19.7 for its acid fragments ac-1 and ac-2, respectively, were calculated from the tryptophan and tyrosine contents (4). Identical values were assumed for HLA-B40.

CD Spectra. These were recorded on a Jasco J-20 spectropolarimeter at 25°C at speeds of 1–5 nm/min and at a sensitivity of 1 millidegree/cm. Cylindrical cells with pathlengths of 1, 5, 10, or 20 mm (Hellma) were used. Spectra were normalized per residue or per mol by using molecular weights and number of residues from the HLA-B7 sequence (4). Secondary structure contents were calculated by a modification (unpublished) of a published procedure (21). A nonlinear least squares fit program written by L. Cantley was used on a Data General Nova 1200 minicomputer.

RESULTS

CD Spectra of HLA and Its Component Chains. *Near-UV region.* Near-UV CD spectra of two different HLA antigens (B7, B40) and urinary β_2m (reproduced from ref. 13) are shown in Fig. 1. All three spectra show a series of bands that arose from electronic transitions of aromatic chromophores in an asymmetric environment. For both HLA-B7 and HLA-B40, peaks with positive ellipticity were seen at about 288 and 295 nm, which may be ascribed to tryptophan transitions. At lower wavelengths (250–275 nm), where tyrosine and phenylalanine transitions usually occur, the two spectra were more different. β_2m had a similar series of positive peaks at different wavelengths. The intensity of the CD bands of β_2m was roughly half that of HLA on a per residue basis. Because β_2m comprises one-fourth of the HLA molecule by weight, its contribution to the total molar ellipticity of HLA is roughly one-eighth, assuming no radical changes in its spectral properties on binding to the HLA heavy chain. Therefore, most of the intensity of the peaks in the near-UV spectrum of HLA was from the spectral component of the HLA heavy chain. Comparison of the HLA-B7 and -B40 spectra suggests conservation of the environment of tryptophan relative to tyrosine and phenylalanine.

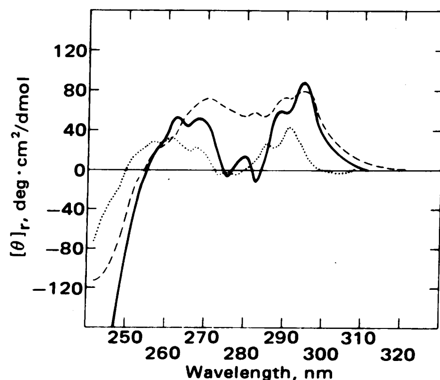

FIG. 1. Near-UV CD spectra of HLA of two different specificities and of β_2m, expressed as ellipticity per mean residue (θ_r). —, HLA-B7 (0.36 mg/ml in a 20-mm-pathlength cell); - - -, HLA-B40 (0.78 mg/ml in a 10-mm-pathlength cell); ·····, urinary β_2m (reproduced from ref. 13).

This is consistent with the known amino acid sequences for the heavy chains of HLA-B7 (residues 1–180), HLA-A2 (residues 1–100) (4), and their murine counterpart H-2K^b (22) (residues 1–173). For these NH_2-terminal regions, where most alloantigenic variation is thought to occur (4), only one substitution at positions occupied by tryptophan was observed (5 positions compared), whereas substitutions at 7 positions occupied by tyrosine or phenylalanine (20 positions compared) were seen.

The aromatic CD spectra of β_2m and HLA heavy chain were not expected to be similar because their tryptophan, tyrosine, and phenylalanine compositions are quite different (4, 12). Despite these differences, the overall features of the near-UV CD spectra of the two chains are quite similar. This is probably fortuitous but may also reflect some common structural features of the two chains.

Far-UV region. Fig. 2 shows the far-UV CD spectra of HLA-B7 and of urinary β_2m. The β_2m spectrum is similar to that previously reported (13, 14). Both spectra are dominated by a negative band at 217–219 nm which crosses over to positive values around 210 nm. These features are characteristic of transitions associated with peptide bonds in the β-pleated sheet structure (21). The positive band at 232 nm in the β_2m spectrum has been ascribed to a tyrosine transition (13), and it is possible

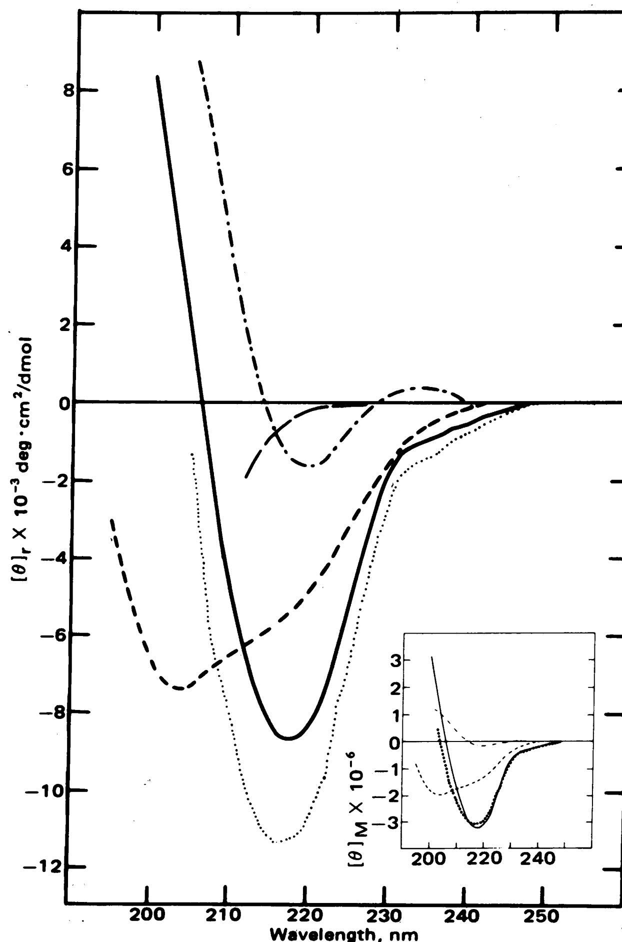

FIG. 2. Far-UV CD spectra of HLA and its constituent chains, expressed as ellipticity per mean residue. —, HLA-B7 (0.14 mg/ml); - - -, the Gdn·HCl-separated HLA-B7 heavy chain (0.13 mg/ml); -·-, urinary β_2m (0.19 mg/ml); ·····, the β_2m-complexed heavy-chain spectrum calculated by subtracting the molar spectrum (*Inset*) of β_2m from that of HLA-B7 and rescaling to per-residue; – –, the Gdn·HCl-separated HLA-B7 heavy chain (0.13 mg/ml) in 4.5 M Gdn·HCl. All spectra were taken in a 1-mm cell.

that the same is true for the shoulder seen in the HLA-B7 spectrum in the same region. The far-UV CD spectra of HLA-B40 and HLA-A2 were also measured and were found to be very similar to that of HLA-B7.

The spectral component contributed by the heavy (Fig. 2) chain was calculated by subtracting the molar ellipticity of β_2m from that of HLA-B7 (Fig. 2 *inset*). As before, it was assumed that the spectrum of urinary β_2m is identical to that of the HLA light chain when complexed with the heavy chain. This assumption is supported by reports that isolated β_2m has a stable conformation which may be fully recovered after denaturation in 6 M Gdn·HCl (13) and that certain monoclonal antibodies recognize both forms of β_2m equally well (23). In addition, it may be seen that on a molar basis the spectral contribution of β_2m to HLA was very small. Thus, even relatively large differences between the spectra of free and heavy chain-bound β_2m would have little effect on the calculated heavy chain spectrum. This calculated spectrum for the HLA heavy chain in association with β_2m was also dominated by spectral features characteristic of the β-pleated sheet conformation, having a negative peak at around 218 nm and an apparent crossover to positive values at around 205 nm.

The relative content of the three main types of secondary structure was calculated from HLA-B7, β_2m, and calculated heavy chain spectra and shown in Table 1. The whole HLA antigen contained about 80% β-pleated sheet, with small amounts of α helix and unstructured conformation. β-Pleated sheet was also the predominant feature in the secondary structure of β_2m and the HLA heavy chain when associated with β_2m. Because problems were encountered in the analysis of the β_2m spectrum (see legend to Table 1), any interpretation of the difference in random structure content between HLA and its component chains should be considered with caution.

Far-UV CD Spectrum of Isolated HLA Heavy Chain. HLA-B7 heavy and light chains were separated by gel exclusion chromatography in 6 M Gdn·HCl and then allowed to renature by dialysis against Tris/NaCl. The far-UV CD spectrum of the renatured heavy chain is shown in Fig. 2. Two features of this spectrum are apparent: (*i*) it it different from the spectrum of the same chain in 6 M Gdn·HCl, indicating that the renatured chain is not in a completely unfolded state, and (*ii*) it is significantly different from the calculated spectrum of the heavy chain when complexed with β_2m, having a negative peak at 204 nm and an extrapolated crossover to positive values below 195 nm. Table 1 shows that the separated, renatured heavy chain has less than half of the β-pleated sheet and 3 times as much random structure as the β_2m-complexed heavy chain. No significant differences in the low α helix content were observed.

HLA-B7 heavy chain was also prepared by using 3 M sodium thiocyanate to separate the chains. This preparation displayed a slightly different spectrum with a negative peak at about 212 nm. A second preparation of Gdn·HCl-separated heavy chain displayed a negative peak at 210 nm. However, for all renatured preparations of the HLA heavy chain, both the negative peak and the crossover points to positive ellipticity were at significantly shorter wavelengths than in the spectra of HLA and its calculated heavy chain component. In all the heavy chain spectra, a shoulder at 218 nm, probably due to a β-pleated sheet component, was observed. The slight variations between spectra of different heavy chain preparations may be related to the conformational lability of the HLA heavy chain. However, the main spectral features are invariant, which suggests the existence of some regions of well-defined secondary structure in the denatured, separated, and renatured HLA heavy chain.

Further Characterization of Isolated HLA Heavy Chain: Alloantigenic Properties. Preparations of HLA-A2, -B7, and -B40 heavy chains, separated under denaturing conditions and allowed to renature, were 1/20th to 1/1000th as active as inhibitors of specific HLA alloantisera than was the intact HLA antigen as exemplified in Fig. 3. Assuming that the nonpolymorphic β_2m does not interact directly with alloantibodies, this would suggest that a major conformational change has occurred, in agreement with the CD results. In a recent study (24), it was found that Gdn·HCl-separated HLA-A2 heavy chain had residual affinity towards an HLA-A,B,C specific monoclonal antibody (W6/32), which suggests that the low activity observed here may be a bulk property of the heavy chain preparations rather than due to low levels of contaminating HLA.

That the denaturing conditions used to separate the HLA heavy and light chains need not irreversibly destroy the alloantigenic properties of HLA was shown by the following type of experiment. HLA-A2 antigens were made 6 M in Gdn·HCl

Table 1. Relative content of α helix, β-pleated sheet, and unstructured conformation in HLA antigens and their component chains

Protein	α helix	β sheet	Random
HLA	0.05 (0.08)	0.79 (0.89)	0.16 (0.03)
β_2m*	— (0.10)	— (0.65)	— (0.25)
Heavy chain$_{calc}$	— (0.09)	— (0.70)	— (0.21)
Heavy chain$_{obs}$	0.16 (0.04)	0.27 (0.33)	0.57 (0.63)
ac-1	0.29 (0.10)	0.19 (0.31)	0.52 (0.60)
ac-2	0.19 (0.14)	0.17 (0.16)	0.64 (0.70)
ac-1 + ac-2	0.24 (0.07)	0.20 (0.31)	0.55 (0.62)

Calculated fractions of the three major secondary structural types in HLA and its component chains and fragments. The values are considered to be significant to within ±15%. Coefficients are calculated in wavelength ranges of 200–250 nm and, in parentheses, for 208–250 nm. Differences between the two modes of calculation arise from the relatively high error involved in measurements at 200–208 nm, both in the spectra presented here and in those of the model polypeptides used as spectral modes (21).

* The computer fits were unsatisfactory for the β_2m spectrum and those values are significant only to ±25%. This is probably due to the very high wavelength (214 nm) point of crossover to positive values which, although very reproducible, does not conform with any of the three spectral modes used in the analysis.

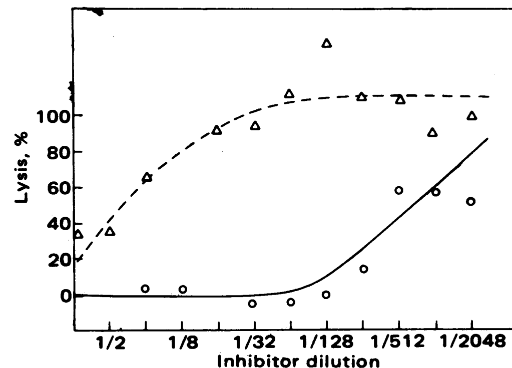

FIG. 3. Inhibition of complement-mediated anti-HLA-B40-dependent lymphocytotoxicity of LB cells by HLA-B40 (O) and its 3 M NaSCN-separated heavy chain (Δ). Original (1:1) antigen concentrations were: HLA-B40, 0.079 mg/ml; heavy chain, 0.068 mg/ml.

and then divided in two parts. One part was subjected to gel exclusion chromatography at 25°C to separate chains, whereas the other part was incubated at 25°C. Separated heavy and light chain fractions and the Gdn·HCl-treated mixture of heavy and light chains were then dialyzed against Tris/NaCl and tested for specific alloantigenic activity. As shown in Table 2, the renatured mixture of heavy and light chains possessed 50% of the specific activity of the buffer-treated control, whereas the separated heavy chain had only 1–2% of this activity. Furthermore, anti-β_2-microglobulin affinity chromatography (25) of the renatured mixture of heavy and light (β_2m) chains showed that most of the heavy chains had reassociated with the light chains. However, the separated heavy and light chains did not readily associate when mixed in buffer.

Characterization of Acid-Cleavage Fragments of HLA Heavy Chain. The separated heavy chain has a higher content of random structure and a lower content of β-pleated structure than the same chain when complexed with the light chain (β_2m). It has been suggested (15) that the main site of structural change is α1, the NH_2-terminal region. To further check this point, fragments of the heavy chain were produced. Proteolysis was not successful, because the highly susceptible heavy chain was invariably degraded to small peptides with no observable intermediates (7) and, thus, chemical methods were used. The heavy chain of most HLA specificities, including B7 and B40, can be specifically cleaved at a single aspartyl-proline bond into two large fragments. In HLA-B7, this cleavage occurs between residues 183 and 184, between the two disulfide loops, and near the putative boundary between the α2 and α3 regions (4, 11, 19). Because α3 resembles a constant immunoglobulin domain in its primary and possibly its secondary and tertiary structure, it was considered most likely that in the isolated heavy chain this region would contain much of the β-pleated sheet structure, whereas the α1 and α2 regions would contain much of the random structure.

Treatment with 70% formic acid (19) of the Gdn·HCl-separated HLA-B40 heavy chain gave two well-defined fragments: the larger, ac-1, includes the α1 and α2 regions; the smaller, ac-2, roughly corresponds to the α3 region of the heavy chain.

The far-UV CD spectra of the two acid-cleaved fragments are shown in Fig. 4. By comparison with the spectrum of the heavy chain in 6 M Gdn·HCl, it is seen that none of the fragments is completely unfolded. The ac-1 fragment appears to

Table 2. Alloantigenic activity of denatured and renatured HLA preparations

Inhibitor	HLA-B7 activity*	HLA-A2 activity*	A_{280}	HLA-A2 specific activity†
Separated, renatured HLA-A2 heavy chain (α)	0	210	0.61	660
Separated, renatured light chain (β_2m)	0	160	0.12	—
Gdn·HCl-treated, renatured HLA-A2 (heavy and light chains	0	2100	0.23	23,600
Buffer-treated HLA-A2 (heavy and light chains)	10	4400	0.24	46,700

* Dilution of antigen required to give 50% inhibition of immune cytolysis.

† Activity of a solution of the inhibitor containing 1 mg of heavy chain per ml.

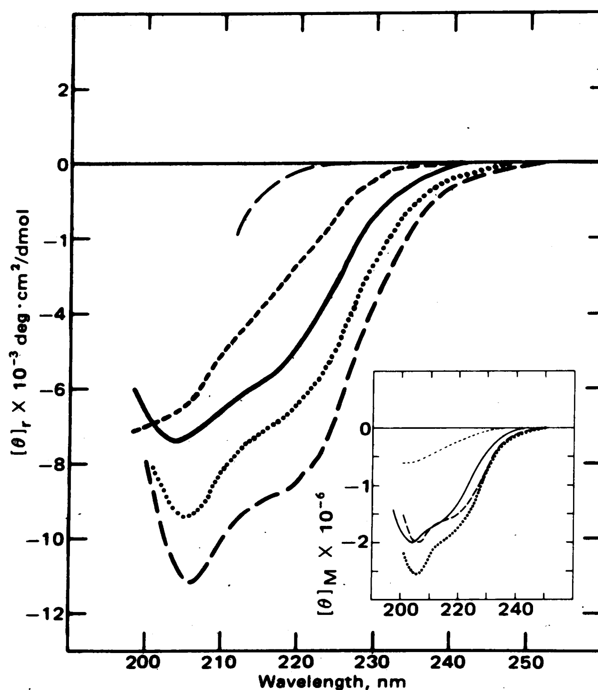

FIG. 4. Far-UV CD spectra of the acid-cleaved fragments of Gdn·HCl-separated HLA-B40 heavy chain, expressed at ellipticity per mean residue. (– – –), the large NH_2-terminal acid-cleaved fragment ac-1, containing the α1 and α2 regions; - - -, the small, COOH-terminal acid-cleaved fragment ac-2, corresponding to the α3 region; ·····, the sum of the molar ellipticity spectra (*Inset*) of the two fragments. For comparison, the spectrum of the Gdn·HCl-separated HLA-B7 heavy chain in Tris/NaCl (—) and in 6 M Gdn·HCl (— —) are reproduced from Fig. 2. Fragments were 0.030 mg/ml and spectra were taken in a 5-mm cell.

have a more ordered structure than the ac-2 fragment (Table 1). The spectrum of ac-1 resembles that of the whole heavy chain, indicating possible preservation of heavy chain structural features in this fragment. Such similarity is less apparent for ac-2. The sum of the spectra of the two fragments has a similar shape to that of the whole heavy chain (Fig. 4), but shows some differences in intensity. Considering the errors (5–10%) involved in the process of normalization of the three spectra, a difference of about 20% is within experimental error, and, thus, the spectrum of the heavy chain may be a direct summation of the spectra of its two fragments. This idea is supported by the analysis in Table 1. Assuming that the spectral component of the α3 region resembles that of the ac-2 fragment, these results suggest, contrary to predictions, that the α3 region is more disordered than the combined α1 plus α2 regions.

DISCUSSION

Both the light chain (β_2m) and the α3 region of the HLA heavy chain have amino acid sequence homology to Ig constant domains and to each other (11, 12). The nature of the conserved residues suggested that β_2m and α3 may also resemble Ig domains in their three-dimensional folding. Furthermore, because the α1 and α2 regions are about the same length as the α3 region, both α2 and α3 contain an Ig-like disulfide loop, and α1 and α2 are homologous to each other, it may be hypothesized that all three HLA heavy chain regions correspond to structural domains. The light chain (β_2m) and α3 region of the heavy chain would thus resemble Ig constant domains, whereas α1 and α2 could be Ig-like or of different type. The high β-pleated sheet content of the β_2m-bound heavy chain is consistent with at least two regions being rich in this secondary structure and

similar to Ig-like domains in at least this respect. A feature of Ig structure is the lateral noncovalent interactions between domains. It is tempting to speculate that in HLA similar interactions occur between the homologous, Ig-like β_2m and α3 regions. However, no direct evidence exists to support this hypothesis and one report (26) suggests that β_2m may interact with the α1 and α2 regions. It is possible that all three heavy chain regions interact with β_2m which may correlate with the effect of β_2m on the heavy chain conformation.

Spectroscopic and immunochemical analysis show that the HLA heavy chain, when renatured after isolation by denaturants, has a strikingly different conformation than when in the complex with β_2m. Much of the β-pleated sheet structure present in the complexed heavy chain is lost in the dissociated heavy chain, and the results obtained with the acid-cleaved fragments indicate preferential loss in the α3 region. This was unexpected, because the homologous, isolated Ig $C_\gamma 3$ (27) domain and β_2m (13) independently refold after Gdn·HCl denaturation. One explanation is that the acid or papain cleavage points deprive this region of a segment essential for correct refolding of the isolated chain.

Treatment with 6 M Gdn·HCl does not produce an irreversible loss of conformation, suggesting that the altered conformation of the isolated heavy chain is not an artifact of preparative procedures. This conclusion is substantiated by the similarity between HLA heavy chain produced by denaturation and biosynthetically produced HLA heavy chain, when not associated with β_2m (ref. 28; M. S. Krangel, personal communication). Both species are recognized by an antiserum raised against the isolated HLA heavy chain but do not react with alloantibodies and monoclonal antibodies raised against the whole HLA molecule. Both heavy chain preparations are highly susceptible to proteolysis, whereas the heavy chain–β_2m complex is not. Also, biosynthetically produced heavy chain does not readily associate with β_2m under some conditions (28), similar to the Gdn·HCl separated chain.

The significance of the radical conformational change that β_2m produces in the HLA heavy chain can only be speculated upon. One hypothesis is that, in the process of evolving to produce a highly polymorphic molecule, the heavy chain has lost some stabilizing structural features and become dependent upon β_2m for its native conformation. Such a situation may be advantageous in allowing further mutation with fewer structural restraints. Alternatively, the β_2m-induced conformational change may serve as a control in biosynthesis, thus explaining the inability of non-β_2m-associated heavy chain to be expressed at the cell surface (28). There is also the possibility that the conformational lability of the HLA heavy chain may be related to the apparent ability of these molecules to interact with a wide range of foreign antigens to permit recognition by specific immune T cells.

Note Added In Proof. Recently, CD spectra similar to some of those presented here were reported elsewhere (29). A discrepancy in calculated β-pleated sheet contents may be ascribed to their different method of analysis.

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