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Localization of the Sites of Iodination of Human β_2-Microglobulin: Quaternary Structure Implications for Histocompatibility Antigens†

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ABSTRACT: Human urinary β_2-microglobulin (β_2m) and papain-solubilized human histocompatibility antigen HLA-B7 were iodinated with iodogen and the sites of iodination determined. In the case of free urinary β_2m, four of the six tyrosines were modified to some degree. Two of these were heavily iodinated (tyrosine-63 and -67) while two were lightly iodinated (tyrosine-10 and -26). In the case of β_2m iodinated in the intact HLA-B7 complex, only one of these tyrosines was modified substantially (tyrosine-67). β_2m iodinated at either of the two major sites exchanged into the HLA-B7 complex, whereas β_2m iodinated at either of the two minor sites did not exchange at all. The relationship of these findings to the quaternary structure of HLA is discussed.

Histocompatibility antigens of the class I type contain two noncovalently associated polypeptides of molecular weights 44 000 and 12 000 [see Ploegh et al. (1981) for a recent review]. The light chain, β_2-microglobulin (β_2m),[1] is also found free in the blood and the urine. The heavy chain, which spans the plasma membrane, is found on the plasma membrane of all nucleated cells in association with β_2m and is highly polymorphic, as defined by both antibodies and cytotoxic T cells.

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[1] Abbreviations: β_2m, β_2-microglobulin; HLA, class I human histocompatibility antigen; u-β_2m, urinary β_2m; c-β_2m, β_2m isolated from HLA-B7; HLA_{pap}, papain-solubilized HLA; TSAO8, 10 mM Tris, pH 8.0/140 mM NaCl/3 mM NaN_3/0.1 mg/mL ovalbumin; HPLC, high-performance liquid chromatography; $NaDodSO_4$, sodium dodecyl sulfate; Ig, immunoglobulin; C_L, Ig light chain constant region; C_H1, Ig heavy chain first constant region; Tris, tris(hydroxymethyl)aminomethane; TLC, thin-layer chromatography; EDTA, ethylenediaminetetraacetic acid.

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A heavy chain–β_2m complex, which retains all of the serological activity, can be solubilized from the membrane by papain. The immunological activity of the heavy chain is lost upon separation from β_2m, and this activity has not been successfully restored by reassociation of the separated chains. However, free β_2m can exchange into the complex at 37 °C (Hyafil & Strominger, 1979), presumably because the free heavy chain intermediate is stable for short periods of time.

The amino acid sequences of both the heavy chain of HLA–B7 and β_2m have been determined. The α_3 domain of the heavy chain and β_2m are both homologous to immunoglobulin domains (Peterson et al., 1972; Cunningham et al., 1973; Orr et al., 1979). Although the quaternary structures of many IgG domains have been solved by X-ray crystallography [e.g., Saul et al. (1978)], no such data have yet been obtained for HLA antigens. In addition, unlike immunoglobulins, whose function at the molecular level is very clear, the molecular role of HLA is obscure. Since Ig and HLA appear to be central molecules in serology and cellular immunology, respectively, it is tempting to speculate that the primary sequence homology reflects a tertiary and quaternary structure homology. This possibility is strengthened by circular dichroism data, which suggest that both the HLA heavy chain and β_2m have large amounts of β structure, which is typical of IgG domains (Isenman et al., 1975; Lancet et al., 1979; Trägårdh et al., 1979b). Hence, β_2m and the α_3 domain of HLA may interact together as Ig domains, perhaps with the four-stranded faces together as in Ig constant domains.

On the basis of these ideas, two independent models that predict a particular strand alignment for the β-pleated sheets of β_2m have been proposed (Becker et al., 1977; Cohen et al., 1980). However, no chemical evidence has yet been obtained that identifies the strands that comprise the putative interdomain interface. One approach is chemical modification studies, which can determine the reactivities of particular residues in different environments. The study of tyrosine iodination is especially useful because all six tyrosines of β_2m are thought to lie on the β strands. In addition, iodinated β_2m can be used to measure the exchange reaction and can be labeled to high specific activity. Since iodination results in a single-atom substitution without alteration of charge, there should be little perturbation of the native structure. Tyrosine residues that are easily iodinated would be expected to have their phenolic functional groups facing into the solvent, instead of facing toward the interior of the molecule. We have found that four of the six tyrosines of urinary β_2m are iodinated to some degree, while only one tyrosine is modified in papain-solubilized HLA–B7. In addition, β_2m that is iodinated at two of these tyrosines is not exchangeable. These data strongly suggest that the four-stranded face of β_2m interacts with the HLA heavy chain, and a model is presented that matches the data to the known structure of C_L and C_H1 of IgG New.

Materials and Methods

Materials. Urinary β_2m was a gift from Arnold Sanderson. Papain HLA–B7 was isolated as described (Lopez de Castro et al., 1979). BBM.1–Sepharose beads were a gift from Peter Parham. Trypsin was from Worthington. Sodium [^{125}I]iodide was from New England Nuclear. Iodogen was from Pierce.

Low Specific Activity Iodination Procedure. A 5-μL aliquot of 13 mM iodogen in chloroform was dried down at the bottom of a 13 × 100 glass tube with nitrogen. A 0.5-mL sample of 25 mM KI/10 mM borate, pH 8.0/1 mCi of Na^{125}I was prepared in a glovebox and 5 μL quenched into 2 mL of 10 mM 2-mercaptoethanol for determination of specific activity. A total of 20 μL of the iodine solution was added to 1.0 mL of 1 mg/mL β_2m that had been dialyzed into 20 mM borate, pH 8.0. The protein solution was then transferred to the iodogen tube and incubated on ice. After 5 min, the solution was quenched by transferral into a tube containing 20 μL of 100 mM 2-mercaptoethanol. The sample was desalted over a 10-mL Sephadex G-15 column equilibrated in 50 mM ammonium bicarbonate.

High Specific Activity Iodination Procedure. Both HLA_{pap} and u-β_2m were dialyzed into 10 mM Tris, pH 8.0/140 mM NaCl. A stock of 0.1 mM iodogen in chloroform was prepared, and 50 μL was dried down with nitrogen at the bottom of a 6 × 50 glass tube. A 5-μg sample of protein was diluted to 50 μL in 20 mM sodium borate, pH 8.0. In a glovebox, the protein solution was transferred into the iodogen tube for 2 min on ice and removed by pipet to another 6 × 50 tube containing 10 μL of 20 mM 2-mercaptoethanol. Later, the iodinated protein was desalted on a 2-mL column of Sephadex G-25 in a Pasteur pipet equilibrated in 10 mM Tris-HCl, pH 8.0/140 mM NaCl/3 mM NaN_3/0.1 mg/mL ovalbumin (TSAO8). The specific activity was calculated from the percentage of ^{125}I used during the iodination and the counts incorporated following desalting, and by this estimate, it always corresponded to less than 0.1 mol of iodine/mol of protein. The protein was then made 0.1% in $NaDodSO_4$ and incubated at room temperature for 10 min in order to separate subunits, and then it was made 1% in NP-40. Monoclonal anti-β_2m beads (BBM.1) that had been recently washed in the elution buffer (see below) were then added to absorb the protein. Often several extractions were required to absorb all of the counts that could bind, even though the beads were in excess. Eventually, most of the β_2m counts could be absorbed in this fashion, as determined by $NaDodSO_4$ gel autoradiography. The absorbed protein was then eluted with 100 mM Tris/NaOH, pH 11.9/10% glycerol/0.1 mg/mL ovalbumin and dialyzed into 10 mM Tris-HCl/140 mM NaCl.

Reduction, Alkylation, and Digestion. In the case of high specific activity I-β_2m, the iodinated protein was diluted in TSAO8 to the desired volume (typically 0.05–2 mL). For both preparations, for each of the following additions, 10% by volume was added, unless specified otherwise: 3.5 M Tris/35 mM EDTA, pH 8.0, 10% $NaDodSO_4$, and 10 mg/mL dithioerythritol. The tube was sealed after a nitrogen sparge under parafilm and incubated for 1–4 h at 37 °C. Iodoacetic acid (30 mg/mL) was added, and the sample was incubated at room temperature in the dark (sealed as before). After 30 min, the sample was quenched by the addition of 1 μL of 2-mercaptoethanol, acetone precipitated by the addition of 6 times the sample volume of acetone, and usually stored in the freezer overnight. After the pellet was spun down, the residual acetone was removed by nitrogen or by lyophilization. The dry pellet was then digested with trypsin or chymotrypsin at an enzyme to protein ratio of from 1/100 to greater than 1/5. The digestion was followed by analytical HPLC runs and stopped after satisfactory patterns were obtained. This typically required overnight digestion at 37 °C.

HPLC Analysis of Peptides. Peptides were separated on a Zorbax TMS reverse-phase column (Du Pont) with a linear gradient over 1 h of 10–40% acetonitrile with an aqueous buffer of 20 mM triethylammonium/formate, pH 3.0 (Seidah et al., 1980) at a flow rate of 1 mL/min in a Waters system. Different aliquots of the same digest ran identically. Samples were collected every 0.5 min and counted in a Searle Model 1185 γ counter. The optical density was often continuously monitored at 214 or 254 nm (Waters). With the preparative

I-β_2m chymotryptic separation, 87% of the counts applied were recovered throughout the gradient. The remainder had to be removed from the column by repetitive blank gradient runs, since extensive isocratic washings at all solvent conditions tried did not eliminate ghost peaks from appearing at the same positions as in Figure 1. Some of the smaller peaks were pooled and rerun over a shallower gradient to separate out contaminants (for example, peak 54 of Figure 1).

Sequence Determination of Tryptic Digests. Entire tryptic digests were sequenced on a Beckman 890B sequenator, with 3 mg of polybrene as a carrier, with a 0.1 M QUADROL program. The entire butyl chloride extract at each step and the residue left in the cup were counted, and sometimes also the solvent washes. Since digestion was never complete, between 10 and 50% of the counts applied were found in the cup. Overlap and washout of counts occassionally contributed extensively to the background.

Sequence Determination of Chymotryptic Fragments. Isolated chymotryptic fragments were sequenced as described (Orr et al., 1979).

Amino Acid Analyses. All amino acid analyses were done on a Beckman 121 M amino acid analyzer. When iodotyrosine was present, it was quantitatively converted to tyrosine during hydrolysis. For specific activity measurements, several microliters were removed and counted immediately prior to loading samples on the analyzer.

TLC Conditions. Samples were digested at 37 °C in 0.1 mg of pancreatin and separated on 0.1 mm MN-cellulose 300 plastic TLC plates (Macherey-Nagel) with distilled water as a solvent. Tyrosine, iodotyrosine, and diiodotyrosine standards were stained with ninhydrin and ethanol, and the plate was autoradiographed. In addition to monoiodotyrosine and diiodotyrosine, species migrating near the dye front were consistently observed for some fractions. These spots were assumed to be inorganic iodine and were not investigated further.

Exchange Conditions. Typically 50-μL aliquots of both radioisotopic β_2m and 0.14 mg/mL HLA-B7 were incubated together for 1–20 h. The mixture was then separated on a 25-mL Sephadex G-75 column over 4 h. The percent exchange was defined as the corrected counts in the excluded peak divided by the sum of the corrected counts in the excluded plus included peaks. When free label was present, it ran as a third peak beyond the free I-β_2m peak and was not used in the calculation.

$NaDodSO_4$ Gels. All gels were 12% Laemmli gels (Laemmli, 1970), with 2-mercaptoethanol always present. Kodak XAR-5 film was exposed to the gels with a fluorescent intensifying screen (Du Pont) until intensities were satisfactory.

Results

Autoradiography of Iodinated β_2m. Samples of iodinated material were run on $NaDodSO_4$ gels, before and after immunoaffinity chromatography employing an anti-β_2m monoclonal antibody (BBM.1). In every sample, most of the label migrated in a sharp band at M_r 12000, including material that did not bind to the column (data not shown). When the HLA–B7 complex was iodinated, β_2m was well labeled, but no labeling of the HLA heavy chain occurred. In addition, there was no evidence of formation of any radiolytic cleavage products. However, a substantial amount of radioactivity remained in the supernatant of the anti-β_2m beads that was acetone precipitable, but that did not appear on film. This material apparently is a low molecular weight contaminant of the HLA preparations.

Analysis of Chymotryptic Digest of Preparatively Iodinated u-β_2m. A total of 42 nmol of u-β_2m was iodinated to a

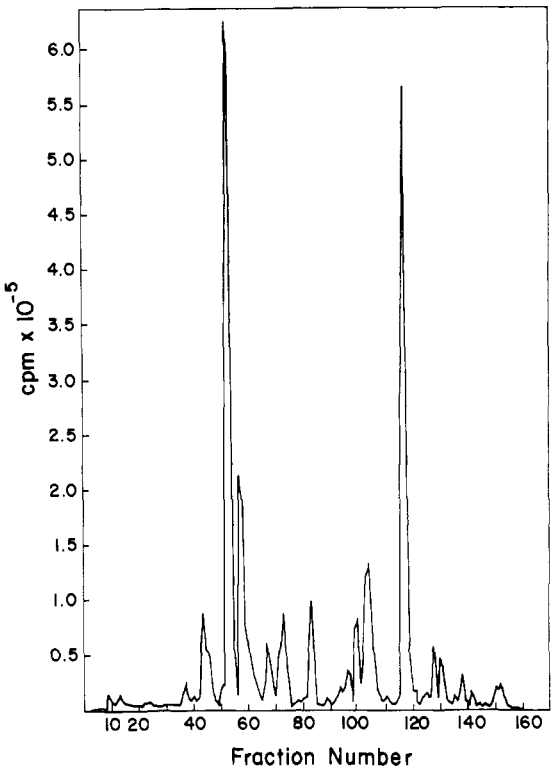

FIGURE 1: HPLC reverse-phase profile of preparatively iodinated u-β_2m, digested with chymotrypsin.

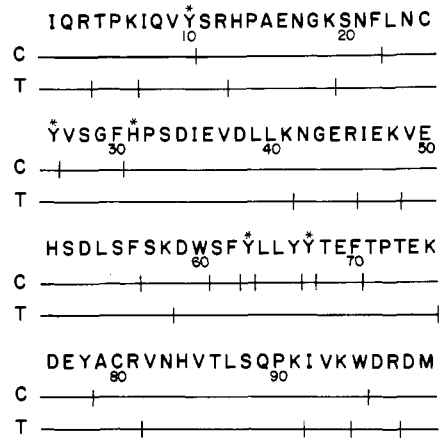

FIGURE 2: Primary structure of β_2m, as reported by Cunningham et al. (1973) and as corrected by Suggs et al. (1981). All expected tryptic and chymotryptic sites are marked. C, chymotrypsin; T, trypsin; *, iodinated residue.

stoichiometry of 1.5 mol of iodine incorporated per mol of β_2m, digested with chymotrypsin, and preparatively separated by HPLC (Figure 1). Because of the iodotyrosine absorbance, each iodopeptide was easily detectable during the course of the separation, and the absorbance pattern was superimposable on the radioactivity profile (data not shown). Aliquots of the radioactive peaks of the preparative run were analyzed for amino acids, and the peptide compositions were matched to the primary sequence of β_2m where possible (Table I and Figure 2). The two large major peaks in Figure 1 (peaks 52 and 117) have compositions that unambiguously corresponded to the peptides $S^{61}FY$ and $L^{64}LYYTEF$, indicating that Y-63 and Y-66 or Y-67 are primarily labeled. Several peptides have compositions that include at least two tyrosines, viz., $L^{64}LYYTEF$ (which was isolated in two forms; in the major peak 117 and in the minor peak 104), $L^{64}LYY$ (peak 83), and $Y^{67}TEFTPTEKDEY$ (peak 56A). From the primary se-

Table I: Amino Acid Composition of Iodopeptides and Corresponding Sequences[a]

amino acid	52[b]	56A[c]	56B[c]	83	100	104	117
			Amino Acid Composition				
D	1[d]	7.8 (1)[e]	3.0	4.4	14.4 (2)	6.2	
T		21.8 (3)	11.2 (1)	4.9		9.0 (1)	13.4 (1)
S	30 (1)		4.1	4.2	13.5 (2)	4.7	
E	3	25.7 (3)	19.9 (2)	6.6	7.5 (1)	14.7 (1)	14.6 (1)
P		8.8 (1)	9.0 (1)		6.9 (1)	2.8	
G	3	1.6	3.1	3.5	8.1 (1)	4.3	
A		0.5	1.1		0.9	0.6	
V		0.9	6.5 (1)		15.1 (2)	4.3	
I		1.9	13.0 (2)	3.4	7.1 (1)	2.6	
L		0.3	0.9	34.6 (1)	14.7 (2)	19.3 (2)	29.6 (2)
Y	28 (1)	12.5 (2)	7.5 (1)	36.6 (1)	0.7	16.1 (2)	27.5 (2)
F	35 (1)	8.4 (1)	2.8	1.7	7.9 (1)	0.6 (1)	15.0 (1)
K		8.2 (1)	8.3 (1)			1.9	
H		0.3	0.5		1.9	2.6	
R		1.2	9.1 (1)			1.2	

Sequences Corresponding to Iodopeptides

52[b]	SFY*[f]
56A	Y*TEFTPTEKDEY
56B	IQRTPKIQVY*
83	LLYY*
100	VSGFH*PSDIEVDLL
104[g]	LLYY*TEF
117[g]	LLYY*TEF

[a] The only peptides listed are those that had unequivocal compositions, including the two major labeled peptides (peaks 52 and 117) and some of the minor peptides. [b] Numbers correspond to fractions from Figure 1. [c] Obtained by second HPLC separation (see Materials and Methods). [d] Expressed in % of total recovered residues. [e] Number in parentheses corresponds to assumed integral value. [f] An asterisk (*) indicates iodinated residue (deduced from the rest of the data). [g] This peptide consistently ran in two different positions.

Table II: Specific Activities

52[a]	80 000 (±50 000)[b]
57	90 000
67	120 000
73	230 000
83	80 000
100	1 000 000
104	40 000
117	90 000
theoretical[c]	110 000

[a] Numbers correspond to fractions from Figure 1. [b] Units of cpm/nmol of tyrosine (see Materials and Methods). [c] Theoretical specific activity of a single iodotyrosine.

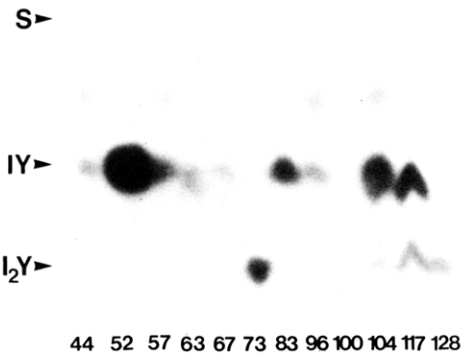

FIGURE 3: Thin-layer chromatography of pancreatin-digested aliquots of peaks from Figure 1, with distilled water as solvent. S marks the solvent front; IY marks the mobility of iodotyrosine; and I_2Y marks the mobility of diiodotyrosine.

quence, it is clear that these peptides are from overlapping rather than distinct regions of the molecule; and they all share Y-67. The simplest explanation for this pattern of cleavage products is that chymotrypsin does not always cleave molecules to the same end products. This is hardly surprising since there are six potential cleavage sites in the region F^{62} YLLYY^{67}. Since iodotyrosine is itself one of the plausible chymotryptic cleavage sites, the pattern of iodination might also influence the pattern of chymotryptic digestion.

Three approaches have been taken to determine which residues are actually iodinated or diiodinated in the peptides identified in Table I. A rough idea of the extent of iodination can be obtained by calculating the specific activity of each peptide (Table II). However, these data are not definitive because of the accuracy of the calculation is not better than a factor of 2, probably because of impurities. The second approach is to determine the extent of diiodination by enzymatically digesting each peptide to free amino acids with pancreatin and measuring the amounts of monoiodotyrosine and diiodotyrosine (Figure 3). These data conclusively demonstrate that there is no diiodinated material in any of the peaks in Table I except as a minor component in peak 117. However, the minor peaks 73 and 128 contain mostly diiodotyrosine and therefore may be identical in sequence with some of the monoiodotyrosine peptides. The third approach is to sequence directly each peptide, measuring both amino acid phenylthiohydantoin derivatives and radioactivity. For peaks 83 and 117, all of the radioactivity came off at the fourth cycle, while the material released at the third cycle could be identified as the phenylthiohydantoin derivative of tyrosine, establishing that in both cases only Y-67 was iodinated. However, it was observed that the recovery of both tyrosine derivatives from L^{64}LYY was lower than that for L^{64}LYYTEF, suggesting that direct sequencing would be unreliable for peptides ending in radioactive tyrosine, due to sequenator losses.

Multiple isolations of an iodopeptide of identical composition (as was observed for the peptide L^{64}LYYTEF in peaks 117 and 104) can be explained by isomeric iodination (iodination at either of two tyrosines), diiodination at a single tyrosine, or even peptide dimerization (Yamada et al., 1981). In the case of the peptide L^{64}LYYTEF, diiodination at either tyrosine is excluded by the pancreatin data. Iodination of both tyrosines

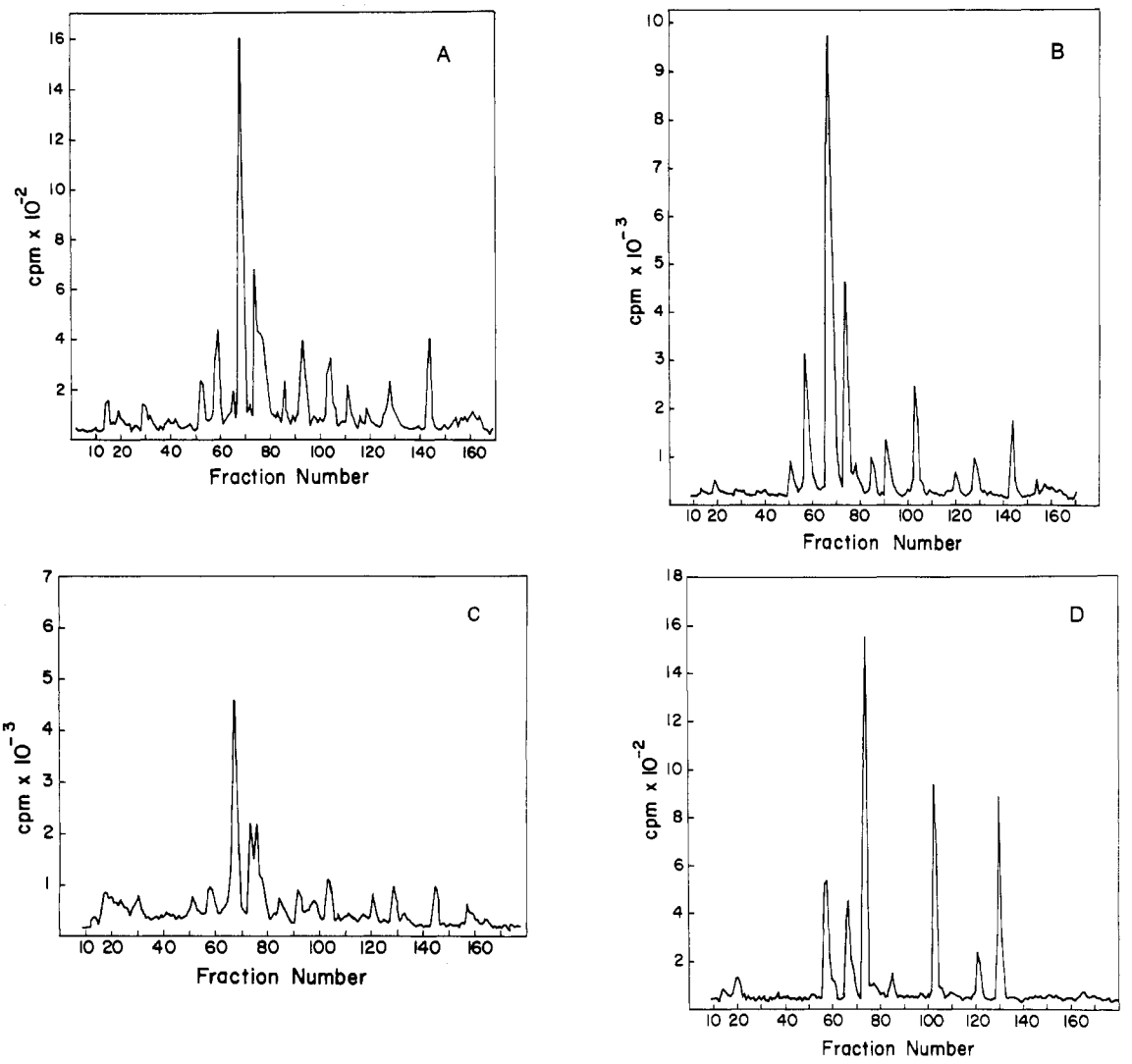

FIGURE 4: HPLC reverse-phase profile of chymotrypsin-digested tracer iodinated β_2m: (A) u-β_2m; (B) u-β_2m exchanged into HLA-B7 complex; (C) u-β_2m that does not exchange into HLA-B7 complex; (D) β_2m isolated from iodinated HLA-B7 (c-β_2m).

of peptide L^{64}LYYTEF in peak 117 is impossible because of the sequencing results. In peak 104, iodination of both tyrosines is unlikely from the specific activity data; in addition, a diiodinated peptide would be expected to migrate more slowly upon reverse-phase chromatography than the homologous monoiodinated peptide. Moreover, none of the data presented below for lightly labeled β_2m suggest that Y-66 is ever iodinated appreciably.

The remaining peptides in Table I can be explained very simply. Peak 56B corresponds to the N-terminal chymotryptic fragment, indicating that Y-10 can be labeled to some extent. Peak 100 is anomalous because there is very little tyrosine present by amino acid composition, causing the specific activity to appear very high (Table II). The composition corresponds exactly to the peptide V^{27} SGFHPSDIEVDLL, except that there is very little histidine, and instead, a new peak is visible that elutes before lysine. The pancreatin data confirm that neither iodotyrosine nor diiodotyrosine is present. The peptide is also unusual in that it contains an internal phenylalanine and a terminal leucine. Apparently, cleavage at F-30 is inhibited by iodohistidine, and cleavage occurs at the secondary chymotrypsin site L-40. This peptide is the only one identified that contains a terminal leucine, suggesting that there might also be an overlapping peptide that extends to F-56. Such a peptide would be 30 residues long, and might be recovered poorly.

Y-26 is also labeled (see below) but is not accounted for by any of the peptides isolated. Presumably the yield after proteolytic digestion and HPLC of a peptide containing this residue (for example, L^{23}NCY or L^{23}NCYVSGF, both of which contain cysteine) was too low to isolate.

Comparison of u-β_2m and Analysis of β_2m Exchange by Peptide Mapping. Since HPLC had been used with excellent results to separate tryptic peptides of unlabeled β_2m (Parker & Strominger, 1982), and for chymotryptic iodopeptides (Figure 1), tracer peptide maps were generated in order to determine how the iodination pattern of β_2m isolated from HLA-B7 (c-β_2m) differs from u-β_2m and whether iodination of specific residues in β_2m blocks subunit exchange. Chymotrypsin was used, as in Figure 1, instead of trypsin or staphylococcal V8 protease so that the primary residues of interest, Y-63 and Y-67, would reside in distinct iodopeptides. Ovalbumin was used as a carrier protein in order to enhance the recovery of radioactivity from the column, and the chymotryptic fragments of ovalbumin were used as an internal standard for comparison between samples. Figure 4A is an HPLC peptide map generated from u-β_2m. The radioactivity profiles in Figures 1 and 4A are different because carrier protein buffers proteolytic activity and because the u-β_2m in Figure 1 is much more heavily iodinated. The largest radioactive peak in Figure 4A–C is peak 67. It was found to be the same material as peak 52 of Figure 1 (S^{61}FY) by means

of a coelution experiment (data not shown). When iodinated u-β_2m is incubated with an excess of HLA–B7, two radioactive peaks are obtained following Sephadex G-75 chromatography, due to exchange of the radioactive β_2m with the endogenous β_2m in the HLA–B7 complex (Hyafil & Strominger, 1979). Peptide maps can be generated from the material that gets incorporated into complex (Figure 4B) and the material that does not get incorporated (Figure 4C). If iodination of β_2m had no influence on exchange into the heavy chain, at equilibrium, greater than 95% of the radioactivity should be in the HLA complex, since the iodinated β_2m is present in tracer amounts. In fact, even in the best preparations, only 60% of the I-β_2m becomes associated, as was found previously (Hyafil & Strominger, 1979). One explanation for this observation is that iodination of some residues prevents exchange. If this were the case, there should be peaks in the nonexchanged material that are not present in exchanged material. There is a shoulder peak at tube 77 (Figure 4A) that is prominent as a peak in Figure 4C and virtually absent in Figure 4B and thus fulfills this criterion. It is the peptide containing Y-10 (residues 1–10). Its elution position with respect to peak 67 is the same as the elution position of peptide I^1QRTPKIQVY with respect to S^{61}FY in Figure 1.

If direct obstruction of exchange by iodination were the only explanation for the incomplete exchange observed, then there should be no peaks in common between parts B and C of Figure 4, since part C should contain only peaks that correspond to iodinated β_2m species that prevent β_2m exchange. However, except for peak 77, all of the peaks of Figure 4A can also be found in Figure 4C as well as in Figure 4B. This apparent partial exchange of a particular iodinated β_2m species would occur if the stability of the modified HLA–B7 complex was lessened because of the iodination. However, in this case, there should be a greater amount of exchange when the HLA–B7 concentration is increased, and this was not observed (data not shown). A second explanation, covalent modification of distal parts of iodinated molecules induced from radiolytic damage of iodogen oxidation, would also cause peaks to be present in both parts B and C of Figure 4. A third explanation, iodination of multiple sites on the same molecule, is not likely, due to the low stoichiometry of labeling and the near perfect homogeneity by ion-exchange chromatography (data not shown). Although all of the above explanations might contribute to incomplete exchange, the presence of a peak in Figure 4B indicates that modification of the corresponding tyrosine does not prevent exchange if it represents the sole modification. For example, peak 67, which corresponds to modification at Y-63, is present in both parts B and C of Figure 4; therefore iodination of Y-63 does not block exchange.

Finally, ^{125}I-β_2m that was isolated from the HLA–B7 complex, digested with chymotrypsin, and separated by HPLC is shown in Figure 4D. The major difference between parts A and D of Figure 4 is that peak 74 rather than peak 67 is the largest. In addition, the shoulder of peak 74 that is largest in Figure 4C is absent. The background is lower, suggesting that a smaller number of tyrosines is labeled in β_2m in the HLA–B7 complex than in free β_2m. All of the peaks in Figure 4D are also present in parts A or B of Figure 4, suggesting that all of the β_2m tyrosines that can be labeled in HLA–B7 can also be labeled in free β_2m. However, the pattern in Figure 4D is still so complex that it was necessary to utilize alternative techniques to elucidate which tyrosines are iodinated under each set of conditions.

Comparison of u-β_2m and c-β_2m and Analysis of β_2m Exchange by Tryptic Digest Sequence Analysis. Although chymotrypsin was useful for the generation of peptide maps, sequence analysis of an unseparated chymotrypsin digest would not yield useful information, due to the presence of multiple peptides with tyrosine at the same position, and to the overlapping peptides. In contrast, in tryptic digests, most of the radioactivity should be on a single peptide from D-59 to R-81 containing Y-63, Y-66, Y-67, and Y-78 since K-75 is known to be resistant to tryptic cleavage (Cunningham et al., 1973). The remaining potential iodination sites, Y-10, Y-26, and H-31, should be on separate peptides, which would not interfere with peptide D-59 to R-81 or with one another, since radioactivity from each tyrosine should be released at a different cycle number (Figure 2). The advantage of sequencing unseparated peptides is that the entire tryptic digest can be analyzed during the same run, with no differential recovery problems upon quantitative transfer of radioactivity to the sequenator cup. Quantitative determination of the percentage of total iodination at each residue is limited only by problems of repetitive yield, overlap, and washout of short peptides.

The sequence pattern for the tryptic digest of u-β_2m is shown in Figure 5A. There are peaks discernible at positions 4, 5, 7, 9, 15, 18, 19, and 22. A comparison of this pattern with the tryptic peptides that would be generated from β_2m (Figure 2) leads to the following assignments for the labeled tyrosine residues: Y-10 (cycle 4, peptide 7–12); Y-63 (cycle 5, peptide 59–81); Y-26 (cycle 7, peptide 20–41); Y-67 (cycle 9, peptide 59–81); Y-63 (cycles 15 and 18); Y-67 (cycles 19 and 22). The last four positions would be generated if complete tryptic cleavage had not occurred at positions 58 and 48. These are reasonable candidates for partial digestion sites, due to flanking acidic residues (Figure 2). The definite occurrence of radioactivity at cycle 7 (Figure 5A,C) can be accounted for only by labeling of Y-26 although the appropriate chymotrypsin peptide was not isolated. Quantitatively, it is apparent that Y-63 (cycles 5, 15, and 18) and Y-67 (cycles 9, 19, and 22) are nearly equally modified, followed by Y-10 (cycle 4) and Y-26 (cycle 7). None of the profiles suggest any labeling at Y-66 (cycle 8) or Y-78 (cycle 20), although in the case of Y-66 minor labeling would be missed due to overlap. It is not clear whether H-31 or other histidines are iodinated from this data because the stability of iodohistidine under sequencing conditions is unknown. There is no evidence for any partial cleavages other than those mentioned above, since in all four profiles in Figure 5, all peaks can be accounted for.

Two conclusions can be drawn from a comparison of the labeling pattern of β_2m which exchanged into HLA complex (Figure 5B) and that which did not exchange (Figure 5C). First, there is no exchange of β_2m containing modified Y-10 or Y-26 (Figure 5B, no labeling at cycles 4 and 7), and instead, the nonexchanged material is enriched in these residues (Figure 5C). Second, there is more radioactivity in the exchanged β_2m at cycle numbers corresponding to the peptides generated from Y-63 than for Y-67 (compare the ratio of cycles 5 to 9 in Figure 5B to that in Figure 5C). Both of these conclusions are consistent with the chymotryptic peptide map data, and the same set of explanations still applies.

Finally, in the case of c-β_2m (Figure 5D), Y-67 (cycles 9, 19, and 22) is primarily labeled, with virtually no labeling of Y-63 (cycles 5, 15, and 18). There is no evidence for any other modifications.

Discussion

Chymotryptic iodopeptide isolation and identification, chymotryptic peptide mapping data, and tryptic peptide sequence data all support the following picture. Primary iodination of u-β_2m occurs at Y-63 and Y-67, with less frequent

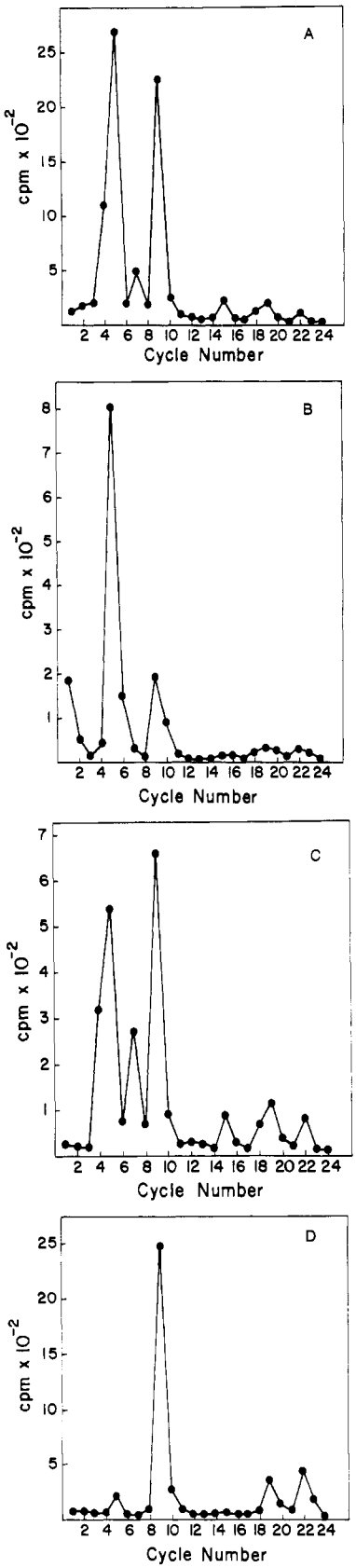

FIGURE 5: Sequence analysis of unseparated tryptic digest of [^{125}I]-β_2m. A–D are as in Figure 4.

iodination at Y-10 and Y-26. Primary iodination of HLA-$B7_{pap}$ occurs at Y-67 of β_2m, with virtually no iodination of Y-63; very little iodination of the heavy chain occurs under the mild conditions used. Iodination at Y-63 and Y-67 does not prevent exchange, whereas Y-10 and Y-26 are detectably iodinated only on material that does not exchange. No iodination is ever observed at Y-66 or Y-78; instead, iodination at H-31 or diiodination is detected when heavily iodinated material is analyzed.

The simplest explanation for the reactivity of Y-63 and Y-67 but not Y-66 is that this region of the molecule is in a β-pleated sheet, with Y-63 and Y-67 facing out and Y-66 facing in. This is consistent with the circular dichroism data, which indicates most of β_2m is in β sheet (Isenman et al., 1975). Moreover, two models that predict secondary structure, based primarily on homology to immunoglobulin (Becker et al., 1977) or primary sequence (Cohen et al., 1980), propose this particular β-strand alignment, when a sequence correction that removes a serine between Y-66 and Y-67 is taken into account (Suggs et al., 1981; Parker & Strominger, 1982). There are two possible explanations for the low level of iodination of Y-63 of β_2m in the HLA–B7 complex. The presence of the heavy chain might directly lower the penetration of solvent to Y-63 by means of steric hindrance (see below). Alternatively, β_2m in association with the heavy chain might be more tightly folded than free β_2m, thus limiting accessibility to Y-63 by means of a conformational change. In either case, the low amount of labeling that was observed could have occurred on β_2m molecules temporarily dissociated from the HLA complex, although the extent of dissociation is thought to be negligible at the low temperatures used for the iodination (Hyafil & Strominger, 1979). A distinction must be made between inaccessibility of a residue to direct iodination and inhibition of the exchange reaction. Clearly, when free β_2m is iodinated at Y-63, it is nevertheless capable of exchanging into HLA–B7 complex, implying that in HLA–B7 complex there is enough room around Y-63 for a bulky iodine atom, even though Y-63 is inaccessible to the outside.

Because Y-78 is two residues from C-80, nonreactivity is expected if one assumes β-sheet architecture, since Y-78 must have its side chain facing in the same direction as the disulfide bridge, which is connected to the other β sheet. The opposite argument can be made for Y-26, which is directly adjacent to C-25 and thus must have its side chain facing out. H-31 would be expected to face in, if it were on the same strand as Y-26, but it is likely to be in between strands, where any reactivity pattern would be consistent with the rest of the data (Figure 6). Finally, Y-10 should face out, since this allows I-7 and V-9 to face in.

There are several explanations for the inability of molecules iodinated at Y-10 and Y-26 to exchange. The most interesting possibility is that they sterically hinder the exchange process. It is useful to consider whether these observations are consistent with the expected structure of HLA. Any model based on Ig homology positions all of the tyrosines except Y-78 on the four-stranded face, quite close to one another. If the four-stranded face of β_2m interacts with the HLA heavy chain as Ig-constant domains interact with each other, accessibility of these tyrosines to solvent should be lowered. If an alignment based on primary sequence homology is combined with the X-ray crystallographic data for the quaternary interactions between domains in the Fab of IgG New (Saul et al., 1978), a model such as presented in Figure 6 can be obtained. The X-ray data say that the α carbon of the residue homologous to Y-10 is within 8 Å of the opposite domain regardless of whether β_2m is more homologous to the C_H1 or C_L domain of IgG New. The homologue of the strand that contains Y-63 and Y-67 is within 8 Å of the opposite domain in C_L but not C_H1, and only at the residues corresponding to L-63, L-64, and Y-65. Therefore, this model would predict that Y-63

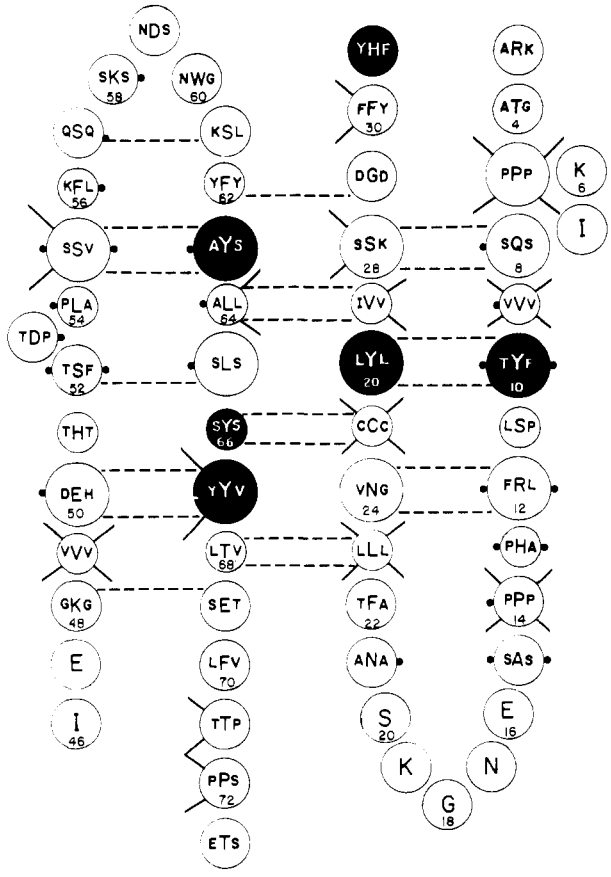

FIGURE 6: Predicted structure of the four-stranded face of β_2m, composed of residues 3–31 and 46–73. The large circles indicate residues that are facing toward the solvent, the small circles indicate residues facing toward the hydrophobic interior, and the intermediate circles indicate residues in between strands, or in irregular parts of β-pleated sheet. The central letters correspond to the sequence of human β_2m and the numbers to the linear sequence. The letters on the left and right correspond to the IgG New primary sequence, C_L and C_H1 domains, respectively. The dotted lines represent hydrogen bonds [from Figure 5; Saul et al. (1978)]. The diagonal lines indicate homology between β_2m and either C_L or C_H1. The small black circles on either side of the labeled circles indicate those residues of either C_L or C_H1 whose α carbons are within 8 Å of the opposite domain [from Figure 9; Saul et al. (1978)]. The large black circles indicate the residues that are most easily iodinated.

should be more shielded by the opposite domain than Y-67, if the β_2m·HLA–B7 interaction is homologous to the C_L·C_H1 interaction. The α carbon of the homologue of Y-26 is further than 8 Å from the opposite domain, but nevertheless, it lies in a pocket between the domains, where its reactivity may be directly influenced by quaternary interactions. Hence, it is possible to propose a reasonable quaternary structure for the HLA–B7 complex that is in accord with all of the experimental observations.

Additional explanations for the lack of exchange of β_2m labeled at Y-10 and Y-26 are possible that are not relevant to the above model. For example, modification of Y-10 and Y-26 may take place only on molecules that are already unable to exchange due to covalent modification of other residues, for instance, due to radiolytic damage (Loring et al., 1982). This possibility is strengthened by the presence of some iodopeptides in the peptide maps of both nonexchanged and exchanged iodo-β_2m (compare parts B and C of Figure 4 and parts B and C of Figure 5). Although urinary β_2m appears chemically homogeneous by ion-exchange chromatography and isoelectric focusing (unpublished observations), a small percentage of defective β_2m cannot be ruled out, and this could be preferentially iodinated. Improperly folded HLA–B7 complex could also explain the small amount of labeling of Y-63 in c-β_2m. A third, intermediate explanation is that iodination of Y-10 or Y-26 stabilizes a conformational state less capable of exchanging, without physical blocking exchange.

The lack of labeling of the HLA–B7 heavy chain was unexpected. It contains 15 tyrosines, predominantly in the N-terminal half of the molecule (residues 7, 9, 27, 58, 66, 84, 85, 99, 116, 118, 123, 159, 171, 209, and 257). Of these, Y-257 in the α_3 domain of HLA-B7 is homologous to Y-78 of β_2m and therefore would not be expected to be labeled. It is possible that all of these residues face in when they are on exposed strands of β-pleated sheet or are blocked by other parts of the molecule (e.g., the carbohydrate at N-86 for Y-84 and Y-85).

The only published data that address the question of what part of the heavy chain associates with β_2m are the following. Limited proteolysis of a mixture of iodinated HLA specificities indicates that a fraction that contains β_2m activity also contains carbohydrate (Trägårdh et al., 1979a). $NaDodSO_4$ gel electrophoresis indicates that this fraction is composed of two polypeptides, of molecular weights 12 000 and 20 000. Since it is known that the carbohydrate is located near the N terminus of the HLA chain (Parham et al., 1977), these data suggest substantial association between β_2m and the N terminus of the heavy chain. Since β_2m could associate with several domains of the heavy chains, these data are not necessarily inconsistent with the model proposed in Figure 6. On the other hand, since the 20 000 molecular weight fragment was isolated in low yield from iodinated heavy chain, it is also possible that its identification as the N terminus of the HLA heavy chain is in error or that the association observed is a consequence of the iodination. In contrast, limited proteolysis data on internally labeled mouse histocompatibility antigens indicate that mouse β_2m and the H-2 α_3 subregion are definitely associated (Yokoyama & Nathenson, 1983).

In conclusion, three residues (Y-10, Y-26, and Y-63) that are accessible to iodination in free β_2m become unreactive in the HLA-B7 complex. Iodination of either Y-10 or Y-26 takes place on molecules that subsequently do not exchange into the HLA–B7 complex. A fourth residue (Y-67) remains accessible in both free β_2m and HLA–B7, whereas a fifth tyrosine (Y-66) is inaccessible in both free β_2m and HLA–B7. All five of these tyrosines are located on the four-stranded face of β_2m, on the basis of primary sequence homology to IgG domains. A sixth tyrosine (Y-78) is on the three-stranded face and, like Y-66, is not labeled. These data can be most easily explained if β_2m interacts with the α_3 subregion of HLA in a fashion homologous to the interaction between C_L and C_H1 of IgG domains, although the data presented here are consistent with close association of the four-stranded face of β_2m with any part of the HLA heavy chain.

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Amino Acid Sequence of the Light Chain Variable Region from a Mouse Anti-Digoxin Hybridoma Antibody†

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ABSTRACT: A hybridoma cell line (26-10) derived from the A/J strain of mice secretes an immunoglobulin (IgG2a-κ) which binds digoxin with an association constant of 1.2 nM. Such high-affinity antibodies have been utilized in clinical radioimmunoassays as well as in the reversal of toxicity due to excess digoxin. The amino acid sequence of the light chain variable region of this antibody was derived by automated sequencing of the following: the intact chain; a fragment beginning C terminal to the tryptophan residue 40, obtained by cleavage with iodosobenzoic acid; a fragment beginning C terminal to arginine residue 82, obtained by trypsin cleavage on the completely reduced, alkylated, and succinylated chain. Difficulties which had previously prevented the automated Edman sequencing of this chain (and, presumably, similar ones of the same subgroup) were overcome by increasing the duration of the cleavage step at proline residues 8 and 12. The sequences of the first two hypervariable and framework regions of this chain are virtually identical with those of the dinitrophenol- and menadione-binding myeloma light chain MOPC 460 (95% homology). This anti-digoxin hybridoma from the A/J strain makes use of a V_κ gene which is similar to that utilized by some BALB/c 2,4-dinitrophenol-binding myelomas.

Mouse hybridoma proteins with anti-digoxin activity are of considerable practical and theoretical interest (Margolies et al., 1981; Mudgett-Hunter et al., 1982a,b). Their practical importance is related to the fact that digoxin, one of a large group of cardiac glycosides, is the drug most frequently prescribed for patients with congestive heart failure. Digoxin intoxication is one of the most prevalent drug reactions encountered in clinical practice, the frequency of digoxin toxicity being related to the narrow margin between therapeutic and toxic doses of the digitalis glycosides. Anti-digoxin antibodies have frequently been used as reagents for radioimmunoassays to monitor levels of circulating digoxin. Fab fragments prepared from sheep anti-digoxin antibodies have been used to rescue victims of otherwise fatal toxicity resulting from digoxin overdose (Smith et al., 1976). In addition to their clinical importance, anti-digoxin antibodies are excellent models for the study of antigen–antibody interactions at the molecular level as digoxin approximates the size of the antigen binding site, and its steroid moiety is conformationally rigid (Figure 1).

We report the complete amino acid sequence of the light chain variable region of an anti-digoxin hybridoma protein with a high binding constant, 1.2 nM (Mudgett-Hunter et al., 1982a,b). The light chain proved to be difficult to sequence because of the presence, at positions 8–9 and 12–13, of prolyl–leucyl and prolyl–valyl bonds, respectively. Some prolyl bonds are known to cleave slowly during Edman degradation (Brandt et al., 1976) and require modification of the cleavage reaction. To our knowledge, the sequence of 26-10 light chain represents the first complete amino acid sequence of the mouse subgroup $V_\kappa 1$ (Potter, 1977) characterized by the Pro^8-Leu^9 and Pro^{12}-Val^{13} residues.

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