The Class I Major Histocompatibility Complex Related Fc Receptor Shows pH-Dependent Stability Differences Correlating with Immunoglobulin Binding and Release†

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Received March 8, 1993; Revised Manuscript Received June 3, 1993

ABSTRACT: Maternal immunoglobulin G (IgG) in milk is transported to the bloodstream of newborn rodents via an Fc receptor (FcRn) expressed in the gut. The receptor shows a striking structural similarity to class I major histocompatibility complex (MHC) molecules, being composed of a related heavy chain and the identical light chain (β2-microglobulin). FcRn binds IgG at the pH of milk in the proximal intestine (pH 6.0–6.5) and releases it at the pH of blood (pH ~7.5). We have compared the stability of a soluble form of FcRn in these two pH ranges and find that the heterodimer is markedly more stable at the permissive pH for IgG binding. Using the rate of β2m exchange as a correlate of heterodimer stability, we find that exchange is more than 10 times slower at pH 6.1 compared to pH 7.8. Thermal denaturation profiles of FcRn heterodimers at pH 8.0 indicate a two-step, sequential heavy-chain (T_m = 52 °C) and β2m (T_m = 67 °C) denaturation. By contrast, at pH 6.0, a single transition is observed, centered at 62 °C, corresponding to denaturation of both chains. The striking difference in stability does not appear to be correlated with the binding of peptide as in class I MHC molecules, because analysis of purified FcRn by acid dissociation and sequencing suggests that FcRn is not associated with cellular peptides. These results are indicative of pH-dependent conformational changes in the FcRn heterodimer, which may be related to its physiological function.

Transfer of immunoglobulin from mother to offspring is important for immune defense before a neonate develops a fully functional immune system. A receptor for the Fc portion of immunoglobulin G (IgG)[1] is expressed in the proximal intestine of rodents during the first 3 weeks after birth (Simister & Rees, 1985). This receptor, FcRn (Fc receptor, neonatal), is responsible for the transport of maternal IgG from ingested milk into the bloodstream of neonatal rats. IgG dissociates from FcRn upon raising the pH from 6.5 to 7.5 (Jones & Waldman, 1972; Rodewald, 1976; Simister & Rees, 1985; Simister & Mostov, 1989a,b), corresponding to the difference in pH between ingested milk on the luminal side of intestinal epithelial cells (pH 6–6.5) where IgG is bound and the basal side where IgG is released into the blood (pH 7.4 or above). FcRn resembles class I major histocompatibility complex (MHC) molecules (Simister & Mostov, 1989a,b), which present peptide antigens to T-cells (Townsend & Bodmer, 1989). Both types of molecules are heterodimers consisting of a membrane-spanning heavy chain noncovalently attached to a light chain that is not directly linked to the membrane. The light chain of both FcRn and class I MHC molecules is β2-microglobulin (β2m) (Simister & Rees, 1985), and the three extracellular domains of the FcRn heavy chain shows sequence similarity to the corresponding α1, α2, and α3 domains of class I molecules (Simister & Mostov, 1989a,b). Both class I MHC molecules and FcRn are anchored to the cell surface via transmembrane regions C-terminal to their heavy-chain α3 domains.

Crystallographic analyses of class I MHC molecules have shown that the α3 and β2m domains resemble immunoglobulin constant domains and that the α1 and α2 domains form a platform of eight antiparallel β-strands topped by two long α-helices. A groove between the helices constitutes the binding site for short peptide antigens (octamers and nonamers) (Bjorkman *et al.*, 1987a,b; Garrett *et al.*, 1989; Madden *et al.*, 1991; Fremont *et al.*, 1992). FcRn is predicted to fold into a heterodimeric structure resembling that of MHC class I molecules (Simister & Mostov, 1989a; Parham, 1989; Gastinel *et al.*, 1992). The functional basis for a similarity in structure is not understood, since the ligand of FcRn is a macromolecule (IgG) rather than a peptide.

To increase our understanding of the similarities and differences between FcRn and class I MHC molecules, and to probe the mechanism of the pH-dependent binding of IgG to FcRn, we have initiated a biochemical characterization of a soluble form of rat FcRn expressed in Chinese hamster ovary (CHO) cells (Gastinel *et al.*, 1992). The soluble form, consisting of the extracellular domains of the heavy chain complexed to β2m, has previously been shown to be functionally active, retaining its ability to bind IgG and reproducing the physiological pH dependence of binding (Gastinel *et al.*, 1992; Huber et al., 1993). In a first comparison of FcRn with class I MHC molecules, we analyzed soluble FcRn for the presence of bound peptides. Purified preparations of class I molecules contain mixtures of endogeneous peptides (Bjorkman *et al.*, 1987a; Rötzschke et al., 1990; Van Bleek &

† Supported by the Howard Hughes Medical Institute (L.N.G. and P.J.B.) and a postdoctoral fellowship from the Cancer Research Institute (M.R.). P.J.B. is a Pew Scholar and has a Young Investigator Award from the Cancer Research Institute.

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[1] Abbreviations: FcRn, Fc receptor, neonatal; MHC, major histocompatibility complex; IgG, immunoglobulin G; β2m, β2-microglobulin; MSX, methionine sulfoximine; CHO, Chinese hamster ovary; ELISA, enzyme-linked immunosorbent assay; HPLC, high-performance liquid chromatography; CD, circular dichroism; T_m, transition midpoint.

0006-2960/93/0432-8654$04.00/0

Nathenson, 1990; Falk *et al.*, 1991), which bind to newly synthesized class I proteins during their transit through the endoplasmic reticulum (Townsend & Bodmer, 1989). Under physiological conditions, endogenous or foreign peptides are required for efficient cell surface expression of MHC class I molecules (Townsend *et al.*, 1989; Ljunggren *et al.*, 1990; Schumacher *et al.*, 1990), and the thermal stability of a class I heterodimer is greatly enhanced by peptide binding (Ljunggren *et al.*, 1990; Fahnestock *et al.*, 1992). We have analyzed soluble FcRn to determine whether it is also complexed with peptides that are present during the assembly of its heavy and light chains in the endoplasmic reticulum. Using techniques that elute peptides from class I MHC molecules, we find no evidence for the association of cellular peptides with FcRn. Because FcRn seems to represent a class I-like molecule that has evolved to be structurally stable in the absence of peptide, we were interested in defining parameters other than the presence of peptides that affect the structural stability of FcRn. Here, we report stability differences in soluble FcRn correlating with its physiological pH-dependent binding of IgG. As the first measure of stability, we find that the dissociation rate of β2m from the FcRn heavy chain is an order of magnitude faster at pH 7.8 than at pH 6.1. Secondly, thermal stability profiles of FcRn show greatly enhanced stability at pH 6.0–6.5 compared to pH 7.5–8.0. These results suggest that the interaction between the two chains of the FcRn heterodimer is altered over this narrow pH range, and these structural changes may correlate with the binding and release of IgG.

MATERIALS AND METHODS

Reagents. Centricon-10 ultrafiltration units were obtained from Amicon (Beverly, MA). CNBr-activated Sepharose for affinity column preparation, a Superose 12 FPLC column, and PD-10 gel filtration columns were from Pharmacia (Piscataway, NJ). Rat Fc was from Jackson ImmunoResearch (West Grove, PA). The anti-rat β2m monoclonal antibody 2B10C11 was a gift of Dr. Lennart Lögdberg (Sandoz Pharmaceuticals). α minimum essential medium (αMEM) and dialyzed fetal bovine serum were obtained from Irvine Scientific (Santa Ana, CA) and GIBCO/BRL (Grand Island, NY), respectively. Methionine sulfoximine (MSX) was obtained from Sigma (St. Louis, MO). The BCA protein assay reagent was obtained from Pierce (Rockford, IL). A water-jacketed cuvette for the CD measurements was purchased from Hellma (Jamaica, NY).

Expression and Purification of Secreted FcRn and H-2K^d. CHO cells expressing a secreted form of FcRn were obtained as previously described by transfecting the genes encoding rat β2m and the rat FcRn heavy chain truncated shortly before its predicted transmembrane domain (Gastinel *et al.*, 1992). Soluble FcRn was isolated in a functional purification procedure by passing pH 6.0 supernatants from transfected CHO cells over a rat IgG column and eluting protein by raising the pH to 8.0. Eluted FcRn protein was shown to be an equimolar heterodimer of the FcRn heavy chain complexed to rat β2m (Gastinel *et al.*, 1992). Soluble H-2K^d was expressed in CHO cells by transfecting the human β2m gene together with a truncated form of the K^d heavy chain (Fahnestock *et al.*, 1992). H-2K^d was purified on an immunoaffinity column as described (Fahnestock *et al.*, 1992).

Acid Elutions of FcRn or a Class I Heterodimer (H-2K^d). Purified FcRn or H-2K^d were analyzed for the presence of bound peptides using established methods (Van Bleek & Nathenson, 1990; Jardetzky *et al.*, 1991). Briefly, about 0.5 mg of FcRn or K^d was concentrated to 100 μL in a centricon-10 (molecular weight cutoff of 10 000) ultrafiltration device. The proteins were diluted with 1.0 mL of 50 mM ammonium acetate (pH 7.5) and concentrated again to 100 μL, and this procedure was repeated. The washed proteins were then treated with 1.0 mL of 10% acetic acid, heated to 100 °C for 5 min, and subsequently concentrated again to 100 μL in the ultrafiltration unit, with the filtrate containing any eluted peptide material. The acid eluates were lyophilized and analyzed by automated Edman degradation using an Applied Biosystems Model 4778 protein sequencer.

Expression and Purification of Rat β2m. The rat β2m cDNA (a gift of Dr. Neil Simister) was subcloned into the expression vector pBJ5-GS (Gastinel *et al.*, 1992), which carries the glutamine synthetase gene as a selectable marker and means of gene amplification, in the presence of the drug methionine sulfoximine (MSX) (Bebbington & Hentschel, 1987). The plasmid was transfected into CHO cells by a calcium phosphate procedure (Stratagene), and cells resistant to 400 μM MSX were selected according to the protocol established by Celltech, modifications of which have been previously described (Gastinel *et al.*, 1992). Transfected CHO cells were grown in α minimal essential medium supplemented with 10% dialyzed fetal bovine serum, 100 units/mL penicillin/streptomycin, and the specified concentrations of MSX. Supernatants from transfected cells were tested for secreted rat β2m with an enzyme-linked immunosorbent assay (ELISA) using the anti-rat β2m monoclonal antibody 2B10C11 and a goat antimouse secondary antibody conjugated to horseradish peroxidase. A high-expressing clone that had been amplified in the presence of 400 μM MSX was introduced into a hollow fiber bioreactor device (Cell Pharm I, Unisyn Fibertec). Supernatants were harvested daily, and rat β2m was purified on a 2B10C11 immunoaffinity column. For this purpose, tissue culture supernatants were brought to 25 mM Tris (pH 8.0) and 0.05% NaN_3 and passed over the immunoaffinity column at a flow rate of 5–10 mL/h. The column was washed with 20 column vol of 50 mM Tris (pH 8.0) before elution with 0.1 M glycine hydrochloride (pH 2.7). The yield of purified protein was approximately 4–6 mg/L cell supernatants, estimated using a BCA protein assay with bovine serum albumin as the standard.

Reductive Methylation of FcRn. The radiolabeling procedures used to methylate FcRn on free amino groups were similar to those previously described for class I MHC molecules used for β2m exchange studies (Hyafil & Strominger, 1979). NaB^3H_4 (100 mCi at 13.37 Ci/mmol) was added to 0.65 mL of FcRn solution (2 mg) in 0.2 M borate buffer (pH 8.7). Formaldehyde was added at 5-min intervals to a final concentration of 1.0 M, and the protein was kept on ice for 1 h. Excess NaB^3H_4 was removed by gel filtration on a PD-10 gel filtration column equilibrated in 50 mM phosphate buffer (pH 6.0). Fractions containing protein were pooled, passed over a rat IgG affinity column, and washed extensively with the pH 6.0 buffer as previously described (Gastinel *et al.*, 1992). Labeled FcRn protein was eluted from the IgG column by raising the pH to 8.0. This purification procedure ensures that any labeled protein used for subsequent β2m exchange experiments was functionally intact, retaining its ability to bind and dissociate from IgG in the known pH-dependent manner.

On the basis of observed specific activities, an average of five methyl groups were incorporated per molecule of FcRn. Upon denaturation of [^{3}H]FcRn with 6 M guanidine hydrochloride, approximately 57% of the total radioactivity migrated with the β2m fraction and the remainder migrated with the

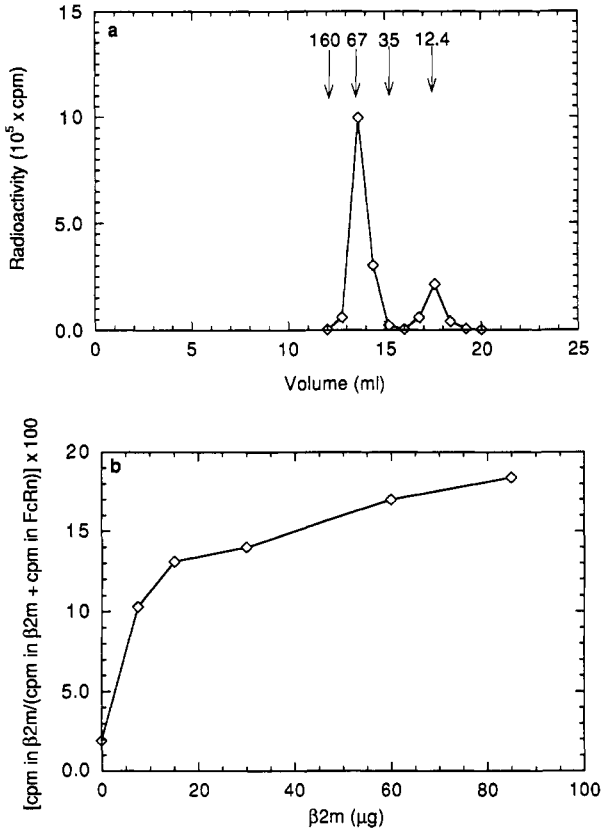

FIGURE 1: Exchange between β2m in [^{3}H]FcRn and free unlabeled β2m. (a) Gel filtration profiles of FcRn and β2m after the β2m exchange reaction. [^{3}H]FcRn (24 μg) was mixed with 85 μg of unlabeled β2m and incubated in 0.2 mL of 25 mM phosphate buffer (pH 6.5) for 18.5 h at 37 °C. The mixture was injected into a Superose 12 column, fractions of 0.8 mL were collected, and levels of radioactivity in each fraction were determined by liquid scintillation counting. The measured radioactivity is plotted versus the volume of elution, and the expected elution volumes for standards of molecular weights 160 000, 67 000, 35 000 and 12 400 are indicated on the plot. The FcRn heterodimer (first peak) is well separated from the β2m peak (second peak). (b) Dose response curve for exchange between β2m in [^{3}H]FcRn and free unlabeled β2m. [^{3}H]FcRn (24 μg) was mixed with increasing amounts of unlabeled rat β2m and incubated at 37 °C in 0.2 mL of 25 mM phosphate buffer (pH 6.5) for 15–18 h. For each incubation with a different amount of unlabeled β2m, the heterodimer was separated from free β2m as described for a. The ratio of counts in free β2m fractions to total counts in both β2m and FcRn fractions is plotted versus the amount of unlabeled β2m incubated with [^{3}H]FcRn.

heavy-chain fraction on a gel filtration column, suggesting that, on average, three sites in β2m and two in the heavy chain were methylated.

Determination of the Rate of β2m Dissociation. Radiolabeled FcRn heterodimer (1 μg) eluted from the rat IgG affinity column at pH 8.0 was diluted with unlabeled FcRn (20 μg, for a total of 0.35 nmol) and incubated with a 15-fold molar excess of rat β2m (65 μg, 5.4 nmol) for various time intervals in 0.2 mL of 25 mM phosphate buffer (pH 6.1 or 7.8) at 37 °C. For each time point, protein mixtures were injected into a Superose 12 FPLC column that had been equilibrated in 0.1 M NH_4HCO_3 (pH 7.0). Free β2m was well separated from the FcRn heterodimer, with each component eluting at 17.5 and 13.6 mL, respectively (Figure 1a). Levels of radioactivity in the FcRn and β2m fractions were determined by liquid scintillation counting using a Beckman Model LS 5000TD counter. Slopes obtained from plots of ln [(cpm in FcRn fractions)/(cpm in FcRn fractions + cpm in β2m fractions)] versus time gave a measure of the rate constant for dissociation (k) according to the following equation: ln [(cpm in FcRn fractions)/(cpm in FcRn fractions + cpm in β2m fractions)] = $-kt + c$. The half-time for dissociation ($t_{1/2}$) was calculated as $0.693/k$. Since ~40% of the total radioactivity resides on the heavy chain, the y-axis [(cpm in FcRn fractions)/(cpm in FcRn fractions + cpm in β2m fractions)] was normalized to take into account the fact that a ratio of 0.4 represents maximal dissociation.

Table I[a]

cycle number	pmol of amino acids from acid elutions	
	K^d	FcRn
1	441	115
2	424	0
3	399	3
4	230	0.5
5	278	2
6	172	3
7	279	9.5
8	178	0.9
9	79	7.5
10	7	0.2

[a] 0.25 mg of K^d or FcRn in 50 mM ammonium acetate (pH 7.5) was treated with 1.0 mL of 10% acetic acid, heated to 100 °C for 5 min, and subsequently concentrated again to 100 μL in an ultrafiltration unit (M_r cutoff: 10 000). The filtrate was lyophilized and subjected to 10 cycles of N-terminal sequencing. The total yield of amino acids from each sequencing cycle is presented. Only those amino acid residues that showed an increase in the absolute amount recovered compared to the previous cycle were considered significant. In the case of K^d, 425 pmol of amino acid was recovered in the second sequencing cycle, of which 293 pmol corresponds to tyrosine, and 86 and 45 pmol were from phenylalanine and leucine, respectively. In the fourth cycle, 230 pmol was recovered, of which 85 pmol was from proline residues. If we assume a typical 30–50% initial yield in the sequencing reaction and that the acid elution procedure releases all of the bound peptide, the starting K^d material appears to have a peptide occupancy of about 20–35%, consistent with previous estimates using other methods (Fahnestock *et al.*, 1992). For FcRn, with the exception of cycle 1 which is typically subject to high backgrounds, the total picomole recovery of amino acid is negligible.

CD Spectra and Thermal Stability Analyses. A Jasco J-600 spectropolarimeter with a 1.0-mm water-jacketed cuvette was used for the CD measurements described. The protein samples were present in the concentration range of 0.25–0.4 mg/mL in 20 mM phosphate buffer at the appropriate pH. For measuring melting curves, the CD signal was monitored at 210 nm while the sample temperature was raised from 25 to 75 °C at a rate of approximately 0.6–0.8 °C/min. Over the range from 25 to 75 °C, the temperature dependence of the pH of phosphate buffer was found to be very slight, increasing by only 0.1–0.2 pH unit from starting pH values of 6.0 and 8.0. T_m's were calculated by estimating the half-point of the ellipticity change between the pure native and pure denatured states.

RESULTS

Purified Soluble FcRn Does Not Contain Bound Peptides. Peptides associated with class I MHC molecules have typically been analyzed by N-terminal sequencing of eluates recovered after ultrafiltration of acid-dissociated heterodimers (Rötzschke *et al.*, 1990; Van Bleek & Nathenson, 1990; Falk *et al.*, 1991; Jardetzky *et al.*, 1991). To determine whether purified FcRn contains noncovalently associated peptides, we compared acid eluates of purified FcRn and the class I MHC moleucle H-2K^d for the presence of low molecular weight peptide material. Table I shows the total yield of amino acids for 10 sequencing cycles of the FcRn and K^d acid eluates. Sequences obtained from K^d eluates corresponded to the previously

characterized motif for peptides bound to this class I allele (Falk *et al.*, 1991), with tyrosine and proline predominating at positions 2 and 4, respectively. The distinct drop in amino acid residues recovered after cycle 9 is in accordance with the previously described preference of class I molecules for binding octamer and nonamer peptides (Falk *et al.*, 1990; Rötzschke *et al.*, 1990; Van Bleek & Nathenson, 1990). By contrast, cycles of Edman degradation of acid eluates from FcRn showed a uniform low background of residues throughout the last nine cycles (the first cycle typically has a high background originating from buffer and other nonspecific sources), similar to the levels of amino acids found in acid eluates from an unrelated protein predicted to contain no bound peptides (ribonuclease; data not shown). Even if 5% of the FcRn molecules used for the peptide analysis experiment were actually peptide-filled and the efficiency of the initial sequencing reactions assumed to be 30–50%, the expected yield in the second sequencing cycle would be approximately 60–100 pmol. Table I shows that neither cycle 2 nor any of the subsequent sequencing cycles for FcRn eluates have total amino acid yields greater than 10 pmol.

The Dissociation Rate of β2m from FcRn Is Dependent upon pH. The β2m light chain of FcRn undergoes an exchange reaction (Figure 1b) similar to the well-documented exchange of β2m in MHC class I molecules (Hyafil & Strominger, 1979; Bernabeu *et al.*, 1984; Bernhard *et al.*, 1988; Kozlowski *et al.*, 1991). The dissociation rate governing this exchange process is a measure of heterodimer stability, and in the case of MHC class I molecules, it has been shown to be dependent on the nature of the bound peptide (Parker *et al.*, 1992). Since the acid elution experiments indicated that FcRn was not associated with peptides, we evaluated the FcRn exchange process for comparisons with class I molecules (Parker *et al.*, 1992) and to ascertain whether the inferred stability showed a pH dependence correlating with physiological IgG binding properties. The kinetics of the FcRn heterodimer dissociation was therefore studied at pH 6.1 and 7.8. For these experiments, radiolabeled FcRn was incubated with an excess of unlabeled rat β2m, and the time course of appearance of radioactivity in free β2m fractions was measured after the separation of β2m/heavy-chain heterodimers by gel filtration (Figure 1a). Rate constants for dissociation were calculated using plots of ln [(cpm in FcRn fractions)/(cpm in FcRn + cpm in β2m fractions)] versus time (Figure 2). The $t_{1/2}$ for dissociation of β2m from the FcRn heavy chain was found to vary markedly with pH, being an order of magnitude longer at pH 6.0–6.5 (1900–2400 min) compared to pH 7.5–8.0 (170–175 min).

Differences in Thermal Stability Profiles of FcRn at pH 6.0 and 8.0. The pH dependence of the β2m dissociation rate suggested conformational differences in the FcRn heterodimer that were a function of pH. In order to gain more insight into the nature of the differences, we used circular dichroism analyses to study structural features and thermal stability of FcRn as a function of pH. The room temperature CD spectra of FcRn were nearly identical at pH 6.2 or 7.6, indicating that there was no major difference in the secondary structure content of the protein at the two pH values (Figure 3). To assess the thermal stability of the FcRn heterodimer as a function of pH, we recorded the circular dichroism spectrum of the protein as a function of temperature at pH 6.2 or 7.6. Figure 3 shows the far-UV CD spectra of FcRn at temperatures ranging from 26 to 70 °C, the extreme temperatures representing the completely native and completely denatured states of FcRn, respectively. Comparisons of the spectra at higher temperatures reveal differences in the thermal stability

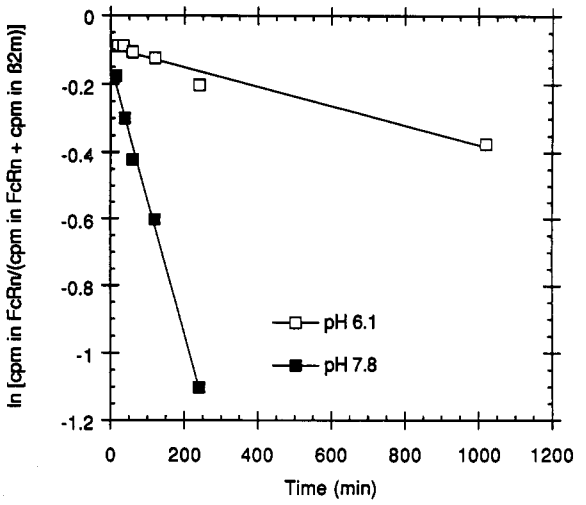

FIGURE 2: pH dependence of the dissociation rate of β2m from the FcRn heterodimer. [^{3}H]FcRn was incubated at 37 °C for various time intervals with a 15-fold molar excess of rat β2m at pH 6.1 (□) or 7.8 (■) in 25 mM phosphate buffer. After the specified time interval, the radioactivity in FcRn and β2m fractions was determined following separation of the proteins on a Superose 12 column. ln [(cpm in FcRn fractions)/(cpm in FcRn fractions + cpm in β2m fractions)] is plotted versus the time of incubation for one experiment (at pH 6.1, the time points were taken at 20, 35, 60, 120, 240, and 1020 min; at pH 7.8, time points were taken at 15, 38, 60, 120, and 240 min). The *y*-axis has been normalized to take into account the fact that a ratio of 0.4 represents maximal dissociation, since ~40% of the total radioactivity resides on the heavy chain. If we assume first-order reactions, the negative slopes of the plots give estimates of the rate constant (k) of the dissociation reactions, and the half-time for dissociation ($t_{1/2}$) is calculated as $0.693/k$. The calculated $t_{1/2}$ values for this experiment are 2400 min at pH 6.1 compared to 170 min at pH 7.8. In an independent experiment, the respective calculated $t_{1/2}$ values were 1900 and 175 min (data not shown).

of the protein at the two pH values. For examples, at 51 °C and pH 7.6, the FcRn molecule appears significantly denatured, whereas at the same temperature and pH 6.2, FcRn has mostly native-like secondary structure.

In order to compare the thermal stabilities of FcRn more precisely, and also to resolve the contributions of the unfolding of each of the polypeptide chains of the heterodimer, the CD signal was monitored at 210 nm while the sample temperature was increased from 22 to 75 °C. A wavelength of 210 nm was chosen because it is close to the circular dichroism minima for the β-sheet and α-helical structures and because there were significant and measurable differences in the CD signal of native and denatured FcRn (Figure 3) at this wavelength. Figure 4 illustrates the melting curves, thus derived, of the FcRn heterodimer and of rat β2m at pH 6.0 and 8.0. The melting curve of free rat β2m varies slightly with pH, showing a single transition with a midpoint (T_m) of 64.5 ± 1 °C at pH 6.0 or 68 ± 1 °C at pH 8.0, values similar to the T_m of 64 ± 1 °C previously observed for the unfolding of human β2m at pH 7 (Fahnestock *et al.*, 1992). The unfolding profiles of the FcRn heterodimer, however, show dramatic differences at the two pH values. At pH 8.0, there are two distinct transitions. The first, centered at 51.5 ± 1 °C, presumably corresponds to the dissociation and concurrent denaturation of the heavy chain, while the second, centered at 67 ± 1 °C, corresponds to the denaturation of β2m at this pH. At pH 6.0, there is a single unfolding transition with a T_m of 62 °C, which we interpret as corresponding to the simultaneous unfolding of both the heavy-chain and β2m polypeptides. This 10 °C shift in the T_m at the two pH values reveals a marked difference in the stability of the FcRn heavy chain/β2m

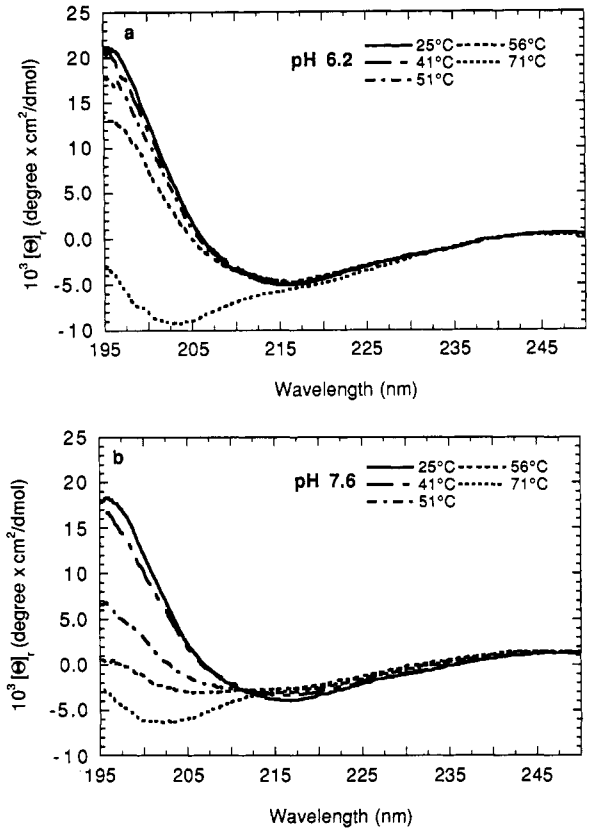

FIGURE 3: pH and temperature dependence of the far-UV CD spectra of FcRn (0.28–0.3 mg/mL) in 20 mM phosphate buffer at pH 6.2 (a) or 7.6 (b). Scans were recorded at the indicated temperatures ranging from 25 to 71 °C. Measurements were made on a Jasco J-600 spectropolarimeter, using a 1.0-mm water-jacketed cell.

complex, correlating with the observed differences in the rate of β2m exchange.

DISCUSSION

A soluble form of the neonatal rat Fc receptor FcRn has been previously characterized as being a heavy-chain/light-chain heterodimer that is capable of binding IgG in a pH-dependent manner (Gastinel *et al.*, 1992; Huber *et al.*, 1993). The heavy chain alone does not appear to bind ligand, on the basis of diminished binding of rat Fc to a lipid-linked form of the FcRn heavy chain expressed on the surface of transfected CHO cells (Gastinel *et al.*, 1992). These results suggest that FcRn is not fully functional in the absence of rat β2m, even when possibly paired with an endogenous hamster β2m protein or bovine β2m derived from the serum in the medium (Gastinel *et al.*, 1992). Calorimetric analysis of binding of human IgG1 to soluble FcRn yields a $K_a \approx 2.0 \times 10^6$ M^{-1} (Huber *et al.*, 1993), compared to K_a values of 2.0×10^7 to 3.0×10^7 M^{-1} obtained by Scatchard analysis of the binding of rat IgG1 or IgG2b to detergent-isolated whole receptors purified from neonatal rat intestine (Hobbs *et al.*, 1987). The affinity difference may reflect a difference in the method, a difference in the ligand (human IgG1 versus rat IgG2a or -2b), or a difference in the binding of IgG by the soluble as compared to the membrane form of FcRn. Irrespective of the absolute value of the measured affinity, secreted FcRn exhibits the same pH-dependent binding of IgG as does the membrane form of the molecule (Gastinel *et al.*, 1992; Huber *et al.*, 1993) and has thus been used in the experiments described here to examine the structural basis of IgG binding and release and for composition comparisons with secreted class I molecules.

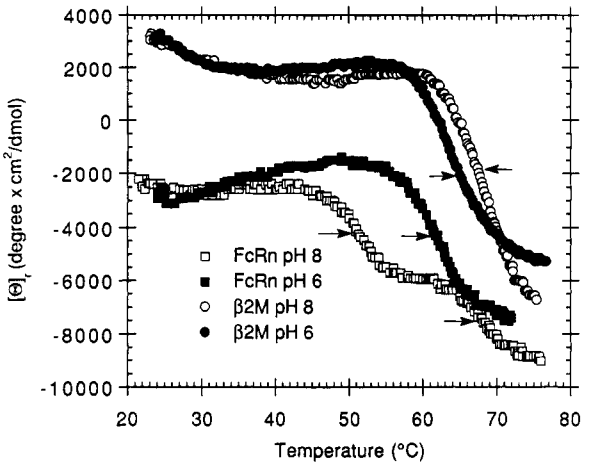

FIGURE 4: Thermal denaturation profiles of FcRn heterodimers or rat β2m monitored by the CD signal at 210 nm. The CD signal of FcRn (0.3 mg/mL) or β2m (0.25–0.35 mg/mL) in 20 mM phosphate buffer at pH 6.0 or 8.0 was monitored in a water-jacketed 1.0-mm cell while the sample temperature was raised from 25 to 75 °C at a rate of 0.6–0.8 °C/min. T_m's were derived by estimating the half-point of the ellipticity change between the beginning and end of each transition from at least two separate experiments. For each T_m reported, the ellipticity values for the pure native (θ_N) and the pure denatured (θ_D) states for the transition are given in parentheses and expressed in units of deg·cm^2/dmol: rat β2m pH 6.0 (●), one transition, $T_m = 64.5 \pm 1$ °C ($\theta_N = 2000$, $\theta_D = -5400$); rat β2m pH 8.0 (○), one transition $T_m = 68 \pm 1$ °C ($\theta_N = 1750$, $\theta_D = -6000$); FcRn pH 8.0 (□), two transitions, $T_m = 51.5 \pm 1$ °C ($\theta_N = -2500$, $\theta_D = -6000$) and $T_m = 67 \pm 1$ °C ($\theta_N = -6000$, $\theta_D = -8500$); FcRn pH 6.0 (■), one transition, $T_m = 62 \pm 1$ °C ($\theta_N = -1600$, $\theta_D = -7400$).

MHC class I molecules bind peptides derived from endogenously synthesized proteins during their assembly in the endoplasmic reticulum (Townsend & Bodmer, 1989), and peptides greatly increase the stability of the class I heterodimer (Fahnestock *et al.*, 1992). Because FcRn is predicted to contain a groove similar to that functioning as the peptide binding site in class I molecules (Simister & Mostov, 1989a,b), we analyzed samples of purified FcRn for the presence of bound peptides. Using conditions that are required to elute peptides associated with class I MHC heterodimers, we find no evidence for the association of peptides with FcRn, results consistent with a previous sequencing analysis of intact FcRn (Gastinel *et al.*, 1992). It is unlikely that the conditions required to elute peptides from FcRn would need to be more stringent than the acid and heat treatment procedures that release peptides from all class I molecules analyzed so far (Rötzschke *et al.*, 1990; Van Bleek & Nathenson, 1990; Falk *et al.*, 1991; Jardetzky *et al.*, 1991). Thus it appears that there is no high-affinity interaction between the majority of soluble FcRn molecules and peptides derived from cellular proteins.

Because peptides were not found in association with purified FcRn, we examined its sequence to determine whether residues involved in conserved contacts to peptides in class I molecules were present in analogous positions in the FcRn sequence. Crystallographic studies of MHC class I molecules have shown that pockets at each end of the peptide binding site contact the main-chain atoms of the N- and C-termini of octamer and nonamer peptides (Saper *et al.*, 1991; Madden *et al.*, 1991; Fremont *et al.*, 1992; Matsumura *et al.*, 1992; Silver *et al.*, 1992). These two pockets (A and F) are lined with residues that are highly conserved in class I sequences. In pocket A, tyrosine residues at positions 7, 59, 159, and 171 form hydrogen bonds to main-chain atoms of the amino terminal end of the peptide (Madden *et al.*, 1991; Matsumura *et al.*, 1992; Silver

et al., 1992). At the analogous positions in the FcRn sequence, the residues are Tyr, Tyr, Phe, and His, respectively. In pocket F of class I molecules, the carboxyl group of the peptide C-terminus hydrogen bonds with the conserved side chains of Tyr 84, Thr 143, and Lys 146 (Madden *et al.*, 1991; Matsumura *et al.*, 1992; Silver *et al.*, 1992), while the analogous residues in FcRn are Gln, Val, and Leu. If pockets A and F existed in FcRn, their chemical nature would be altered such that, in most cases, similar hydrogen bonds with both peptide termini could not be formed, correlating with our observation of the absence of peptides associated with the purified protein.

Many studies have demonstrated that β2m associated with class I MHC heavy chains undergoes an exchange reaction with free β2m (Hyafil & Strominger, 1979; Bernabeu *et al.*, 1984, Kozlowski *et al.*, 1991). The exchange rate has recently been shown to be dependent upon the nature of the bound peptide; thus, the exchange rate of complexes formed with peptides of optimal length and sequence is over an order of magnitude slower ($t_{1/2}$ = 2400 min) than the exchange rate of complexes formed with suboptimal or nonspecific peptides ($t_{1/2}$ = 60 min) (Parker *et al.*, 1992). β2m exchange from FcRn heterodimers had not been previously documented, so we tested for the occurrence of exchange and sought to define the parameters that influence the exchange rate in this MHC-like heterodimer that does not contain peptide. Although FcRn heterodimers secreted from CHO cells do not appear to incorporate endogenous hamster β2m or bovine β2m from the medium (Gastinel *et al.*, 1992), exchange was found to occur in the presence of excess rat β2m. We have subsequently found that the CHO cells transfected with the FcRn heavy chain and rat β2m genes (Gastinel *et al.*, 1992) secrete excess rat β2m into the medium, so that secreted FcRn heterodimers may undergo an exchange reaction that is not detected by N-terminal sequencing of the light chain (M. Raghavan and P. J. Bjorkman, unpublished observations).

The dissociation rate of β2m from the FcRn heterodimer was found to be markedly pH-dependent, being increased at pH 7.8 ($t_{1/2}$ = 170–175 min) as compared to pH 6.1 ($t_{1/2}$ = 1900–2400 min). Such an increase in the dissociation rate could be explained by the specific titration of charged residues at the heterodimer interface, or it could be indicative of conformational changes affecting the subunit interaction. Far-UV CD spectra taken of FcRn at room temperature were nearly identical at the two pH values; therefore, there is no evidence for changes in secondary structure as a function of pH. The fluorescence emission spectra following excitation at 280 nm were also nearly identical at the two pH values (data not shown), indicating a conservation of the environment of aromatic residues. Thus, the pH dependence of β2m exchange is likely to be due to differences in the heterodimer interface at the two pH values, with the interchain interactions being less stable at pH 8.0 than at pH 6.0. The pH dependence of the β2m exchange rate was also investigated for the class I molecule HLA-B7 (Parker & Strominger, 1985). Over the pH range from 6.0 to 8.0, the $t_{1/2}$ for dissociation was observed to increase by over an order of magnitude. Although it is difficult to evaluate these data because the HLA-B7 heterodimers used in this study presumably contained a mixture of endogenous peptides, HLA-B7 appears to be less stable at pH 6.0 compared to pH 8.0. It was suggested that these results may be relevant to the dissociation of class I MHC heterodimers during recycling through acidic compartments (Parker & Strominger, 1985). Thus the effect of pH on the stability of HLA-B7 over this pH range is the opposite of the effect on FcRn, perhaps reflecting the differences in the physiological function of the two types of molecules.

Studies of heat-induced unfolding of FcRn at pH 6.0 and 8.0 monitored by the CD signal at 210 nm confirmed that the FcRn heterodimer is less stable at pH 8.0 than pH 6.0. By contrast, neither the class I MHC molecule H-2K^d nor the FcRn ligand (rat Fc) showed significant thermal stability differences in the pH range 6.0–8.0 (M. L. Fahnestock, I. Tamir, M. Raghavan, and P. J. Bjorkman, unpublished results). In combination with exchange rate measurements, these results suggest that the nature of the FcRn heavy-chain/β2m association is altered over a narrow pH range, with the heterodimer being considerably more stable at the permissive pH for IgG binding. At pH 8.0, the unfolding pattern of FcRn is similar to that observed for the class I MHC molecule H-2K^d at pH 7.0 (Fahnestock *et al.*, 1992).[2] Two transitions were observed during thermal denaturations of K^d, the first corresponding to the dissociation and denaturation of the heavy chain and the second corresponding to the denaturation of β2m. The T_m for the first transition was dependent upon whether the class I molecule was empty or peptide-filled, occurring at 45 ± 1 or 57 ± 1 °C, respectively, and illustrating that a peptide-filled class I molecule was significantly more stable than an empty molecule (Fahnestock *et al.*, 1992). Here we show that, at pH 8.0, the thermal stability of FcRn is less than that observed for peptide-filled K^d, but greater than that of empty K^d. At pH 6.0, however, the FcRn heavy chain has enormously enhanced stability compared to its stability at pH 8.0 and is somewhat more stable than peptide-filled K^d. Since the primary function of class I MHC molecules is the binding of endogenously synthesized peptides, enhanced stabilities of peptide-filled class I molecules compared to empty molecules would appear to be a functional requirement. FcRn, however, is a surface receptor for IgG with no known functional requirement for binding peptides; thus, the expectation is that the heterodimeric unit comprising the receptor is inherently stable in the absence of peptide. Our experiments demonstrate that pH appears to have as significant an influence on the stability of the FcRn heterodimer as the presence or absence of peptide does on the stability of a class I molecule.

Since imidazole side chains of histidines usually have pK_a values in the range 6–7, histidine residues have been suggested to be present at the Fc binding site of FcRn to account for the pH dependence of IgG binding (Simister & Mostov, 1989b; Parham, 1989). However, mutagenesis experiments in which all FcRn heavy-chain histidine residues predicted to be surface accessible (and would therefore have the potential to contact Fc directly) were changed to the corresponding residues found in class I molecules demonstrated that histidines 168, 250, and 251 (corresponding to the class I residues 171, 255, and 256) of FcRn are not essential for the pH dependence of IgG binding (M. Raghavan and P. J. Bjorkman, unpublished results). These results suggest that the observed pH dependence of the interaction between FcRn and IgG (Jones & Waldman, 1972; Simister & Rees, 1985; Simister & Mostov, 1989a,b) does not necessarily reflect the direct involvement

[2] Melting curves reported previously for H-2K^d were measured at 223 nm, where changes in ellipticity resulting from denaturation of free β2m are opposite in sign to those resulting from denaturation of the heavy chain in the heterodimer (Fahnestock *et al.*, 1992). The FcRn melting curves were measured at 210 nm, where the β2m and heavy-chain denaturations both result in increased negative ellipticity values; thus, the K^d and FcRn denaturation profiles differ in appearance but correspond to similar unfolding phenomena.

of histidine residues in the IgG binding site of FcRn, but could occur as a consequence of pH-induced structural changes in parts of the molecule distinct from the binding site. Here we report pH-dependent differences in the stability of FcRn indicative of structural changes that may be responsible for the abrogation of IgG binding at high pH.

There are at least two possible models for how the stability differences we observe in FcRn relate to the binding and release of IgG. These are as follows: (i) Destabilization of the FcRn heavy chain–β2m interface owing to titration of charged residues at pH values above 7.5 results in an altered ligand binding site, causing the release of IgG. (ii) Structural changes at a position distinct from the heterodimer interface in the FcRn molecule result in the release of IgG and also indirectly affect the strength of interaction of FcRn subunits. Using the structure of the class I MHC molecule HLA-A2 (Bjorkman *et al.*, 1987a; Saper *et al.*, 1991) as a model for the structure of FcRn, we examined the chemical properties of the residues assumed to be located in the FcRn heterodimer interface. A histidine at position 10 in FcRn (corresponding to Phe 8 in class I molecules) is predicted to be involved in contacts between the α1 domain and β2m. Other residues at the α1α2–β2m interface are largely conserved between FcRn and class I molecules, whereas there are significant differences in the α3–β2m interface region. Among the observed differences in the α3–β2m interface is residue 237 (corresponding to residue 242 in class I molecules), which is a histidine in FcRn and a conserved glutamine in class I sequences. In class I molecules, Gln 242 forms hydrogen bonds to the main-chain carbonyl groups of β2m residues 11 and 12 (Saper *et al.*, 1991). If a histidine at this position in FcRn is involved in similar contacts, deprotonation of its imidazole ring at pH values above 7.5 could disrupt hydrogen-bonding contacts to β2m residues. Such a loss of hydrogen bonding could account in part for the pH dependence we have observed in FcRn for its thermal stability and β2m dissociation rates. Whether the same changes also account for a loss of ligand (IgG) binding above pH 7.5 remains to be tested using mutagenesis studies of potential subunit interface residues such as His 237 (class I 242), His 10 (class I 8), and other proximal amino acids.

ACKNOWLEDGMENT

We thank Dr. Margaret Fahnestock for providing purified H-2K^d for the comparative acid elutions, Drs. Fahnestock and Ilana Tamir for performing experiments to assess the pH dependence of H-2K^d stability, the Caltech and USC Microchemical Facilities for sequence analyses, Dr. Lennart Lögdberg for the anti-rat β2m monoclonal antibody, and R. M. Renny Feldman for modeling the FcRn structure based on the structure of HLA-A2. We also thank Dr. Michael Blum for earlier contributions to the FcRn project in our laboratory. We are grateful to Drs. Neil Simister, Margaret Fahnestock, Wilhelm Burmeister, and Roland Strong for critical reading of the manuscript.

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