Isolation and Partial Characterization of a β_2-Microglobulin-Containing, H-2 Antigen-Like Murine Serum Protein†

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ABSTRACT: β_2-Microglobulin occurs in three size classes in normal mouse serum. Apart from β_2-microglobulin in free form, it occurs associated with H-2 antigen fragments as well as with a high-molecular-weight protein complex. The latter material has been isolated in highly purified form. During the isolation procedure, the high-molecular-weight β_2-microglobulin-containing serum material dissociated to yield a component of considerably smaller size. The highly purified material was comprised of molecules composed of two subunits. The small subunit displayed size, charge, and antigenic characteristics which made it indistinguishable from β_2-microglobulin derived from regular H-2K and D antigens. The large subunit had an apparent molecular weight of about 42 000 and was, thus, slightly larger than the water-soluble H-2 antigen heavy-chain fragment produced by limited proteolysis. The serum-component heavy chain had a lower isoelectric point than the corresponding H-2 antigen chain and the serum material exhibited less charge heterogeneity than the H-2 antigens. Whereas papain-solubilized H-2 antigens were efficient in inhibiting alloantiserum-induced cytotoxicity, the serum component had no such effect even at 1000-fold higher concentrations. With use of similar techniques, the serum component was shown to be devoid of easily recognizable alloantigenic determinants shared with Ia or TL antigens. Antiserum against the H-2 region-controlled Ss protein did not react with the serum material. Rabbit antisera raised against highly purified serum component and H-2 antigen preparations recognized preferentially the immunogens but cross-reacted reciprocally. With use of the rabbit antiserum against the isolated serum component, attempts were made to find out the polypeptide chain composition of the intact high-molecular-weight β_2-microglobulin-containing serum material. Indirect immunoprecipitation of the ^{125}I-labeled, partially purified high-molecular-weight material followed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis revealed the coprecipitation of two polypeptide chains with apparent molecular weights of 65 000 and 75 000 in addition to the 12 000 and 42 000 molecular weight chains. Thus, all data taken together strongly suggest that normal mouse serum contains a high-molecular-weight multisubunit protein complex which contains one polypeptide chain which is similar, but distinct, from the alloantigenic H-2 antigen subunit.

The major histocompatibility complex (MHC)[1] controls a number of immunobiological events (for reviews, see Shreffler and David, 1975; Klein, 1974). So far, the molecular mechanisms underlying the MHC-controlled events are but poorly understood. However, immunogenetic analyses have revealed that the MHC region, in the murine system called the H-2 complex, emcompasses several distinct loci that control the expression of cell-surface antigens. According to their basic structure, two distinct types of MHC-controlled cell-surface antigens can be distinguished. The H-2K, H-2D, TL, Qa-2, and H-2D′ antigens are composed of two types of polypeptide chains. The larger chain, with an apparent molecular weight of about 45 000, carries the particular alloantigenic determinants, whereas the smaller chain, with a molecular weight of 12 000, is invariant and has been identified as β_2-microglobulin (Rask et al., 1974; Silver and Hood, 1974; Natori et al., 1974; Östberg et al., 1975; Vitetta et al., 1975; Michaelson et al., 1977; Hansen et al., 1977). The other MHC-controlled family of cell-surface molecules, the Ia antigens, are probably derived from three or more genes in the MHC region. All these molecules seem to be dimers comprising dissimilar subunits with the apparent molecular weights of 27 000 and 35 000 (see Cullen et al., 1976). It is as yet unclear if both or only one of the subunits express alloantigenic determinants. However, all the described molecules have been discovered due to their genetic polymorphism, as reflected by their expression of alloantigenic determinants.

Recently, we raised a heteroantiserum directed against highly purified H-2 K and D antigens. This antiserum seemed to react with several H-2 antigen-like molecules like the TL and possibly the Qa-2 and H-2D′ antigens but also with H-2 antigen-like components present in normal mouse serum (Kvist et al, 1978). Natori et al. (1976) and Natori and Hansen (1977) have previously examined some of the properties of H-2 antigen-like substances present in serum. The present communication reports the isolation of an H-2 antigen-like serum component in highly purified form and some of its physicochemical and immunological properties are described.

Materials and Methods

Serum. Normal mouse serum was obtained from B10.A mice maintained in our own mouse colony.

Antisera. Two alloantisera directed against H-2 antigens were used in this study. One of the antisera, against H-2K^k antigens, was obtained by injecting (B10.D2 × B10)F$_1$ mice (H-2K^{d}D^d × H-2K^{b}D^b) with B10.A (H-2K^{k}D^d) spleen and lymph node cells. The antiserum against H-2 D^d antigens was the same as used earlier (Kvist et al., 1977) and was raised by injecting (B10.BR × B10)F$_1$ mice (H-2K^{k}D^k × H-2K^{b}D^b) with B10.A (H-2K^{k}D^d) spleen and lymph node cells. An antiserum against Ia^k antigens was obtained by injecting male and female A.TH mice with spleen and lymph node cells from A.TL mice. The anti-TL 1.2.3 antigen serum has been described previously (Anundi et al., 1975). A rabbit antiserum raised against highly purified Ss protein was obtained as reported (Curman et al., 1975). The reactivities of rabbit antisera directed against highly purified β_2-microglobulin and papain-solubilized H-2 antigens have been described elsewhere (Peterson et al., 1975; Kvist et al., 1978). A rabbit antiserum

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[1] Abbreviation used: MHC, major histocompatibility complex.

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against a sample of highly purified serum component was produced with repeated lymph node and foot pad injections. The full characterization of this antiserum will be described in a subsequent communication (Kvist, S., and Peterson, P. A., in preparation).

H-2 Antigens. Highly purified H-2 antigens, solubilized by limited papain digestion, were isolated from a crude membrane fraction derived from B10.A mouse spleens. The purification procedure and the characteristics of the isolated antigens were identical to those previously described (Kvist et al., 1977).

Special Materials. Sephadex G-100 and G-200, DEAE-Sephadex A-50, and Sepharose 6B (Pharmacia Fine Chemicals AB, Uppsala, Sweden) were prepared according to the instructions supplied. Guanidine hydrochloride was obtained from Sigma and treated with activated charcoal prior to use. All other chemicals were of analytical grade or better.

Concentration of Proteins. Protein in fractions containing the β_2-microglobulin serum component was concentrated by ultrafiltration (Berggård, 1961). The recoveries of the serum component were always greater than 85%.

Immunological Techniques. Indirect immunoprecipitations of radioactively labeled protein were carried out according to a previously described procedure (Ostberg et al., 1976). The inhibition of alloantiserum-induced lymphocytotoxicity was measured by the ^{51}Cr-release method (Sanderson, 1964; Wigzell, 1965). Radioimmunoassay procedures for the determination of β_2-microglobulin and the heavy chains of the serum component and of the H-2 antigens were developed according to the technique outlined before (Klareskog et al., 1978). Highly purified preparations of ^{125}I-labeled heavy chains of the two types were obtained by subjecting ^{125}I-labeled serum component and H-2 antigens, respectively, to gel chromatography on Sephadex G-200 columns after the interaction between the subunits had been abolished by KSCN. The procedure has been detailed before (Kvist et al., 1977).

Electrophoresis and Isoelectric Focusing. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate was performed according to the procedure of Laemmli (1970). Isoelectric focusing in polyacrylamide gels was carried out as described by O'Farrell (1975). When radioactively labeled components were separated, the gels were segmented into 2-mm-wide slices by a Gilson automatic gel fractionator after completed electrophoresis. Chemical amounts of protein were detected by staining with Coomassie brilliant blue, and the stained protein zones were recorded in a Gilford linear-transport gel scanner.

Analytical Gel Chromatography. The molecular weights of the serum component and the separated subunits were determined by gel chromatography on a column (120 × 1 cm) of Sepharose 6B (Fish et al., 1969). Stokes' molecular radii were estimated by analytical gel chromatography on columns (100 × 1 cm) of Sephadex G-200, G-100, and G-75 equilibrated with 0.02 M Tris-HCl buffer (pH 8.0) containing 0.15 M NaCl. The detailed procedure was the same as that of Karlsson et al. (1972).

Determinations of Diffusion Coefficients, Sedimentation Constants, Molecular Weights, and Frictional Ratios. Apparent diffusion coefficients ($D_{20,w}$) were computed from the Stokes' radii obtained from gel chromatography by the use of the Stokes–Einstein equation (see Gosting, 1956). The sedimentation constants were determined by ultracentrifugation in linear sucrose gradients from 5 to 20% (w/v) in 0.02 M Tris-HCl buffer (pH 8.0) containing 0.15 M NaCl. Molecular weights were calculated from sedimentation constants, diffusion coefficients, and partial specific volumes by the equation of Svedberg. Frictional ratios (f/f_0) were calculated from

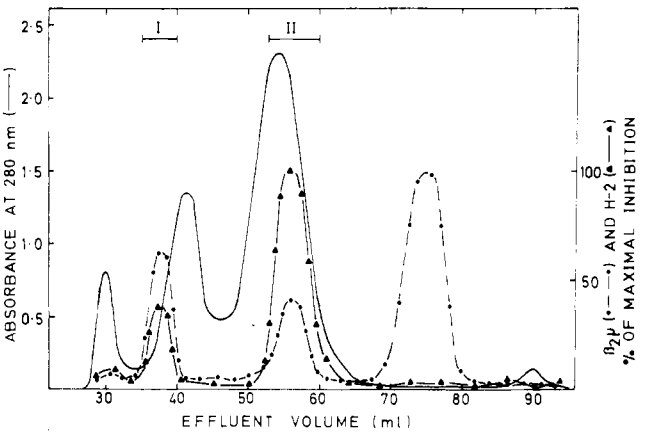

FIGURE 1: Gel chromatography on Sephadex G-200 of 0.6 mL of normal serum. The column (100 × 1 cm) was equilibrated with 0.02 M Tris-HCl buffer, pH 8.0, containing 0.15 M NaCl. Fractions of 1.2 mL were collected at 15-min intervals. The distribution in the effluent of β_2-microglobulin (● - - - ●) and H-2 antigen heavy chain reacting material (▲ - - ▲) was monitored by radioimmunoassay methods using specific rabbit antisera. Fractions denoted by the bars were pooled and concentrated.

Stokes' radii, sedimentation constants, molecular weights, and partial specific volumes. Since scarcity of material precluded actual measurements of the partial specific volume, $\bar{v}$ was arbitrarily chosen to 0.72. The relevant equations employed are found in Svedberg and Pedersen (1940).

Other Methods. Protein was labeled with ^{125}I and ^{131}I by a slight modification of the chloramin T procedure of Hunter and Greenwood (1962). Immunosorbent chromatography columns containing covalently bound IgG fractions were prepared as outlined by Cuatrecasas (1970). The IgG fractions were isolated from rabbit antisera directed against highly purified H-2 antigen preparations by a procedure involving Na_2SO_4 precipitation and gel chromatography on columns of Sephadex G-200. The immunosorbent columns were equilibrated with 0.02 M Tris-HCl buffer (pH 8.0) containing 0.15 M NaCl. Bound protein was desorbed by the addition of 3 M KSCN to the equilibrating buffer. All spectrophotometric analyses were carried out on a Beckman DU-2 spectrophotometer. Protein concentrations in unpurified fractions were estimated by the Folin procedure of Lowry et al. (1951) with bovine serum albumin as the reference substance. More highly purified fractions were quantitatively assayed for protein by measuring the absorbance at 280 nm. The extinction coefficient at 280 nm for the serum component was arbitrarily chosen to be 1.5 for a 1 mg/mL solution. During the entire isolation procedure, the serum component was monitored by its content of β_2-microglobulin. The latter protein was detected by the radioimmunoassay method outlined above.

Results

Identification of H-2 Antigen-Like Molecules in Serum. Normal mouse serum was subjected to gel chromatography on a column (100 × 1 cm) of Sephadex G-200 equilibrated with 0.02 M Tris-HCl buffer (pH 8.0) containing 0.15 M NaCl. As expected, the protein resolved into three peaks, as can be seen in Figure 1. The distribution in the effluent of β_2-microglobulin and of H-2 antigen heavy-chain-reacting material was independently monitored by sensitive radioimmunoassay techniques. It is evident from the figure that β_2-microglobulin occurred in three from each other well separated elution positions corresponding to K_{av} values of about 0.10, 0.42, and 0.74, respectively. The latter two K_{av} values are identical to those obtained for papain-solubilized H-2 antigens

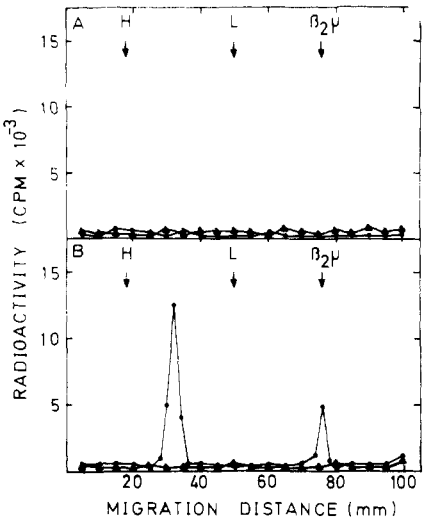

FIGURE 2: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of ^{125}I-labeled molecules in fraction I (A) and II (B) of normal mouse serum (cf. Figure 1), reactive with a mixture of anti-H-2K^k and anti-H-2D^d alloantisera (● — ●) and normal mouse serum (▲ — ▲), respectively. Immune complexes were isolated by indirect immunoprecipitation, reduced, dissolved, and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The arrows denote the migration position of ^{131}I-labeled IgG heavy (H) and light (L) chains and β_2-microglobulin ($\beta_2\mu$).

and for free β_2-microglobulin (Kvist et al., 1977). The β_2-microglobulin-containing peak emerging at a K_{av} value of 0.10 (in the figure denoted I) did not seem to correspond to the elution position of any of the classical molecules containing β_2-microglobulin. Material reacting with the rabbit anti-H-2 antigen heavy-chain antiserum occurred in the chromatogram at positions identical to those for the two largest β_2-microglobulin-containing components, as revealed by the radioimmunoassay. It is noteworthy that the relative amounts of β_2-microglobulin and H-2 antigen-reacting material varied for the two elution positions.

To further analyze the H-2 antigen-like material present in serum, the two fractions, denoted I and II in Figure 1, were separately pooled and concentrated. Aliquots from each fraction were labeled with ^{125}I and material isolated by indirect immunoprecipitation was analyzed by NaDodSO$_4$-polyacrylamide gel electrophoresis. Figure 2 shows that fraction II, but not fraction I, contained molecules reactive with the relevant alloantisera directed against H-2K and D antigens. The precipitated molecules displayed the expected polypeptide patterns, i.e., one molecule with the apparent molecular weight of 12 000 and another molecule with the apparent molecular weight of 37 000. From these data it appeared reasonable to conclude that normal mouse serum contains a β_2-microglobulin-associated molecule that does not exhibit the typical H-2K or D alloantigenic determinants.

Isolation of the High-Molecular Weight β_2-Microglobulin-Containing Molecule from Normal Mouse Serum. The high-molecular-weight β_2-microglobulin-containing serum component was isolated from 200- to 500-mL batches of normal mouse serum. A typical isolation procedure is summarized in Table I.

The serum was centrifuged at 105 000g for 60 min to remove particulate material. The supernatant, containing about 13 000 mg of total protein, was subjected to gel chromatography on a column (112 × 8 cm) of Sephadex G-200 equilibrated with 0.02 M Tris-HCl buffer (pH 8.0) containing 0.15 M NaCl. The chromatogram was similar to that depicted in Figure 1. The elution position for the high-molecular-weight

TABLE I: Purification of the β_2-Microglobulin-Containing Serum Component.

fraction	total protein (mg)	β_2-microglobulin[a] (%)	purity (%)
mouse serum	13 000[b]	100	0.025
Sephadex G-200	668[b]	87	0.42
Na_2SO_4 precipitation	154[b]	65	1.4
DEAE-Sephadex, pH 6.5	45[b]	51	3.8
Sephadex G-200	4.2[b]	36	28
affinity chromatography	1.3[c]	26	66
Sephadex G-200	0.55[c]	17	100

[a] Measured by a radioimmunoassay procedure. The quantitative measurements had to be performed in the presence of 0.1% sodium dodecyl sulfate to release β_2-microglobulin from the associated polypeptide chains.[2] [b] Determined by the Folin technique. [c] Estimated from the optical density at 280 nm.

β_2-microglobulin-containing component corresponded to a K_{av} value of 0.10, as evidenced by the β_2-microglobulin radioimmunoassay procedure, and fractions comprising this peak were pooled and concentrated (cf. Figure 1).

Most of the protein in the gel-chromatography fraction containing the β_2-microglobulin-associated component consisted of serum immunoglobulin. The β_2-microglobulin-containing fraction obtained from the Sephadex G-200 chromatography step, containing 668 mg of total protein in 40 mL of the Tris buffer, was therefore made 18% (w/v) with regard to sodium sulfate. After incubation at 37 °C for 30 min, the precipitate was removed by centrifugation for 20 min at 10 000g. The supernatant, comprising more than 75% of the β_2-microglobulin-associated component, was exhaustively dialyzed against 0.01 M sodium phosphate buffer (pH 6.5) containing 0.025 M NaCl.

The dialyzed material, containing 154 mg of total protein, was subjected to ion-exchange chromatography on a column (10 × 2 cm) of DEAE-Sephadex equilibrated with the same phosphate buffer. Elution was performed at pH 6.5 with a 800-mL linear gradient of NaCl from 0.025 to 0.60 M. Apart from the breakthrough material, the applied protein resolved into two main peaks. β_2-Microglobulin gave rise to a single broad peak which did not coincide with any of the main protein peaks. The β_2-microglobulin-containing fractions, which represented the trough between the two main protein peaks, were pooled and concentrated.

The β_2-microglobulin-containing serum-component material obtained from the DEAE-Sephadex chromatography step was subjected to gel chromatography on a column (100 × 2 cm) of Sephadex G-200 equilibrated with 0.02 M Tris-HCl buffer (pH 8.0) containing 0.15 M NaCl. The major part of the applied protein emerged close to the void volume of the column but small amounts of protein were eluted at a K_{av} value of about 0.4. The occurrence of β_2-microglobulin in the chromatogram was exclusively confined to the latter-eluted protein peak. This result was surprising, since during the first gel-chromatography step the β_2-microglobulin-associated component displayed a K_{av} value of 0.1 (cf. Figure 1). However, preliminary experiments have demonstrated that the β_2-microglobulin-associated component is broken down during the DEAE-Sephadex chromatography step (Kvist, S., and Peterson, P. A., in preparation) (see below). Fractions comprising β_2-microglobulin were pooled and concentrated.

The purity of the β_2-microglobulin-containing serum component was assessed after this purification step. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed the

TABLE II: Physical Properties of the Highly Purified β_2-Microglobulin-Associated Serum Component.

	serum component	H-2 antigens[a]
sedimentation constant (s)	4.1	3.9
Stokes' molecular radius (Å)[b]	33	33
diffusion constant ($\times 10^7$ cm^2 s^{-1})	6.3	6.3
frictional ratio (f/f_0)	1.22	1.24
mol wt		
sediment.—Stokes' radius	54 000	51 500
gel chromatography[c]	40 000; 12 000	38 000; 12 000
electrophoresis[d]	42 000; 12 000	37 000; 12 000

[a] The data are taken from a previous article (Kvist et al., 1977). [b] Estimated by analytical gel chromatography. [c] Determined in 6 M quanidine hydrochloride on reduced and alkylated polypeptide chains. [d] Data from sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

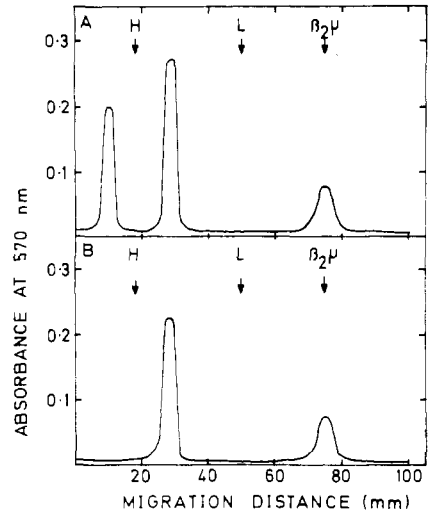

FIGURE 3: Absorbance profiles of Coomassie Brilliant Blue stained sodium dodecyl sulfate–polyacrylamide gels. The separated materials consisted of the β_2-microglobulin-containing serum fraction before the first gel chromatography step (A) and after the last purification step (B) (cf. Table I). The arrows denote the migration positions of IgG heavy (H) and light (L) chains and of β_2-microglobulin ($\beta_2\mu$) run on a separate gel.

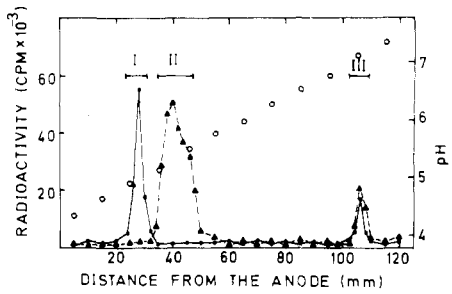

FIGURE 4: Isoelectric focusing in 6 M urea of the ^{125}I-labeled β_2-microglobulin-containing serum component (● — ●) and ^{131}I-labeled, highly purified, papain-solubilized H-2^a antigens (▲ - - - ▲). Radioactive material in the fractions denoted by the bars was extracted and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The pH (○) was measured in alternate fractions.

presence of three distinct polypeptide chains (Figure 3).

Final purification of the β_2-microglobulin-containing serum component was achieved by immunosorbent chromatography on a Sepharose 4B column containing covalently bound rabbit antibodies directed against highly purified H-2 antigens. Protein which did not bind to the column was eluted with 0.02 M Tris-HCl buffer (pH 8.0) containing 0.15 M NaCl. Bound protein was desorbed by the same buffer containing 3 M KSCN. The latter material was dialyzed, concentrated, and subjected to gel chromatography on a column (102 × 1 cm) of Sephadex G-200 equilibrated with 0.02 M Tris-HCl buffer (pH 8.0) containing 0.15 M NaCl. The gel-chromatography step removed minor amounts of aggregated protein, but the main protein peak was symmetrical and emerged at a K_{av} value of about 0.4 The distribution of β_2-microglobulin was coincident with the major protein peak. Evidence will be given below to certify that this material represents a highly purified H-2 antigen-like component with the capacity to bind β_2-microglobulin.

Purity and Physicochemical Characterization of the β_2-Microglobulin-Binding Serum Component. Size homogeneity for the isolated serum component was established by gel chromatography on a column of Sephadex G-200 and by sedimentation velocity analyses. The apparent sedimentation constant of 4.1 S together with the value for the Stokes' molecular radius (33 Å) for the isolated component indicate that the molecular weight is about 54 000.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the isolated material revealed two protein zones, as can be seen in Figure 3, with apparent molecular weights of 12 000 and 42 000, respectively. Likewise, gel chromatography on a column of Sepharose 6B equilibrated with 6 M guanidine hydrochloride showed that the serum component gave rise to two peaks with apparent molecular weights of 40 000 and 12 000, respectively. The subunit separation occurred in the presence, as well as in the absence, of reducing agents, suggesting that the interaction between the two types of polypeptide chains is entirely consistent of noncovalent forces.

Table II summarizes some physical properties of the isolated serum component. For comparison, the corresponding data for papain-solubilized H-2 antigens are presented.

Comparative Studies of the β_2-Microglobulin-Binding Serum Component and Papain-Solubilized H-2 Antigens. The physical properties of the β_2-microglobulin-binding serum component are very similar, if not identical, to those of papain-solubilized H-2 antigens (cf. Table II). Various tests were therefore performed to examine in greater detail the properties of the serum component in relationship to those of papain-solubilized H-2 antigens.

The β_2-microglobulin-containing serum component, labeled with ^{125}I, and papain-solubilized, highly purified H-2^a antigens, labeled with ^{131}I, were mixed and subjected to isoelectric focusing in urea. Figure 4 depicts the result. Material from each peak was separately analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The peaks with apparent pI values of about 7.1 comprised exclusively β_2-microglobulin (not shown). All other peaks also contained components that were homogeneous in molecular weight. Figure 5 demonstrates that the ^{131}I-labeled H-2 antigen heavy chain was slightly smaller than the acidic heavy chain of the β_2-microglobulin-containing serum component. Thus, these data show that the heavy chain of the serum material is somewhat larger, more acidic, and less heterogeneous in charge than the corresponding chain of the papain-solubilized H-2 antigens.

It has previously been shown that the interaction between the H-2 antigen subunits is abolished in the presence of high concentrations of KSCN (Kvist et al., 1977). This procedure was therefore used to separate the subunits of the β_2-microglobulin-containing serum component. The ^{125}I-labeled serum

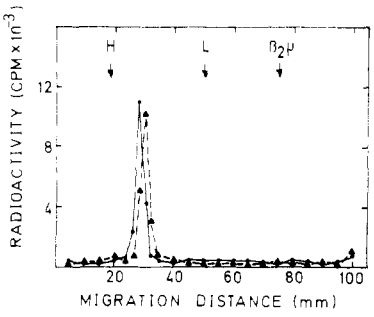

FIGURE 5: Polyacrylamide gel electrophoresis in sodium dodecyl sulfate of material from fraction I (the acidic component of the serum material labeled with ^{125}I) and fraction II (^{131}I-labeled H-2^a antigen heavy chain) obtained from the isoelectric focusing separation depicted in Figure 4. The arrows denote the migration positions of IgG heavy (H) and light (L) chains and β_2-microglobulin ($\beta_2\mu$) run on a separate gel. ^{125}I radioactivity (● — ●); ^{131}I radioactivity (▲ - - - ▲).

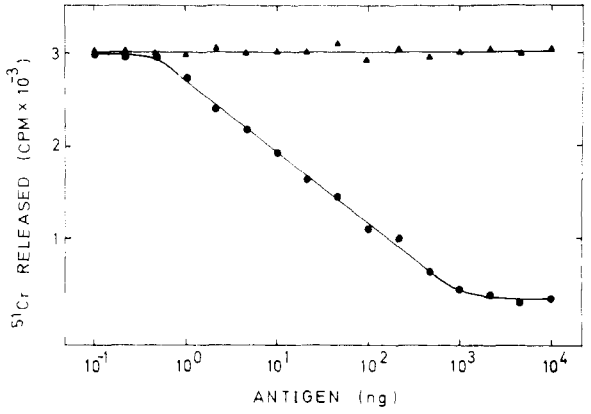

FIGURE 6: Inhibition of cytotoxicity of an anti-H-2D^d alloantiserum as measured by ^{51}Cr release from labeled B10.A spleen cells. The antiserum was diluted 640-fold to yield 80% of maximal cytotoxicity. Various amounts of highly purified H-2^a antigens (● — ●) and β_2-microglobulin-containing serum component derived from B10.A serum (▲ — ▲) were mixed with the appropriate concentration of the antiserum for 30 min before the mixture was incubated with the cells. At a 10-fold lower dilution than used the alloantiserum did not react with Ia antigens as measured by cytotoxicity on ^{51}Cr-labeled B10.A spleen cells after the antiserum had been absorbed on B10.A thymocytes.

material was mixed with ^{131}I-labeled H-2^a antigens and subjected to 6 M KSCN for 3 h. The mixture was then applied to a Sephadex G-100 column (100 × 1 cm) equilibrated with 0.02 M Tris-HCl buffer (pH 8.0) containing 0.15 M NaCl. Prior to the application of the sample, 10 mL of 6 M KSCN was allowed to enter the column. Elution was carried out with the Tris-HCl buffer. The two size classes of subunits were well separated and the radioactive elution profiles of the two isotopes were superimposable. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis on material recovered from the two peaks demonstrated that each type of subunit was free from contamination of the other type. Thus, fractions comprising the ^{125}I-labeled heavy chain of the serum component and the ^{131}I-labeled heavy H-2 antigen chain were pooled and concentrated.

The capacity of the isolated chains to rebind exogeneously added β_2-microglobulin was examined. Unlabeled β_2-microglobulin in a large excess was added to the labeled chains and the mixture was incubated at 37 °C for 60 min. After this period of time antibodies against β_2-microglobulin were added and the resulting immune complexes were isolated. It can be seen in Table III that, whereas only negligible amounts of the H-2 antigen chain were coprecipitated by this procedure, a

TABLE III: Binding of β_2-Microglobulin to the Isolated Heavy Chains from Papain-Solubilized H-2^a Antigens and the Serum Component.[a]

serum	additive	% radioactivity precipitated: H-2 antigen	% radioactivity precipitated: serum component
anti-β_2-M	β_2-M	8.2	48.4
anti-β_2-M		7.6	6.9
anti-H-2 antigen		72.2	69.8
anti-serum component		68.4	70.6
NRS	β_2-M	7.8	5.6
NRS		8.9	8.1

[a] ^{125}I-labeled serum component heavy chain and ^{131}I-labeled H-2^a antigen heavy chain were isolated as described in the text. To mixtures containing 68 000 cpm of ^{125}I and 13 000 cpm of ^{131}I, either no protein (no entry) or 100 μg of β_2-microglobulin (β_2-M) was added. Radioactive molecules reactive with antibodies against β_2-microglobulin, H-2 antigens, and the serum component, respectively, were isolated by indirect immunoprecipitation. Normal rabbit serum (NRS) served as the control.

significant portion of the serum component heavy chain had obviously recombined with the β_2-microglobulin. Control experiments demonstrated that in the absence of β_2-microglobulin insignificant amounts of the serum component chain reacted with the anti-β_2-microglobulin serum. Neither did normal rabbit serum display any reactivity with the labeled material. Thus, these experiments show that, under the conditions employed, the serum component retains at least in part its capacity to bind β_2-microglobulin in contrast to the heavy H-2 antigen chain.

Initial attempts failed to disclose the presence of H-2 alloantigenic determinants on the high-molecular-weight β_2-microglobulin-associated serum component (cf. Figure 2). It is, however, well known that chemical modifications, like iodination, may derange the alloantigenic determinants (Pancake and Nathenson, 1972). Therefore, the isolated β_2-microglobulin-containing serum component and H-2^a antigens were separately examined for their ability to inhibit anti-H-2K^k and anti-H-2D^d sera-induced lymphocytotoxicity. Figure 6 shows that an appreciable inhibition of the anti-H-2D^d serum induced cytotoxicity was obtained with less than 10 ng of the H-2 antigen preparation, whereas 1000-fold more of the serum component did not impede the cytotoxic activity of the antiserum. The results were similar when the cytotoxicity was induced with the anti-H-2K^k serum (not shown). These data are, thus, fully consistent with the view that the β_2-microglobulin-containing serum component does not exhibit any of the classical alloantigenic determinants.

TL antigens display a gross structure similar to that of H-2 antigens (see Anundi et al., 1975). However, the serum component material did not exhibit measurable ability to inhibit the cytotoxicity caused by an anti-TL1.2.3 serum when measured by ^{51}Cr release from B10.A labeled thymocytes. In similar types of tests it was shown that the serum component did not display any recognizable Ia-antigenic determinants in as much as the highly purified material did not impede the cytotoxic acitivity of an anti-Ia^k serum against ^{51}Cr-labeled B10.A splenocytes. The H-2 region controlled Ss protein is a serum component (see Curman et al., 1975), but seems to be unrelated to the isolated β_2-microglobulin-containing material. Thus, a rabbit antiserum raised against highly purified Ss protein did not measurably react with the ^{125}I-labeled serum component as measured by indirect immunoprecipitation tests. These data make it reasonable to conclude that the highly purified serum component does not express any of the partic-

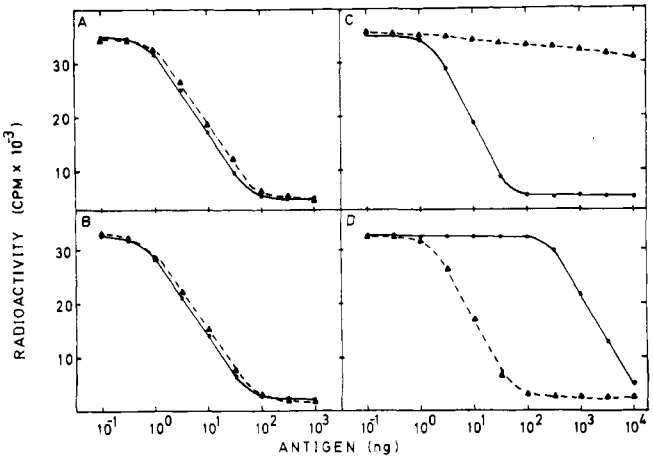

FIGURE 7: Inhibition of H-2 antigen and serum component radioimmunoassays. In A, 50 μL of rabbit antibodies against H-2 antigens, diluted 300-fold, was mixed with 48 000 cpm of ^{125}I-labeled β_2-microglobulin derived from the H-2 antigens. Various concentrations of unlabeled H-2 antigens (● — ●) and unlabeled serum component (▲ - - - ▲) were added to abolish the binding of the ^{125}I-labeled β_2-microglobulin to the antibodies. The radioactivity bound to the antibodies was measured after indirect immunoprecipitation. In B, 50 μL of rabbit antibodies against the serum component, diluted 2000-fold, was mixed with 45 000 cpm of ^{125}I-labeled β_2-microglobulin derived from the serum component. Otherwise the protocol and the symbols are exactly the same as described in A. In C, 50 μL of rabbit antibodies against the H-2 antigens, diluted 700-fold, was mixed with 54 000 cpm of ^{125}I-labeled, separated H-2 antigen heavy chain. The experimental protocol and the symbols are the same as in A. In D, 50 μL of the rabbit antibodies against the serum component, diluted 2000-fold, was mixed with 50 000 cpm of ^{125}I-labeled, separated serum component heavy chain. The experimental procedure and the symbols are the same as in A.

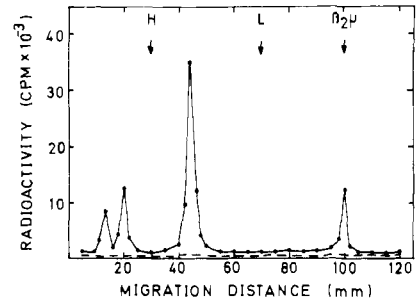

FIGURE 8: Polyacrylamide gel electrophoresis in sodium dodecyl sulfate of ^{125}I-labeled, high-molecular-weight serum proteins reactive with a rabbit antiserum raised against highly prufied papain-solubilized H-2 antigens (● — ●). Normal rabbit serum (● - - - ●) served as the control. The labeled molecules were isolated by indirect immunoprecipitation. See text for further details.

ular antigenic determinants which specify the known products of the H-2K,I,S,D and TL-loci.

To analyze in greater detail the obvious xeno-antigenic relationship between the H-2 antigens and the serum component, a rabbit was immunized with the highly purified β_2-microglobulin-containing serum component. The resulting antibodies displayed excellent reactivity both with the separated serum component subunits and with the separated H-2 antigen subunits as revealed by indirect immunoprecipitation analyses. These results show that antibodies raised against the serum component cross-react with regular H-2 antigens. The extent of the immunological cross-reactivity was examined by quantitative radioimmunoassay procedures. Figure 7 summarizes the results. It is evident from the figure that the two rabbit antisera, which contained antibodies against serum component derived and H-2 antigen derived β_2-microglobulin, respectively, did not distinguish between β_2-microglobulin from the two sources (Figures 7A and 7B). However, the antibodies raised against the heavy H-2 antigen chains reacted so well with the ^{125}I-labeled heavy H-2 antigen chain that even more than a 1000-fold excess of the serum component could not measurably impede this interaction (Figure 7C). Likewise, the antibodies raised against the heavy chain of the serum component reacted much better with the serum component than with the H-2 antigens. Figure 7D shows that the interaction between the ^{125}I-labeled heavy chain from the serum component and the rabbit antibodies against the serum component was abolished both by unlabeled serum component and unlabeled H-2 antigens. However, about 1000 times more of the H-2 antigens had to be added to react the same degree of inhibition as could be attained with the serum component.

Polypeptide Chain Structure of the High-Molecular-Weight β_2-Microglobulin-Associated Serum Component. From the foregoing data it is obvious that during the isolation procedure the high-molecular-weight β_2-microglobulin-associated serum component (material in fraction I of Figure 1) was altered in such a way that the isolated material displayed a much smaller size as judged by gel chromatography (cf. Figure 5). To analyze if the isolated material represented one subunit of several identical ones or one subunit among other dissimilar subunits, the high-molecular-weight material was purified through the first two purification steps (cf. Table I). The material was then subjected to gel chromatography on a column of Sephadex G-200 to ensure that β_2-microglobulin was still associated with the high-molecular-weight material. Fractions comprising β_2-microglobulin and occurring at a K_{av} of about 0.10 were pooled, concentrated, labeled with ^{125}I, and subjected to indirect immunoprecipitation with the rabbit antisera raised against papain-solubilized H-2 antigens, the highly purified serum component and β_2-microglobulin, respectively. Figure 8 depicts the electropherogram of sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the isolated immune precipitate obtained with the rabbit anti-H-2 serum. It is clearly evident that this antiserum precipitated, in addition to the expected 12 000 and 42 000 dalton polypeptide chains, two components with approximate molecular weights of 65 000 and 75 000, respectively. The antisera directed against the serum component and β_2-microglobulin reacted with identical molecules (not shown). It can be seen in the figure that normal rabbit serum did not react with any labeled molecules. Although, the exact relationship between the precipitated polypeptide chains remains unclear, the data strongly suggest that β_2-microglobulin and the 42 000-dalton chain, which were isolated as a complex, occur bound to other types of polypeptide chains in serum.

Discussion

The purification procedure adopted for the β_2-microglobulin-containing serum component was very reproducible. However, the use of the immunosorbent step, which involves desorption of the material under rather drastic conditions, was mandatory to remove one contaminating protein. Despite this rather harsh treatment, the isolated protein appeared "native" in as much as it did not display any tendency to aggregate, it was highly immunogenic, and its antigenic characteristics were similar to if not identical with those of the serum component in fresh serum. Moreover, previous analyses in this laboratory have shown that H-2^a antigens treated under identical conditions retain their alloantigenicity (Kvist et al., 1977). This suggests that thiocyanate may not be too much of a denaturant for H-2 antigens and the serum component since expression of alloantigenic determinants is a sensitive criterion for the

native state of the H-2 antigens (see Pancake and Nathenson, 1972).

The isolated serum component is composed of two noncovalently linked polypeptide chains with apparent molecular weights of 42 000 and 12 000, respectively. Natori et al. (1976) isolated an H-2 antigen-like serum component in radiochemical amounts. They subjected the high-molecular-weight serum component to limited papain digestion and thereby obtained a serum component consisting of 37 000- and 12 000-dalton polypeptide chains. Detergent-solubilized H-2 antigens are composed of polypeptide chains with apparent molecular weights of about 45 000 and 12 000 (Nathenson and Cullen, 1974), whereas the papain-solubilized H-2 antigen chains display molecular weights of 37 000 and 12 000 (see Rask et al., 1974). Detailed studies are required to find out if the serum component heavy chain, which on sodium dodecyl sulfate–polyacrylamide gel electrophoresis is slightly larger than the papain-solubilized H-2 antigen chain, contains a hydrophobic tail like the detergent-solubilized H-2 antigen chain.

The serum component and the papain-solubilized H-2 antigens have several properties in common. It was therefore of importance to establish if the isolated serum material represented H-2 antigens that might have been shedded from cells to occur in serum. Several lines of evidence make it likely that this is not the case. Thus, the isolated serum component heavy chain displays less charge heterogeneity than the corresponding H-2 antigen subunit. The charge heterogeneity of the H-2 antigens is mainly due to a variable content of sialic acid (Kvist et al., 1977). The serum component is distinctly more acidic than the H-2 antigen chain. Natori et al. (1976) have obtained similar data. Moreover, Natori et al. (1976) reported that their material lacked H-2 alloantigenic determinants. They examined ^{125}I-labeled serum component. This chemical modification might, however, have deranged alloantigenic determinants (Pancake and Nathenson, 1972). Therefore, we explored the possible presence of alloantigenic determinants on the "native" serum component. However, also the "native" material appeared to lack alloantigenic determinants since the serum component had no ability to inhibit alloantiserum-induced lymphocytotoxicity. Neither did the serum component impede anti-TL antigen serum induced cytotoxicity. Armeding et al. (1975) have reported that the allogeneic effect factor is H-2 antigen-like in structure but displays Ia-antigenic determinants. However, the serum component described here did not measurably impede anti-Ia antigen serum-induced lymphocytotoxicity. Also antiserum against the Ss protein did not react with the serum component. The antigenic analyses described here together with those of Natori et al. (1976) strongly suggest that the serum component does not display any easily recognizable alloantigenic determinants.

The serum component shares xenoantigenic determinants with H-2 antigens. It is clear from the present data that the β_2-microglobulin associated with the serum component is antigenically indistinguishable from the H-2 antigen-derived β_2-microglobulin. Also the heavy chain of the serum component displays antigenic relationship with the H-2 antigen heavy subunit. It is, thus, evident that, although antisera raised against the two types of materials preferentially react with the immunogens, they display crossreactivity. Natori and Hansen (1977) have shown that a rabbit antiserum against H-2 antigens react weakly with the serum component, which is in agreement with the present findings.

The dissociation of the high-molecular-weight β_2-microglobulin associated serum component to a molecule of considerably smaller size during the isolation procedure raised the question whether the isolated material was part of a more complex macromolecule or just had desaggregated. Indirect immunoprecipitations of the partially purified high-molecular-weight serum component suggested that the two isolated polypeptide chains are bound to unrelated polypeptide chains of larger molecular weights. Attempts are now being made to isolate the coprecipitated polypeptide chains so that the polypeptide chain composition of the intact β_2-microglobulin-associated high-molecular-weight serum component can be determined.

It is yet much too early to speculate about the origin of the β_2-microglobulin-associated serum component. It seems reasonable to assume, however, that, since related molecules of similar type, i.e., the H-2K, H-2D, TL, Qa-2, and H-2D′ antigens, are cell surface proteins, the serum component may very well fulfill its function at the cell surface.

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Symmetry of Binding Sites of a Mouse IgA Myeloma Protein (MOPC 315)†

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ABSTRACT: We have investigated the mechanism of monovalency of the 7S subunit of a mouse IgA myeloma protein (MOPC 315) against a large antigen. This subunit, although it clearly can bind two molecules of a small hapten, fails to precipitate or hemagglutinate the relevant multivalent antigen. In an equilibrium Farr assay, we have shown that the subunit has only one valence for a univalent 40 000 molecular weight antigen (dinitrophenyl-dextran). We have investigated how various levels of affinity labeling quantitatively affect (a) the valence observed in the equilibrium Farr assay against a large antigen, and (b) the binding of the MOPC 315 to an insoluble antigenic matrix. Our results indicate that the Fab regions of the 7S subunit are arranged symmetrically and that the inactivity of one of them toward a large antigen is probably due to steric hindrance caused by the antigen bound to the adjacent site.

The concept that every Fab moiety of an antibody molecule provides one antigen-binding site, and that the sites in any one molecule bind hapten identically, is clearly supported by a large body of structural and binding data with isolated fragments and intact molecules (Nisonoff et al., 1975). There are, however, curious and so far unexplained exceptions in the case of some IgM and IgA molecules: when tested against large antigens, the 7S subunits of such antibodies are functionally monovalent, although they contain two Fab fragments, both of which bind hapten identically. For example, the 7S subunit of the mouse IgA myeloma protein MOPC 315 neither precipitates multivalent dinitrophenyl (Dnp[1]) protein conjugates nor agglutinates Dnp-coated sheep red blood cells (SRBC) (Eisen et al., 1968; Potter, 1972). In addition, equilibrium binding data indicate that half of the Fab fragments in the intact 7S subunit of an IgM rheumatoid factor are not active (Stone & Metzger, 1968).

Steric hindrance of a potentially active site by a large antigen bound to a neighboring site seems the most likely explanation for this apparent monovalence, but other explanations can be imagined. Chavin and Franklin, for example, proposed (Chavin & Franklin, 1969) that it is possible for identical Fab fragments to be arrayed asymmetrically in the 7S molecule, such that the binding site of one would face out and be able to bind a large antigen, and the other would face in and would not. On the other hand, the *in* site could be sufficiently unencumbered to accept a small ligand as efficiently as the *out* site. Such a molecule would not have a twofold axis of symmetry as has been shown for a crystallized IgG molecule (Terry et al., 1968), but rather would have superimposable Fab fragments.

Another alternative explanation for the observed monovalence is an allosteric effect whereby the binding of one ligand alters the conformation of the active site of the neighboring Fab fragment such that it can no longer accept a large ligand. This explanation could further be refined by specifying whether a large or small ligand must bind to the first site in order to inactivate the second site. This third hypothesis seems to be the least appealing.

We have approached this question through the use of affinity labels and an IgA myeloma protein (MOPC 315). We have confirmed (a) that 7S MOPC 315 has two apparent binding sites for a small monovalent hapten and one for a large monovalent antigen; and we have shown (b) that blocking binding sites with a small affinity labeling hapten, bromoacetyl-Dnp-lysine (BADL), quantitatively reduces the binding of large molecules in a manner strongly favoring a model of a symmetric 7S molecule, in which the binding of a large ligand to one site inhibits an adjacent site from binding a second large ligand.

Materials and Methods

Proteins. MOPC 315 was purified from reduced and alkylated ascites by affinity chromatography on Dnp-lysine agarose as described (Goetzl & Metzger, 1970). [^{14}C]MOPC 315 was similarly prepared by using [^{14}C]iodoacetamide for the alkylation step. Rabbit anti-Dnp was prepared by using

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[1] Abbreviations used: SRBC, sheep red blood cells; Dnp, dinitrophenyl; BADL, bromacetyl-Dnp-lysine; EACA, ϵ-aminocaproic acid; Pnp, *p*-nitrophenyl; HGG, human γ-globulin; Dnp-OH, dinitrophenol; NHS, *N*-hydroxysuccinimide ester; BBS, borate buffered saline; SAS, saturated ammonium sulfate.

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