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Hepatitis B Surface Antigen Polypeptides: Artifactual Bands in Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis Caused by Aggregation

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Hepatitis B surface antigen, subtype *ad*, was purified and studied by sodium dodecyl sulfate-polyacrylàmide gel electrophoresis under reducing conditions. Two major bands with molecular weights of 23,500 and 27,500 and several weaker bands with higher molecular weights were observed. When the low-molecular-weight bands and the group of high-molecular-weight bands were excised from the gel, eluted, and reelectrophoresed, neither the low-molecular-weight bands nor the high-molecular-weight bands ever appeared alone, but both high- and low-molecular-weight bands always appeared. It was concluded that the apparently high-molecular-weight bands represented aggregates of the two small polypeptides whose monomers formed the major bands. The preparation thus contained only two polypeptides.

The number and sizes of polypeptides coded by the hepatitis B virus (HBV) genome are not completely known. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions has been used to study the polypeptide compositions of purified preparations of HBV and hepatitis B surface antigen (HBsAg) particles. The latter have a diameter of 16 to 25 nm and are thought to be composed of the lipoprotein material that forms the outer coat of HBV (14). Two or three main polypeptides have been found in the HBV core, whereas estimates of the number and sizes of polypeptides in the surface antigen particles vary strikingly. Amounts of two to nine polypeptides have been reported, with molecular weights ranging from 10,000 to 120,000 (14). A common feature of many reports is the finding of two major polypeptides in the molecular-weight region of 22,000 to 32,000 (2, 6, 13, 17, 19). These two polypeptides have been chemically characterized to some extent, and it seems likely that the only difference between them is that one is a glycosylated form of the common polypeptide backbone (13, 17, 19). The HBV gene coding for this polypeptide has been sequenced recently (23). The aim of the present study was to explore the relationship between the apparently high-molecular-weight polypeptides and the two major low-molecular-weight polypeptides.

MATERIALS AND METHODS

Isolation of HBsAg. HBsAg (*ad*) containing plasma from healthy blood donors was pooled after subtyping the individual units with a radioimmunoassay (22). The plasma was stored at −21°C until use.

About 1 liter of plasma was used at a time for the isolation. The isolation procedure was as follows: the plasma was formulated to contain 5.5% polyethylene glycol by adding a 40% solution of polyethylene glycol 6000 in 0.01 M Tris-hydrochloride–0.14 M NaCl (pH 7.2). The mixture was incubated for 1 h at room temperature and centrifuged for 20 min at 2,000 × *g*. The concentration of polyethylene glycol was increased to 11% in the supernatant, which was allowed to stand for 1 h at room temperature and was then centrifuged as described above. The precipitate was dissolved in one-fourth the initial plasma volume of phosphate-buffered saline (pH 7.8) and incubated overnight at 4°C after mixing with an immunoadsorbent consisting of sheep anti-HBs immunoglobulin coupled to Sepharose 4B by the cyanogen bromide method (3). The immunoadsorbent was washed on a glass filter with a volume of phosphate-buffered saline about 30 times the volume of the gel, transferred to a column, and eluted with 5 M NaI in a volume of phosphate-buffered saline about 1.5 times the bed volume. The eluate was concentrated by pressure dialysis to ca. 10 ml and dialyzed at the same time against 0.1 M glycine-hydrochloride (pH 2.3). Pepsin was added to a final concentration of 0.2 mg/ml, and the solution was incubated for 3 h at 37°C. The solution was cleared by centrifuging for 20 min at 10,000 × *g* and applied to a column (5 by 90 cm) of Sephadex G-200 in phosphate-buffered saline (pH 7.8). Fractions corresponding to the only prominent peak at 280 nm appearing in the void volume were combined and concentrated to a protein concentration of about 5 mg/ml.

The HBsAg prepared by this method consisted of spherical particles 15 to 25 nm in diameter, as studied by electron microscopy. The recovery of HBsAg, as determined by a radioimmunoassay (10), was about 40%. The preparation gave no immunoprecipitate against horse antiserum to normal human serum proteins in agar gel double diffusion.

SDS-PAGE. For electrophoresis, the samples were dialyzed or dissolved in sample buffer or sample buffer containing 8 M urea, and sucrose, SDS, and bromophenol blue were added to 6, 2, and 0.03% final concentrations. For reducing a sample, 2-mercaptoethanol was added to a 4% (vol/vol) final concentration. Before electrophoresis, samples were heated for 2 min at 100°C. Analytical and preparative PAGE was performed in gel slabs (2 by 80 by 140 mm). The buffer system was that described by Laemmli (11). The stacking gel contained 4% acrylamide, the separating gel contained 12% acrylamide, and the ratio of acrylamide to bisacrylamide was 37:1. Electrophoresis was at 40 mA per gel. After electrophoresis, the gels were stained with Coomassie brilliant blue.

Protein corresponding to individual bands or groups of bands was isolated in the following way: HBsAg was divided into several slots and electrophoresed. The gels were stained briefly to make the bands visible. The bands were cut out with a scalpel and transferred to large test tubes. The slices were rinsed overnight with destaining solution (methanol-water-acetic acid; 5:5:1) and lyophilized. A fivefold amount of the wet volume of the slices of 0.1% SDS in 0.05 M NH_4HCO_3 was added to the tubes, which were incubated for 2 days at 37°C. The liquid was changed, and the elution was continued overnight. The combined eluates were lyophilized, dissolved in a minimal volume of water, and dialyzed in sample buffer. The eluates were prepared for reelectrophoresis as described above.

Molecular weights were determined by comparing with standard proteins run in parallel. The standard protein mixture was the Low Molecular Weight calibration kit of Pharmacia Fine Chemicals, Inc., Uppsala, Sweden.

Reduction and alkylation. For reducing and alkylating a sample of HBsAg, the sample was dialyzed in Laemmli gel buffer (0.75 M Tris-hydrochloride [pH 8.8]–0.2% SDS) containing 8 M urea, SDS was added to 2%, and dithiothreitol was added to a 0.02 M final concentration. The sample was heated at 100°C for 1 min and incubated for 1 h at room temperature. Iodoacetamide was added to 0.03 M, and the solution was incubated for 30 min in the dark and dialyzed to the desired buffer.

RESULTS

The effects of urea and reduction were first studied to find out what conditions are needed to dissociate HBsAg into its components. When HBsAg was prepared for SDS-PAGE by boiling for 2 min in the presence of 8 M urea and 2% SDS without reduction, most of the staining material remained at the top of the 4% stacking gel, and no staining material appeared in the 12% separating gel (Fig. 1a). When a sample was prepared with reduction but without urea, the pattern seen in Fig. 1b was obtained. There were two strong bands whose molecular weights were estimated at about 23,500 and 27,500, a triplet of weaker bands, the apparent molecular weights of which were about 41,000, 46,000, and 51,000, and a very faintly stained region whose middle point corresponded to a molecular weight of about 72,000. Some stained material appeared on top of the separating gel. When 8 M urea was included in the sample in addition to SDS and 2-mercaptoethanol, practically all the stain appeared in the separating gel.

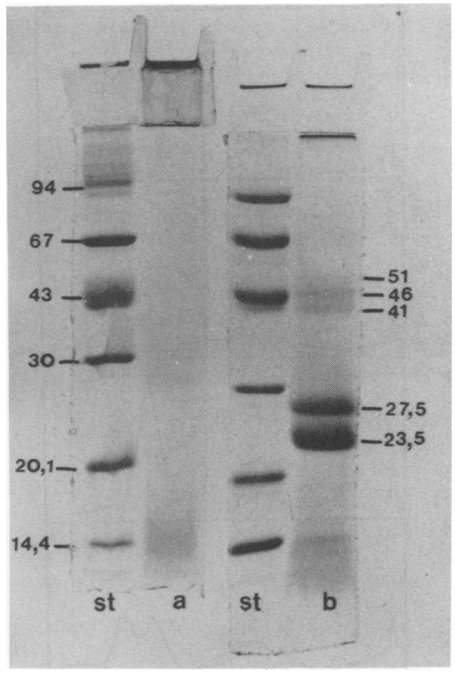

FIG. 1. *SDS-PAGE of purified HBsAg. The samples (150 μg of protein) were prepared by heating for 2 min at 100°C in sample buffer with the following additions: (a) 2% SDS, 8 M urea; (b) 2% SDS, 4% 2-mercaptoethanol. The bands marked st are the following standard proteins (molecular weight × 10^{-3} in parentheses): phosphorylase b (94), bovine serum albumin (67), ovalbumin (43), carbonic anhydrase (30), soybean trypsin inhibitor (20.1), and α-lactalbumin (14.4). The molecular weights of the standards and the apparent molecular weights of the sample components are also indicated in the figure.*

For studying the relationship of the high-molecular-weight bands to the two main bands, a reduced sample was then electrophoresed preparatively, and the gel was divided into three slices. Each of two slices contained one of the major bands, and one contained all the apparently high-molecular-weight bands from a molecular-weight region of about 30,000 upwards. The peptides eluted from the slices as described above were reelectrophoresed. The results are shown in Fig. 2. When the major polypeptides were reelectrophoresed, there appeared, in addition to the main band, an apparently higher-molecular-weight band in each case. The molecular weights were estimated at about 41,000 and 51,000 for the smaller and larger main bands,

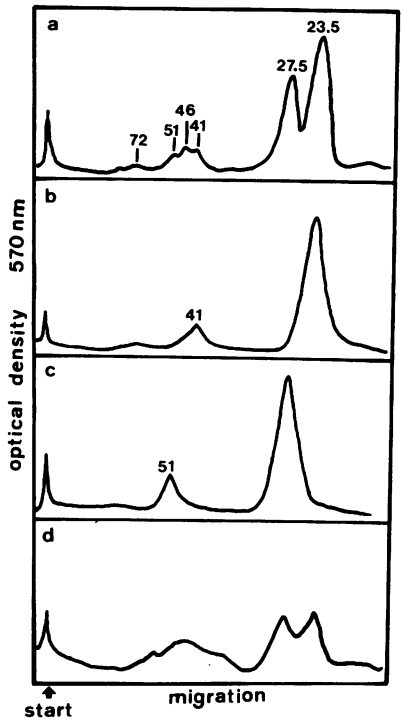

FIG. 2. *SDS-PAGE of HBsAg and reelectrophoresis of eluted polypeptides. Densitograms of a Coomassie brilliant blue-stained gel were made with a Helena Laboratories (Beaumont, Tex.) Auto Scanner Flur-Vis densitometer equipped with a 570-nm filter. (a) HBsAg; (b) reelectrophoresis of eluted polypeptide of molecular weight 23,500; (c) reelectrophoresis of eluted polypeptide of molecular weight 27,500. (d) reelectrophoresis of eluted apparently high-molecular-weight polypeptides. Estimated molecular weights* $\times 10^{-3}$ *are indicated.*

respectively (Fig. 2b and c). When the eluted, apparently high-molecular-weight bands were reelectrophoresed, both of the low-molecular-weight bands appeared again (Fig. 2d). Thus, it seems that the triplet of bands seen above the major bands represent dimers of two monomeric peptides forming the major bands, the middle one of the triplet bands being an aggregate of both. The faintly stained region above the triplet is presumably formed by trimers.

A similar pattern of bands was obtained with a reduced and alkylated HBsAg preparation, suggesting that covalent complexes formed during electrophoresis are not responsible for the findings.

The inclusion of 6 M urea in the stacking and separating gels did not affect the formation of aggregates.

DISCUSSION

The coding capacity of HBV DNA has been estimated at about 125,000 daltons of the primary gene product (9, 21). In addition to the HBsAg polypeptides, there has to be coding capacity for the polypeptides in the HBV core. Because of this, and because HBsAg antigenic determinants have been found in many or all of the polypeptides isolated from HBsAg particles (1, 4, 7, 16, 18), it seems that either the polypeptides of various sizes carrying HBsAg determinants are split products of a common primary polypeptide or the (apparently) high-molecular-weight bands found in SDS-PAGE are formed by the aggregation of smaller ones. Evidence in favor of the latter hypothesis is presented here. It was shown that two HBsAg polypeptides whose molecular weights were estimated to be about 23,500 and 27,500 also appeared in SDS-PAGE as aggregates, mostly as dimers. As the apparently high-molecular-weight bands could be shown to be aggregates of the two small polypeptides, these were the only true polypeptides in the preparation, as far as could be detected by Coomassie brilliant blue staining. The apparent molecular weights of the dimers were less than exact multiples of those of the monomers, but aggregates may not behave like single chains in electrophoresis. If HBsAg was not reduced, urea and SDS were unable to dissociate the particles to components small enough to penetrate even the 4% stacking gel. It thus seems that either the polypeptides are cross-linked through disulfide bonds in the native particles or intrachain disulfide bonds maintain a conformation that enables the polypeptides to interact noncovalently extremely well. As pointed out by Valenzuela et al. (23), who determined the sequence of the gene coding for the major HBsAg polypeptide, the protein contains an exceptionally long hydrophobic sequence, consisting of 19 amino acids. A hydrophobic sequence of similar length has also been found in some integral membrane proteins, e.g., glycophorin A, which spans the human erythrocyte membrane (5). This analogy may be important as a membrane-like ultrastructure could explain the finding of normal plasma proteins in some HBsAg preparations after treatment with detergents (12, 20). These could have been trapped within the particles as they were formed (Fig. 3), analogous to

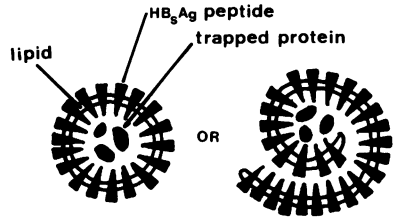

FIG. 3. *A hypothetical model for the structure of an HBsAg particle.*

the incorporation of proteins within liposomes (15). An ordered orientation of molecules must in any case be the basis of the rather complex morphology of HBsAg (8).

It is possible that the high-molecular-weight HBsAg polypeptides reported earlier can be explained by the aggregation of the small polypeptides and adsorption on or trapping of normal plasma or liver constituents within the particles. The molecular simplicity of the preparations used in this study may be due to the rather drastic isolation conditions, i.e., low pH, pepsin digestion, and 5 M NaI treatment, which could have removed extraneous components initially present in the particles. Virus-coded peptides might also have been removed during the process, but this is unlikely. Because the basic property of HBsAg, antigenicity, was preserved, there is reason to believe that the two small polypeptides found are the true basic HBsAg polypeptides.

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