

Multiple chemical forms of hepatitis B surface antigen produced in yeast

(protein structure/disulfide bonds/vaccine/thiocyanate)

D. E. WAMPLER*, E. D. LEHMAN*, J. BOGER†, W. J. MCALEER*, AND E. M. SCOLNICK*

Departments of *Virus and Cell Biology and †Medicinal Chemistry, Merck, Sharp & Dohme Research Laboratories, West Point, PA 19486

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ABSTRACT Hepatitis B surface antigen (HBsAg) has been extracted from yeast cells that produce HBsAg. These cells contain the gene for surface antigen carried on a plasmid that replicates in the cells. Analysis of the yeast-derived HBsAg by sucrose gradient centrifugation and by polyacrylamide gel electrophoresis shows that the antigen that is initially released from yeast cells is a high molecular weight aggregate of the fundamental M_r 25,000 subunit. Unlike HBsAg derived from human plasma, the yeast antigen is held together by noncovalent interactions and can be dissociated in 2% NaDodSO₄ without the use of reducing agents. During *in vitro* purification of the yeast antigen, some disulfide bonds form spontaneously between the antigen subunits, resulting in a particle composed of a mixture of monomers and disulfide-bonded dimers. Treatment with 3 M thiocyanate converts the 20-nm particles into a fully disulfide-bonded form that is not disrupted in NaDodSO₄ unless a reducing agent is added. This disulfide-bonded particle resembles the naturally occurring, plasma-derived surface antigen particle, and the *in vitro* formed particle has been used to prepare a vaccine for humans against hepatitis B virus infection.

Hepatitis B surface antigen (HBsAg) derived from human blood is a complex particle, about 20 nm in diameter, composed of protein, carbohydrate, and lipid (1, 2). The protein portion, which carries the antigenic determinants (3), is composed of two types of subunits that share a common polypeptide sequence but that occur either as a nonglycosylated, M_r 25,000 molecule or as a glycosylated, M_r 28,000 form (4-6). In the HBsAg particle these polypeptides are joined by disulfide linkage into dimers and higher multimers. Purified, formaldehyde-treated antigen, isolated from human plasma, is an effective vaccine for the prevention of hepatitis B infection (7). Recently, the HBsAg gene has been cloned in yeast (8-11) and a vaccine has been made that is safe and immunogenic in humans (12, 13) and is effective in preventing hepatitis B infection in chimpanzees (14).

The production of useful proteins by recombinant DNA techniques requires not only that the correct polypeptide be expressed but also that the polypeptide assume the correct three-dimensional structure. For multimeric proteins such as insulin, immunoglobulin, and HBsAg, tertiary structure is maintained by intrachain and interchain disulfide bonds.

Although the HBsAg particle purified from yeast has the form of a 20-nm particle (14-16), it has not been clear whether this structure is assembled in the yeast cell or is a product of the purification process. In addition, it was not known whether the interchain and intrachain disulfide bonds were formed in the cell or after extraction.

In this communication we present evidence that HBsAg produced in yeast is initially released as a large particle in

which the subunits are held together by noncovalent interactions (form I). *In vitro*, this form can be converted to a second form in which individual polypeptides are joined together by disulfide bonds into dimers (form II). Finally, disulfide bonds may be formed *in vitro* between dimers to create a disulfide-linked particle (form III) that is similar to the plasma-derived antigen in appearance and chemical and immunological properties.

MATERIALS AND METHODS

Source of Antigen. Human plasma antigen was isolated by the procedure that is used to manufacture vaccine (17). Antigen was taken before the addition of formaldehyde.

Two yeast strains were used as a source of HBsAg. The first is that described by Valenzuela *et al.* (15). The second used a glyceraldehyde-3-phosphate dehydrogenase (GAP) promoter instead of the alcohol dehydrogenase (ADH) promoter. Cells were grown as described by Carty *et al.*† and harvested by continuous-flow centrifugation through a Sharples AS16 centrifuge (10.2-cm bowl; flow rate, 3 liters/min). The cell paste (3-5 kg) was suspended in an equal volume of hypertonic phosphate buffer (0.1 M sodium phosphate, pH 7.2/0.5 M NaCl). Phenylmethylsulfonyl fluoride (0.2 M in isopropanol) was added to a final concentration of 2 mM and the cells were disrupted by seven or nine passes through a high-pressure homogenizer (Gaulin, Everett, MA).

Antigen Purification. The crude extract (32-70 mg of protein per ml) was diluted with 4 vol of 0.01 M phosphate buffer (pH 7.5) containing 0.1% Triton X-100 (Rohm and Haas). Cell debris was removed by continuous-flow centrifugation as before, and the supernatant solution was concentrated 5-fold in a hollow fiber unit (H10X100, Amicon) and diafiltered with 2 vol of phosphate-buffered saline [P_i /NaCl (7 mM sodium phosphate, pH 7.2/0.15 M NaCl)]. Triton X-100 was removed by using XAD-2 beads (Rohm and Haas; see ref. 18) and the solution was clarified by centrifugation for 35 min at $9000 \times g$ and 4°C. HBsAg was partially purified by adsorption and elution from fused silica (Aerosil 380, Degussa, Teterboro, NJ; see ref. 19).

Partially purified antigen, processed through the silica treatment step, was routinely stored at -70°C. When these samples were thawed, an insoluble precipitate formed. The precipitate was removed by centrifugation at $8000 \times g$ for 45 min and the resulting clarified product was purified by hydrophobic chromatography on butyl-agarose (Miles-Yeda, Rehovot, Israel) (unpublished data). Alternatively, the crude yeast cell extract was clarified by Sharples centrifugation and purified by immunoaffinity chromatography as described (20).

Abbreviations: ADH, alcohol dehydrogenase; GAP, glyceraldehyde-3-phosphate dehydrogenase; HBsAg, hepatitis B surface antigen.

†Carty, C. E., Kovach, F. X., McAleer, W. J. & Margetter, R. Z., Eighty-Fourth ASM Meeting, March, 12, 1984, St. Louis, MO, abstr. 030, p. 194.

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Antigen concentration was measured by using a commercial RIA kit (AUSRIA, Abbott). Protein was measured by the Lowry method (21).

PAGE. Antigen samples were disrupted by heating at 100°C for 15 min under nonreducing conditions (2% NaDodSO₄/62 mM Tris-HCl, pH 6.8/10% glycerol/6 ppm of bromphenol blue) or under reducing conditions (10 mM dithiothreitol added) as indicated and were separated by electrophoresis through 12.5% acrylamide slab gels as described by Laemmli (22). After electrophoresis, HBsAg polypeptides were visualized by an immunological protein blotting method similar to that described by Burnette (23). Transfer to nitrocellulose sheets (Millipore type HAHY, 0.45 μm) was done in 25 mM sodium phosphate (pH 6.5) for 90 min at 1.5 A. After transfer, the nitrocellulose was saturated by incubation for 2 hr at room temperature in a solution containing 20% gamma globulin-free calf serum (GIBCO), 0.15 M NaCl, 50 mM Tris-HCl (pH 7.6), and 0.1% NaN₃ and then incubated for 90 min with antiserum from rabbits that had been immunized with reduced and denatured plasma-derived HBsAg. Following incubation with ¹²⁵I-labeled protein A (70–100 μCi/μg; 1 Ci = 37 GBq; New England Nuclear) the blots were exposed to Kodak SB5 x-ray film held between two Cronex Lightning Plus intensifying screens (DuPont).

RESULTS

Although it was known that HBsAg purified from yeast is an aggregate of *M_r* 25,000 subunits, it was not clear whether these aggregates were present in crude extracts or were formed during purification. To distinguish between these two possibilities, a sample of yeast extract was subjected to velocity sedimentation through a sucrose gradient. Fractions were analyzed for HBsAg by RIA and the sucrose concentration was measured by refractometry. Plasma-derived HBsAg was centrifuged in a companion tube (data not shown). Both the plasma- and yeast-derived antigens sedimented to a density of 25–35% sucrose. Although the position in the sucrose gradient (Fig. 1A) indicates that the yeast-derived HBsAg is a high molecular weight particle, NaDodSO₄/PAGE analysis under nonreducing conditions (Fig. 1B) shows the particles to be composed of *M_r* 23,000 monomers and *M_r* 37,000 dimers. NaDodSO₄/PAGE analysis under reducing conditions (data not shown) revealed only monomers, indicating that the dimers were held together by disulfide bonds.

Purification by treatment with silica and chromatography over butyl-agarose did not appreciably change the covalent structure of the antigen since most of the immunoreactive polypeptide continued to migrate as monomer and dimer (Fig. 2, compare lanes 7, 8, and 9). By contrast, HBsAg purified from yeast by immunoaffinity chromatography did not enter the running gel under these conditions (Fig. 2, lane 3).

Since purification by immunoaffinity chromatography (which uses 3 M ammonium thiocyanate to elute the antigen) resulted in disulfide-bonded particles, whereas purification by butyl-agarose chromatography yielded non-disulfide-bonded particles, we investigated the possibility that the conversion to disulfide-bonded particles is catalyzed by thiocyanate. NaDodSO₄/PAGE analysis of the clarified yeast extract showed a major band of antigen at *M_r* 24,000, whether or not a disulfide reducing agent was used (Fig. 3, lanes 1). When this clarified extract was treated with 3 M ammonium thiocyanate for 16 hr at 5°C, the antigen no longer entered the running gel unless it had been reduced (Fig. 3, lanes 2). During the preparation of the silica-treated product, part of the antigen was converted to a form that migrated as a mixture of monomer and dimer under nonreducing conditions but still migrated as a monomer when 10 mM dithiothreitol was included in the sample disruption buffer (Fig. 3, lanes 3).

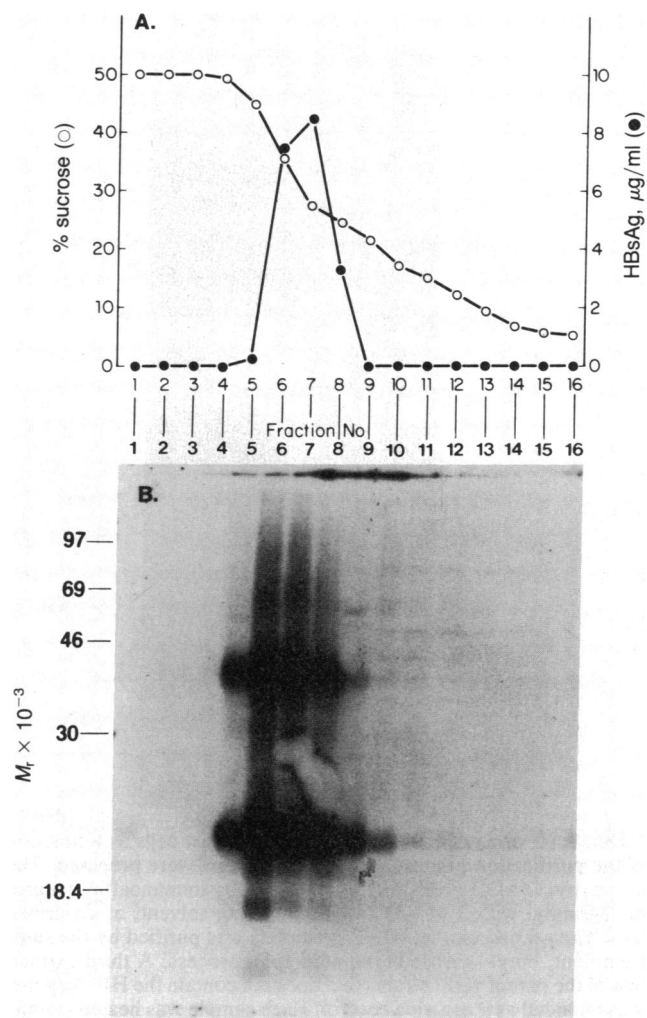


FIG. 1. Sucrose gradient centrifugation of a clarified yeast extract. A 5-ml portion of yeast extract (GAP promoter) was clarified by centrifugation for 15 min at 10,000 × *g*. One milliliter (30 mg of protein) of the clear solution between the pellet and the fatty layer was applied to a 35-ml sucrose gradient (5–50% sucrose), overlaid with 2 ml of P_i/NaCl, and centrifuged for 18 hr at 19,000 rpm (5°C) in a Beckman SW28 rotor. Fractions (2.5 ml) were collected from the bottom of the tube. Each fraction was tested for sucrose concentration by refractive index and for HBsAg concentration by RIA and by nonreducing NaDodSO₄/PAGE. (A) Elution position in the sucrose gradient. ○, Sucrose concentration; ●, HBsAg concentration measured by RIA. (B) NaDodSO₄/PAGE under nonreducing conditions. A 25-μl sample of the indicated fraction was mixed with an equal volume of disruption buffer and heated at 100°C for 15 min. Half of the disrupted sample was applied to a slab gel composed of a 3% acrylamide stacking gel and a 12.5% running gel. Separated polypeptides were transferred to nitrocellulose, allowed to react with rabbit antiserum against denatured human plasma HBsAg, followed by ¹²⁵I-labeled protein A, and visualized by autoradiography. ¹⁴C-labeled molecular weight standards include phosphorylase b (*M_r* 97,000), bovine serum albumin (*M_r* 69,000), ovalbumin (*M_r* 46,000), carbonic anhydrase (*M_r* 30,000), and lactoglobulin A (*M_r* 18,400).

Additional evidence for the role of thiocyanate in the conversion is given by the concentration dependence shown in Fig. 4. Antigen purified by butyl-agarose chromatography is composed of a mixture of monomers and dimers (Fig. 4, lane 1). After incubation with 1.5 M thiocyanate a majority of the antigen still migrated into the running gel (Fig. 4, compare lanes 1 and 2). After treatment with 3 M thiocyanate the conversion was complete (Fig. 4, lane 3) and essentially all of the antigen was excluded from the running gel.

Fig. 4 also shows that the conversion can be affected by

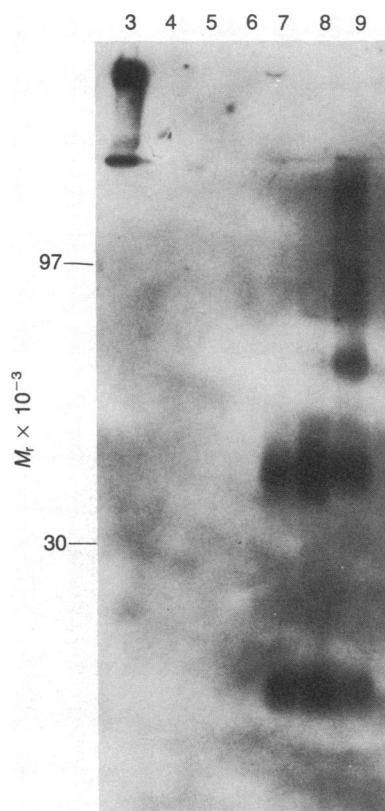


FIG. 2. Forms of HBsAg isolated from yeast cells as a function of the purification process. Three cell extracts were prepared. The first extract (ADH promoter) was purified by immunoaffinity chromatography, with 3 M KSCN as the eluting solvent, as described (20). The second extract (GAP promoter) was purified by the silica treatment, butyl-agarose chromatography process. A third extract, one of the parent yeast strains that does not contain the HBsAg gene, was included as a negative control. Each sample was heated (15 min at 100°C) in disruption buffer under nonreducing conditions and analyzed by NaDodSO₄/PAGE. After electrophoresis, samples were transferred to nitrocellulose and HBsAg was detected by immunoblot with rabbit antiserum to denatured human HBsAg followed by reaction with radiolabeled protein A and autoradiography. Lanes 5, 7, and 8 contained 5 μ g of total protein; lanes 3 and 9 contained 1 μ g of total protein. The lanes were loaded as follows: lane 3, HBsAg from yeast purified by immunoaffinity chromatography; lane 4, empty; lane 5, extract of the parent yeast strain without the HBsAg gene; lane 6, empty; lane 7, clarified yeast extract after treatment with silica; lane 8, clarified silica-treated product; lane 9, butyl-agarose product. Molecular weight standards as in Fig. 1.

elevated pH (Fig. 4, lanes 4–7). This approach has the disadvantage, however, that the resulting antigen has lost much of its immunoreactivity. At pH 11.5 and pH 12.5 the antigen lost 29% and 92%, respectively, of its reactivity in RIA.

Thiocyanate might promote interchain disulfide bond formation by facilitating disulfide exchange (intrachain to interchain) within an already oxidized polypeptide. In some cases (24, 25), the chemical- or enzyme-catalyzed disulfide exchange has been shown to be the rate-limiting process in protein renaturation rather than the oxidation itself. Thiocyanate participation in such a reaction might resemble the Na₂SO₃/O₂ sulfitolysis of disulfides to *S*-sulfonated derivatives that can be followed in a second step by thiolysis to reform disulfide bonds (26), a process used in the production of insulin.

To investigate the possibility that thiocyanate was acting as a catalyst for disulfide exchange, two experiments were conducted with low concentrations of thiol reagents. In the first experiment, samples of butyl-agarose-purified antigen were made 0.1 mM or 1 μ M in either 2-mercaptoethanol or

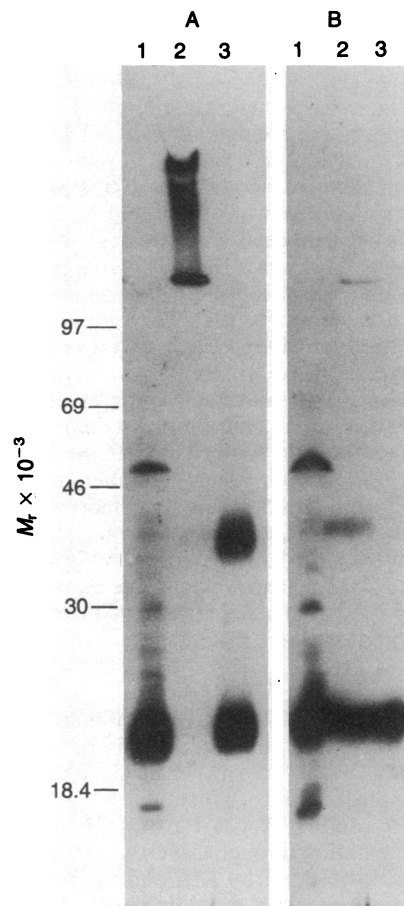


FIG. 3. Formation of intramolecular bonds by 3 M KSCN. A crude yeast extract was prepared from 3.3 kg of yeast cells (ADH promoter). A 100-ml portion of the extract (70 mg of protein per ml) was clarified by centrifugation for 30 min at 36,000 \times *g*. A 5-ml portion of the clarified extract was dialyzed overnight (5°C) against 1 liter of 0.1 M sodium phosphate buffer (pH 7.2) containing 3 M NH₄SCN and 0.5 M NaCl. The precipitate that formed was removed by centrifugation (30 min at 10,000 \times *g*) and the supernatant solution was dialyzed against two 1-liter changes of P_i/NaCl. The remainder of the crude extract (3.7 liters) was processed through the silica treatment step. Samples were heated (15 min at 100°C) in disruption buffer under nonreducing conditions (A) or reducing conditions (10 mM dithiothreitol added) (B). Disrupted samples were applied to a slab gel composed of a 3% acrylamide stacking gel and a 12.5% running gel. Separated polypeptides were transferred to nitrocellulose, allowed to react with rabbit antiserum against denatured human plasma HBsAg, followed by ¹²⁵I-labeled protein A, and visualized by autoradiography. Lanes 1, cell extract clarified by centrifugation, 348 μ g of total protein; lanes 2, same as lanes 1 after thiocyanate treatment, 45 μ g of protein; lanes 3, partially purified antigen without thiocyanate treatment, 3.5 μ g of total protein. Molecular weight standards as in Fig. 1.

glutathione. These samples were held for 24 hr at 4°C. The NaDodSO₄/PAGE results (not shown) showed no increase in interchain crosslinking. In the second experiment, portions of a clarified extract were treated with mixtures of reduced and oxidized glutathione at a 10:1 ratio (4.5 mM reduced and 0.45 mM oxidized glutathione or 45 μ M reduced and 4.5 μ M oxidized glutathione). This treatment also failed to increase the extent of interchain disulfide crosslinking.

DISCUSSION

The work reported here shows that the mature form of HBsAg, held together by disulfide bonds, is not present in the initial yeast cell extract but rather must be made outside the cell by some chemical process. After cell lysis, the clarified

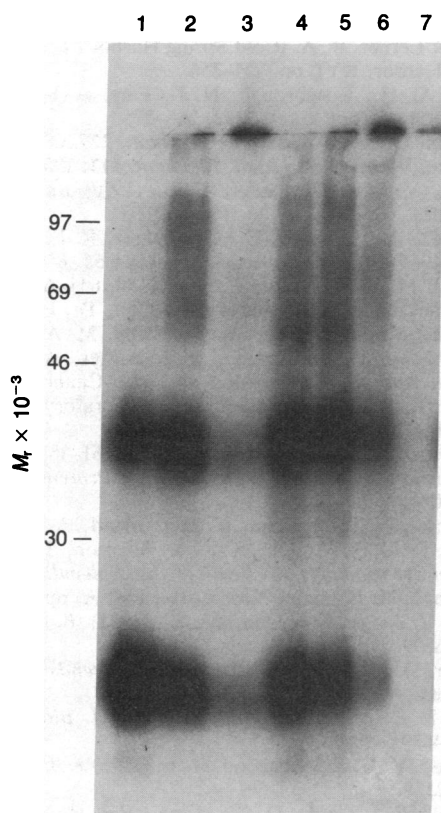


FIG. 4. Conversion to form III at different thiocyanate concentrations or elevated pH. A sample of HBsAg, purified by butyl-agarose chromatography from cells containing the GAP promoter, was divided into four portions. Each portion (0.5–1.5 ml, 0.57 mg of protein per ml) was mixed with an equal volume of $P_i/NaCl$ or of 3 M KSCN (final concentration, 1.5 M) or of 6 M KSCN (final concentration, 3 M). To one of the portions (1.5 ml) that had been mixed with $P_i/NaCl$, 1 M NaOH was added dropwise, with stirring, and samples were removed at the indicated pH. After 16 hr at room temperature, a 5- μ l portion of each sample (2.4 μ g of protein) was removed and diluted 1:5 with deionized water. This diluted sample was mixed with an equal volume of disruption buffer and heated with 100°C for 15 min. Half of the disrupted sample was applied to a slab gel composed of a 3% acrylamide stacking gel and a 12.5% running gel. Separated polypeptides were transferred to nitrocellulose, allowed to react with rabbit antiserum against denatured human plasma HBsAg, followed by ^{125}I -labeled protein A, and visualized by autoradiography. Lane 1, control, mixed with $P_i/NaCl$ only; lane 2, treated at 1.5 M thiocyanate; lane 3, treated at 3 M thiocyanate. Samples in lanes 4–7 were treated with NaOH instead of thiocyanate. Lane 4, pH 9.5; lane 5, pH 10.5; lane 6, pH 11.5; lane 7, pH 12.5. Molecular weight markers as in Fig. 1.

extract contains HBsAg particles that have the same sedimentation coefficient as mature particles but are not held together by covalent bonds. We call this initial particle form I. It is characterized by its migration as a M_r 25,000 monomer in NaDodSO₄/PAGE under nonreducing conditions. During early phases of purification, some interchain disulfide bonds form so that the antigen migrates as a mixture of monomer and dimer under nonreducing conditions. We call the disulfide-linked dimers, again recognized in nonreducing NaDodSO₄/PAGE, form II. After treatment with concentrated ammonium or potassium thiocyanate, additional interchain disulfide bonds form between dimers, and the fully crosslinked particle is formed. We call this mature particle form III. It is recognized by its inability to enter the running gel of the NaDodSO₄/PAGE system unless a reducing agent is used in the disruption step.

It should be emphasized that forms I–III do not represent differences in aggregation state (particle size). Since the

NaDodSO₄/PAGE procedure uses antibody to fully denatured antigen, it detects all of the HBsAg polypeptide, regardless of conformation or aggregation state. Such NaDodSO₄/PAGE analysis of sucrose gradient fractions (Fig. 1) shows no evidence for free monomers. This result agrees with the observations of Valenzuela *et al.* (27), who reported that the antigen travels as a high molecular weight aggregate in size-exclusion chromatography. On the other hand, Heitzeman *et al.* (15) reported that only a small fraction of the HBsAg in yeast cells is aggregated into particles. Their conclusion was based on a comparison of autoradiograms with RIA titers. The difference between these two estimates of HBsAg content was attributed to the presence of monomers that were detected in the autoradiograms but were nonreactive in the RIA. They did not attempt any physical separation of high molecular weight and the proposed low molecular weight forms.

The primary structure of HBsAg contains 14 cysteines per 226 amino acid monomer (8), providing opportunity for numerous different disulfide bonds. Such disulfide bonds contribute to protein stability and proteins with many disulfide links are often resistant to nonreducing denaturing conditions (28, 29). Disulfide links seem particularly important in the stabilization of structural proteins, cell receptors, and extracellular proteins such as immunoglobulins (29). The high cysteine content of HBsAg may explain its unusual heat stability.

Although the “spontaneous” (i.e., O₂) oxidation of protein thiols can take place extracellularly, intracellular disulfide formation either may not occur at all or may be carried out by a variety of mechanisms, including chemical exchange with a pool of oxidized and reduced glutathione (30), various disulfide interchange enzymes coupled with NADPH-dependent glutathione oxidases (31, 32), or sulfhydryl oxidases (33, 34). Heterologous proteins expressed in *Escherichia coli* may remain in a reduced state or may have incorrectly paired disulfide bridges (28, 35, 36). The problem of forming the “correct” disulfide links becomes even more complicated when interchain disulfide bonds are necessary, as in the formation of active insulin from separate A and B chains (see, for example, ref. 37).

Several methods have been used in other laboratories to facilitate the correct disulfide bond formation (23, 38). Saxena and Wetlaufer (24) showed that a mixture of oxidized and reduced glutathione accelerated the reformation of active lysozyme. Cabilly *et al.* (39) and Boss *et al.* (40) used this glutathione mixture to form functional immunoglobulin from polypeptides synthesized in *E. coli* after denaturing the polypeptides in guanidine and urea and then allowing the disulfide bonds to reform. Our results, which failed to show increased disulfide crosslinking after glutathione treatment, suggest that disulfide exchange may not be the mechanism of interchain bond formation in HBsAg from yeast. Instead, these results favor an oxidative mechanism for the thiocyanate conversion.

The oxidation of albumin sulfhydryls by micromolar concentrations of thiocyanogen (NCS-SCN) and by its hydrolysis product, hypothiocyanite (⁻OSCN), has been examined by Aune and Thomas (ref. 41; see also ref. 42). It was shown that the resulting protein sulfenyl thiocyanate (R-S-SCN) is in equilibrium with the sulfenic acid (R-S-OH) by hydrolysis and that this sulfenic acid can also react with another thiol to form a disulfide link. In our experiments, small amounts of NCS-SCN could be formed in 3 M thiocyanate (⁻SCN) exposed to air. The R-S-SCN could also react directly with a second sulfhydryl to form the disulfide bond and release thiocyanate and a proton. The details of thiocyanate participation in form III synthesis are a subject for further investigation.

A number of laboratories, including ours, have shown that breaking disulfide bonds in HBsAg reduces the antigenic activity (43–45). In preparing a vaccine, therefore, we have chosen to ensure that the final product is the disulfide-bonded form III. This form of the recombinant antigen, like the antigen from human plasma, has been shown to elicit antibodies in mice, monkeys, chimpanzees, and humans (12–14, 20) and is protective against the experimental hepatitis B virus infection in chimpanzees (14).

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