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Properties of an Ordered Ring Structure Formed by Recombinant *Treponema pallidum* Surface Antigen 4D

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Ultrastructural and biochemical studies of a recombinant *Treponema pallidum* surface antigen designated 4D have been conducted due to its likely biological significance. Electron microscopy demonstrated that the 190-kilodalton (kDa) 4D molecule is an ordered ring structure of 10-nm diameter. The 90-kDa proteinase K-treated 4D is an ordered ring structure of 6-nm diameter. Evidence is presented that the 190-kDa ordered ring is maintained by noncovalent bonds; 19-kDa monomers can reassociate in vitro to reform a 190-kDa molecule. Amino acid composition analysis of 190-kDa 4D showed that the molecule is composed of 45% hydrophobic residues. Evidence relating the structure of the 4D ordered ring to its potential role in the pathogenesis of syphilis is discussed.

The inability to cultivate *Treponema pallidum* in defined media in vitro has hindered studies to biochemically define the molecules which contribute to its virulence. Purification of cloned *T. pallidum* proteins from *Escherichia coli* host cells has provided an opportunity to study the contribution of individual *T. pallidum* molecules to the pathogenesis of syphilis. We recently reported an initial characterization of a cloned *T. pallidum* protein, designated 4D, which has been purified to homogeneity from *E. coli* with yields in excess of 3 mg per liter of stationary-phase culture (10). The 4D molecule is a polymer of 19-kilodalton (kDa) monomers which has an apparent molecular weight of 190,000. Exhaustive treatment of 190-kDa 4D with proteinase K results in a homogeneous limit digestion product of 90 kDa, which retains specific reactivity with syphilitic sera. A 90-kDa molecule indistinguishable in size, charge (pI, 4.6), and antigenicity was isolated from proteinase K-treated *T. pallidum*, suggesting that the recombinant protein was very similar to the native 4D molecule. The nonpathogenic treponeme *T. phagedenis* biotype Reiter does not contain molecules antigenically related to 4D. Rabbit antisera to 190-kDa 4D immobilizes virulent *T. pallidum* in vitro in the *T. pallidum* immobilization test, suggesting that 4D has a native surface location (10). Recently, we have demonstrated that 4D covers the surface of *T. pallidum* in a uniform distribution as judged by immunoelectron microscopy (J. D. Radolf, T. E. Fehniger, F. J. Silverblatt, J. N. Miller, and M. A. Lovett, submitted for publication). Rabbits immunized with 190-kDa 4D have significant but partial protection against intradermal challenge in the experimental syphilis model (T. E. Fehniger, J. D. Radolf, T. M. Cunningham, H. Togashi, B. L. Johnson, L. A. Borenstein, D. R. Blanco, A. Urquhart, J. A. Lake, J. N. Miller, and M. A. Lovett, Fed. Proc. **44**:596, 1985; L. A. Borenstein, T. E. Fehniger, J. D. Radolf, D. R. Blanco, J. N. Miller, and M. A. Lovett, manuscript in preparation).

Because of the potential significance of the 4D antigen in the pathogenesis of syphilis, we have conducted a detailed study of the physical and chemical properties of this treponemal surface molecule. In this report we show that the purified 190-kDa polypeptide designated 4D forms an ordered ring structure and that the ring structure is maintained following proteolytic digestion with proteinase K. Evidence is presented that noncovalent self-association plays an important role in assembly and stability of the ordered ring. Amino acid composition analysis of 4D is also presented, showing that 4D contains a percentage of hydrophobic amino acids higher than that reported for other bacterial membrane proteins.

MATERIALS AND METHODS

SDS-PAGE and immunoblotting. Vertical 1.5-mm-thick sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) slab resolving gels of 10% total acrylamide with 1.3% cross-linking or linear gradient gels of 8 to 20% total acrylamide with 2.6% cross-linking were prepared and run, using the discontinuous buffer systems and stacking gels previously described (10). Samples analyzed under denaturing conditions were mixed with an equal volume of a final sample buffer (FSB; plus 2-mercaptoethanol [2ME]) of 62.5 mM Tris (pH 6.8) containing 2% (wt/vol) SDS, 5% (vol/vol) 2ME, 20% (wt/vol) sucrose, and 0.1% bromphenol blue dye and then placed into a 100°C boiling-water bath for 10 min immediately before SDS-PAGE analysis. Other samples were mixed 1:2 in FSB or 67.2 mM Tris (pH 6.8) containing 4% (wt/vol) SDS, 20% (wt/vol) sucrose, and 0.01% (wt/vol) bromphenol blue dye, incubated at 37 or 25°C for 30 min, and then analyzed by SDS-PAGE (minimal denaturing conditions). Following electrophoresis, gels were either stained by Coomassie blue or immunoblotted and serologically probed as previously described (10).

A two-dimensional gel system utilizing SDS-PAGE in both dimensions was performed under minimal denaturing conditions in the first dimension and either minimal or complete denaturing conditions and increased molecular sieving in the second dimension as specified in Results. SDS-PAGE tube gels of 1-mm thickness with resolving gels of 4% total acrylamide with 1.6% cross-linking and stacking gels of the same concentration were prepared with the buffers described (10). Sample tube gels to be analyzed under minimal denaturing conditions were loaded directly into preformed slots on the second-dimension gel and overlaid with 1% agar (56°C) to secure the gels in place. Sample tube gels to be analyzed under denaturing conditions were placed in a glass

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centrifuge tube, overlaid with FSB plus 2ME, placed in a 100°C boiling-water bath for 10 min, and then loaded on the second-dimension gels. Following electrophoresis, gels were stained by Coomassie blue. Apparent molecular weights of separated proteins were determined with molecular weight standards of cross-linked myosin (obtained from Emil Reisler, University of California, Los Angeles), purified pentameric human immunoglobulin M, and low-molecular-weight (14×10^3 to 93×10^3; Pharmacia Diagnostics) standardized protein mixtures.

Stability of the 4D antigen to dissociating conditions. The 4D polypeptide was purified to homogeneity from *E. coli* RR1 containing plasmid pAW329 as previously described (10). Individual samples of the purified 4D antigen were incubated at a final concentration of 1 mg/ml under the following conditions for 4 h at room temperature: (i) 1% (vol/vol) acetic acid in distilled water, pH 2.5; (ii) 0.1 M sodium acetate buffer, pH 4.0; (iii) 0.2 M Tris base, pH 9.5; (iv) 0.1 M glycine-NaOH buffer, pH 11.5; (v) 4 M urea, pH 8.4; (vi) 5% (vol/vol) 2-ME in 50 mM Tris, pH 8.0; (vii) 0.1 M dithiothreitol in 50 mM Tris, pH 8.0; (viii) 0.1 M cysteine in 50 mM Tris, pH 8.0; (ix) 0.1% hydrogen peroxide in 50 mM Tris, pH 8.0; (x) 50 mM sodium *m*-periodate in 50 mM Tris, pH 8.0; (xi) 5% SDS in 50 mM Tris, pH 8.4, for 5 h at 55°C; (xii) 50 mM Tris, pH 8.4, at 95°C for 10 min; and (xiii) 50 mM Tris, pH 8.4, containing 0.1% SDS at 95°C for 10 min. Each treated sample was then mixed with an equal volume of FSB, and 100-μl aliquots containing 50 μg of protein were analyzed on duplicate 10% SDS-PAGE gels. Following electrophoresis, one of the gels was stained with Coomassie blue, while the other was Western blotted.

^{125}I-labeled proteins. Protein A derived from *Staphylococcus aureus* (Pharmacia) and molecular weight markers (Pharmacia or Bio-Rad Laboratories) were radioiodinated with lactoperoxidase (18). The purified 4D antigen was radiolabeled, using Iodobeads (Pierce Chemical Co.) per the manufacturer's instructions. Briefly, 100 μg of purified 4D in 100 μl of 0.2 M PO_4 was incubated with 0.5 mCi of NaI (Amersham Corp.) for 5 min and separated from free iodine on a column (9.7 by 10 cm) of Sephadex G-25 resin in 10 mM phosphate buffer (pH 7.4) containing 0.58% Nonidet P-40 (Shell) and 150 mM NaCl.

Denaturation and renaturation of the 190-kDa 4D antigen. Samples (200 μl) containing 5×10^6 cpm of ^{125}I-labeled 4D antigen (1.06×10^5 cpm/μg) were mixed with 250 μl of 0.3 M HCl in H_2O (pH 1.0 final) and left at room temperature for 3 h to dissociate the 4D polypeptide subunits. Individual aliquots (75 μl) were brought to 0.5 ml with H_2O, placed into dialysis tubing (Spectrapore 6 [Pierce]; 2×10^3 molecular weight pore exclusion size), and dialyzed exhaustively for 3 days at room temperature against the following buffers: (i) 0.1 M acetate buffer, pH 4.0, plus 0.15 NaCl; (ii) 0.1 M phosphate buffer, pH 6.0, plus 0.15 M NaCl; (iii) 0.1 M phosphate, pH 7.4, plus 0.15 M NaCl; (iv) 0.1 M Tris buffer, pH 8.4, plus 0.15 M NaCl. Following dialysis, the samples were removed from the dialysis tubing, the pH of each sample was determined, and 100 μl containing approximately 4×10^4 cpm of each sample was analyzed by SDS-PAGE. Following electrophoresis, the gel was fixed in 40% ethanol–10% acetic acid, dried onto Whatman 3M paper, and autoradiographed.

Analysis of the 4D antigen for sugar residues. The purified 4D antigen was analyzed chemically to determine if known classes of sugars were associated with the antigen. Total protein was determined by the method of Lowry et al. (16), using crystalline bovine serum albumin (Sigma Chemical Co.) as a standard. Each of the following assays was performed in duplicate, using 200 to 400 μg of bovine serum albumin equivalents of the 4D antigen per assay, along with appropriate sugar standard controls. Free and *n*-acetylhexosamines were determined by the technique of Levvy and McAllen (15). The unhydrolyzed 4D antigen was assayed for neutral hexose by the phenol-sulfuric acid test (3), for uronic acids by the modified carbazole assay (5), for sialic acid by the periodate-resorcinol test (13), and for 2-keto-3-deoxyoctanic acid by the thiobarbituric acid assay (22). Each of the assays for sugars could minimally detect 5 to 10 μg. Therefore, it is possible that up to several moles of each of these sugars per mole of 19kDa 4D could fail to be detected under these conditions.

Amino acid composition analysis of the 4D antigen. Amino acid analysis of the purified 190- and 90-kDa proteinase K-treated 4D antigen was performed with minor modifications of a protocol developed by Waters Associates, Milford, Mass. Samples were analyzed unmodified or following reduction and carboxymethylation by the method of Crestfield and Stein (8), using final concentrations of 0.1 M dithiothreitol in 5 M guanidine thiocyanate in 50 mM Tris buffer (pH 8.2) containing 2% EDTA.

Samples of the proteins (1 mg/ml) were mixed with the above denaturing/reducing buffer in a glass vial, which was evacuated with N_2 and capped for 4 h at room temperature. Iodoacetic acid (sodium salt) in 2 M Tris (pH 8.6) was then added to a final concentration of 1 M iodoacetic acid on ice for 30 min in the dark to carboxymethylate the samples. All samples were exhaustively dialyzed against 10 mM Tris buffer (pH 8.4) with many changes of dialysis buffer for 4 days. Following dialysis, the samples were concentrated by freeze-drying to approximately 1 mg/ml and then hydrolyzed in 6 N HCl at 110°C for 20 h. The hydrolysate was derivitized with phenylisothiocyanate for 20 min at room temperature. The reaction yielded a phenylthiocarbonyl amino acid of both primary and secondary amino acids which were separated by high-pressure liquid chromatography on a PICO-TAG (Millipore Corp.) reverse-phase column eluted with a gradient of acetate-acetonitrile.

Electron microscopy. Samples of purified 190- and 90-kDa proteinase K-treated 4D (overnight digestion with 100 μg of proteinase K per ml in 0.1% SDS–50 mM Tris, pH 7.4 [10]) were prepared for electron microscopy with the single carbon layer method (25), with solutions adjusted to 25 μg/ml in 50 mM Tris (pH 7.4) and negatively stained with 1% uranyl acetate. Grids were examined in a JEOL electron microscope. Average dimensions of the ordered ring structures were determined from measurements in two dimensions of at least 10 full-face projections at a total magnification of 300,000.

RESULTS

The 190-kDa 4D is a protease-resistant ordered ring structure. The 4D antigen was purified to homogeneity and examined by electron microscopy as described in Materials and Methods. Figure 1A shows that the 190-kDa 4D polypeptide formed an ordered ring structure. The average external diameter of the ring was 10 nm. 4D exhaustively treated with proteinase K to create the 90-kDa form of the molecule is shown in Fig. 1B. Although the ordered ring structure was preserved, the diameter of the proteinase K digested molecule was 6 nm, consistent with the idea that the digestion had occurred from the periphery of the ring since the internal diameter of the ring appeared unchanged. The

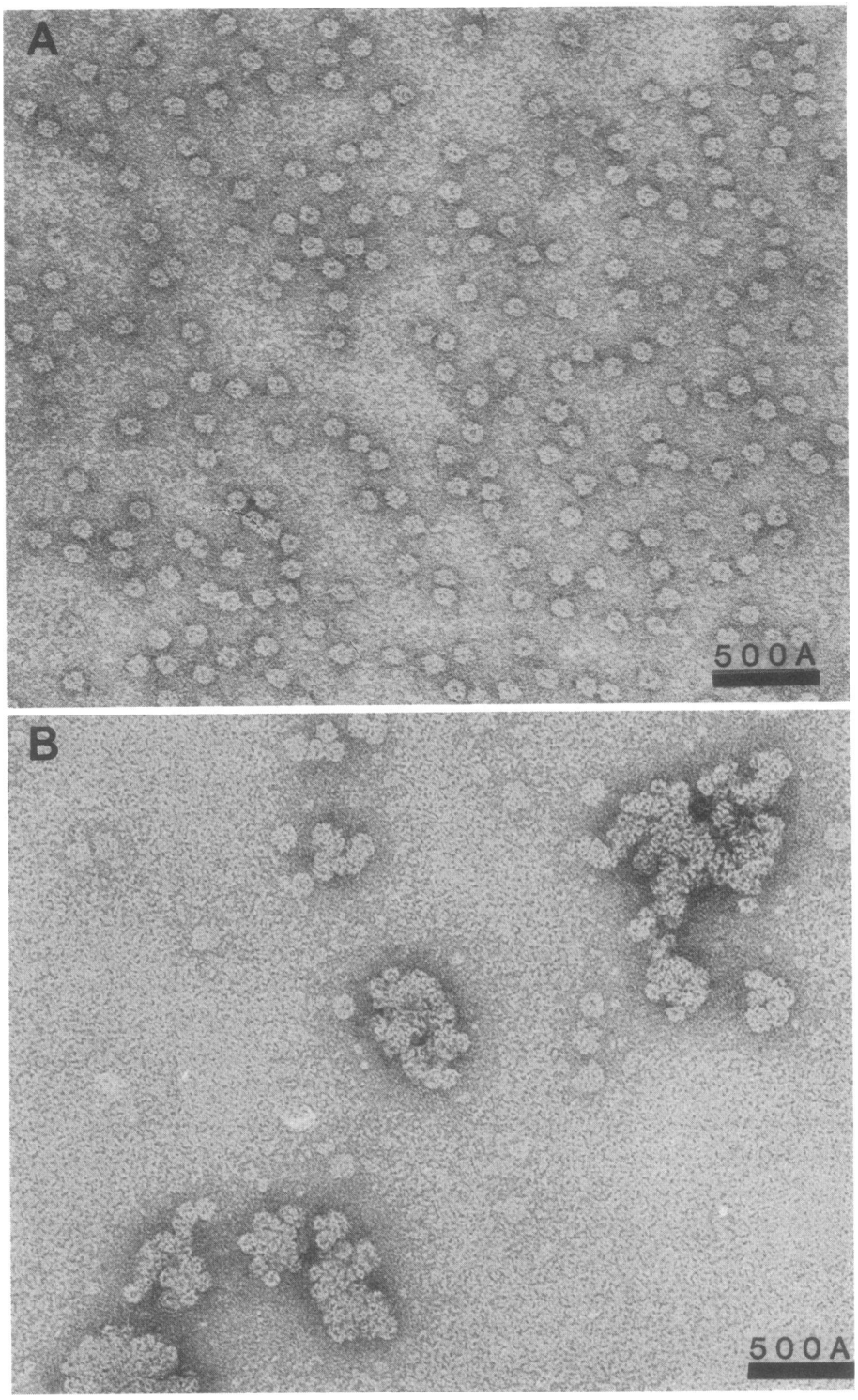

FIG. 1. Electron micrographs of purified 190-kDa 4D antigen before (A) and after (B) digestion with proteinase K. The 190-kDa antigen forms an ordered ring structure 10 nm in diameter; after proteolysis aggregates of 6-nm ring structures are found. (500 Å = 50 nm.)

6-nm rings were found principally as large aggregates, in contrast to the rings of 10 nm.

Stability of 190-kDa 4D to denaturing conditions. To determine the nature of the physical association of the 19-kDa monomers within the 190-kDa ordered ring structure, samples of the purified 4D antigen were treated under conditions which disrupt protein interactions, as described in Materials and Methods, and then were analyzed by SDS-PAGE to determine if denaturation of the 190-kDa polypeptide had occurred. Figure 2A shows a Coomassie blue-stained gel of

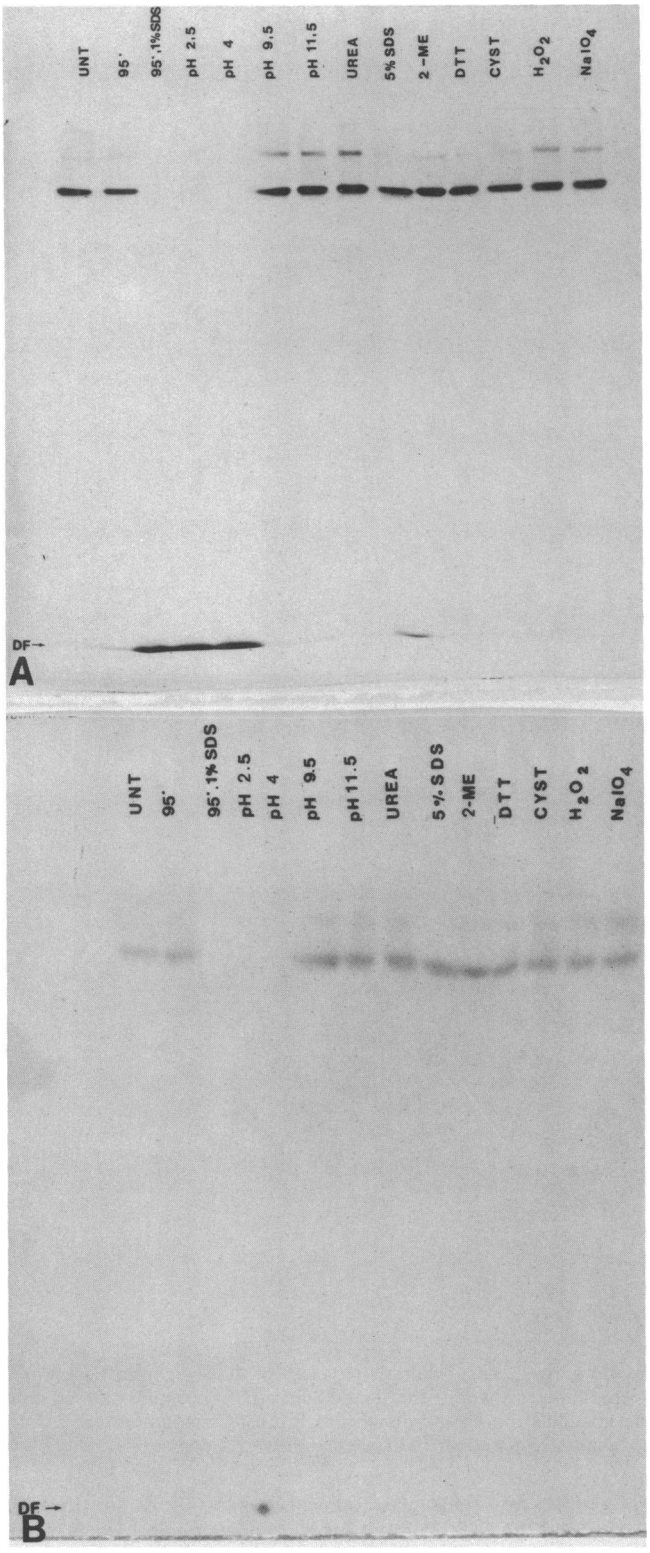

FIG. 2. Stability of the ordered ring structure to different dissociating conditions. Samples of purified 4D antigen were incubated under the conditions indicated. Aliquots were then analyzed by SDS-PAGE and Coomassie brilliant blue staining (A) or transferred to nitrocellulose for immunoblotting with human syphilitic serum (1:100 dilution) and ^{125}I-labeled staphylococcal protein A (B). Only boiling in the presence of SDS or pH ≤ 4.0 dissociated 4D into nonantigenic monomers which migrated at the dye front. UNT, Untreated; DTT, dithiothreitol; CYST, cysteine.

the 4D antigen following the various treatments. The 190-kDa form of the 4D antigen (untreated [UNT]) was maintained when incubated in 5% SDS at 55°C for 6 h in Tris buffer (pH 8.2) or when heated in Tris buffer (pH 8.2) alone at 95°C for 10 min; however, the 190-kDa polypeptide was completely disrupted by heating at 95°C in 0.1% SDS in Tris buffer (pH 8.2) for 5 min to form the previously described 19-kDa monomer (10) as a predominant breakdown product.

The 190-kDa 4D was stable at pH 9.5 and 11.5, but was denatured to constituent 19-kDa monomers at or below pH 4.0. The 190-kDa 4D was stable in 4 M urea–5% SDS–hydrogen peroxide–sodium periodate and in the presence of the following reducing agents: 2-ME, dithiothreitol, and cysteine. The higher-molecular-weight oligomers of the 190-kDa polypeptide, previously identified by immunoblotting with 4D antisera (10), were sensitive to the same treatments as 190-kDa 4D. The 190-kDa 4D and its higher oligomers were also stable to treatment with 20 mM EDTA (data not shown). An identical gel was immunoblotted, reacted with human syphilitic serum and ^{125}I-labeled protein A, and autoradiographed (Fig. 2B). Apparent antigenicity of 4D was unaltered by treatments which preserved the 190-kDa form of the molecule. Treatments which converted 4D to the 19-kDa monomeric form (boiling in SDS and pH ≤ 4.0) destroyed its antigenicity.

Characterization of high-molecular-weight oligomers of the 4D ordered ring. Previous data reported by this laboratory suggested that higher-molecular-weight oligomers of the 190-kDa 4D antigen, which exhibited serological reactivity and heat lability indistinguishable from the 190-kDa polypeptide, were present following purification of the 4D antigen from *E. coli* (10). Utilizing two-dimensional SDS-PAGE gel analysis as described in Materials and Methods, higher oligomers of the 4D antigen could be identified with molecular weights up to 10^6 (Fig. 3A). The oligomers of the purified antigen were separated by electrophoresis under nondenaturing conditions, using a 4% polyacrylamide gel with 1.6% cross-linkage for the first-dimension separation. After the first-dimension separation, an identical tube gel was boiled in final sample buffer before electrophoresis on the second-dimension gel. Figure 3B shows that all high-molecular-weight oligomers seen in Fig. 3A are denatured into indistinguishable 19-kDa monomers after boiling in SDS. Treatment of the first-dimension gel with 0.1 M HCl prior to second-dimension electrophoresis also broke down the high-molecular-weight oligomers to 19 kDa monomers (data not shown).

Reassociation of 4D monomers into a 190-kDa molecule. To determine if 19-kDa monomers of the 4D antigen could self-associate to reform the 190-kDa molecule, 190-kDa 4D was denatured to 19-kDa monomers with acid treatment and dialyzed in various buffers of defined pH as described in Materials and Methods. Following dialysis, each preparation was analyzed by SDS-PAGE to determine if renaturation had occurred. An autoradiogram of the ^{125}I-labeled 4D antigen samples before and after acid denaturation and dialysis is shown in Fig. 4. After incubation in dilute hydrochloric acid (pH 1.5), both the 190-kDa polypeptide and the high-molecular-weight oligomers of the 4D antigen shown in lane 1 were disrupted to form a predominant 19-kDa breakdown product (lane 2). Intermediate molecular weight bands seen between the 19-kDa monomers and the intact 190-kDa polypeptide probably represented various oligomers of the 19-kDa monomers formed by incomplete dissociation of polypeptide. Following dialysis at pH 7.4 in phosphate-buffered saline (lane 5), a partial reassociation of the 190 kDa

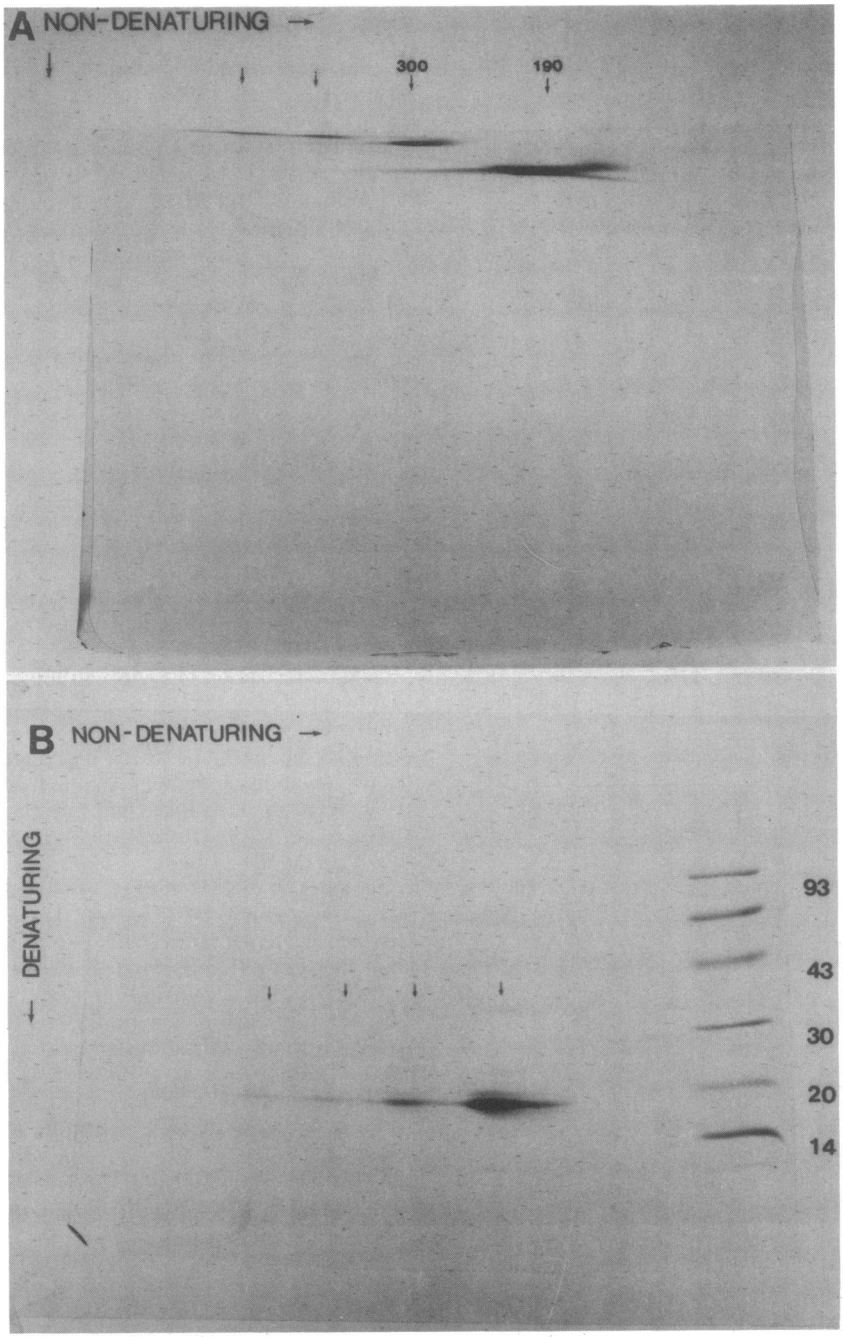

FIG. 3. Characterization of the high-molecular-weight oligomers of the 4D ordered ring. Aliquots (100 μg) of 4D antigen in FSB were electophoresed on 4% SDS-polyacrylamide tube gels. After electrophoresis the tube gels were either loaded directly (A) or boiled for 10 min in FSB + 2ME before loading (B) onto 8 to 20% gradient polyacrylamide slab gels for second-dimension PAGE. First-dimension PAGE separated a series of oligomers (A) which, upon boiling and reduction (B), generated identical 19-kDa breakdown products.

polypeptide was seen. Dialysis at pH 8.4 (0.1 M Tris, 0.15 M NaCl) (lane 6) also resulted in partial reassociation of the 190-kDa form. A faint band at 190 kDa was seen following dialysis at pH 6.0 (0.1 M PO_4, 0.15 M NaCl) (lane 4). There was no detectable reassociation of the 190-kDa form after dialysis at pH 4.0 (0.1 M acetate, 0.15 M NaCl) (lane 3).

Chemical and amino acid analysis of the 4D antigen. The amino acid composition of the 4D antigen is shown in Table 1. The 19-kDa monomer comprising the 190-kDa species (10) contained a relatively high proportion of hydrophobic residues (45%) and polar/charged residues (37.9%). Amino acid analysis of the 14-kDa monomers comprising 90-kDa 4D (10) indicated that proteinase K digestion resulted in a reduction of approximately 21% of the residues recovered; however, the relative proportion of remaining residues was similar to the profile seen for the 190-kDa 4D. This result is in agreement with the calculated 26% difference in molecular weight between the 19- and 14-kDa species shown by SDS-PAGE analysis following boiling in SDS of the 190- and 90-kDa polypeptide molecules, respectively (10). Although

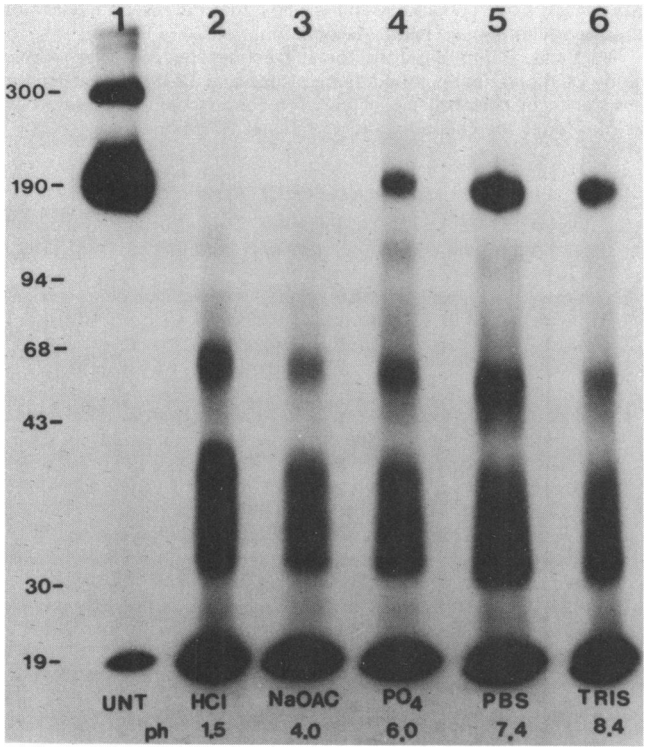

FIG. 4. Reassociation of 4D monomers into a 190-kDa molecule. The 190-kDa 4D and its higher oligomers (lane 1) are converted to a predominant 19-kDa form by treatment at pH 1.5 (lane 2). Partial reassociation of a 190-kDa molecule was evident after extended dialysis at pH 6 (lane 4), 7.4 (lane 5), and 8.4 (lane 6), but did not occur at pH 4.0 (lane 3). UNT, Untreated.

the 19-kDa monomers contained a high proportion of hydrophobic residues, both the 19-kDa monomers and the 190-kDa 4D molecule remained soluble in very dilute aqueous buffers and 45% ethanol (data not shown).

Because of its protease resistance and intense staining with periodate-silver (10), the purified 4D 190-kDa antigen was analyzed for the presence of neutral hexoses, sialic acids, uronic acids, free and *n*-acetylated hexosamines, methylpentoses, and ketodeoxyoctonic acids as described in Materials and Methods. These sugars are not detected in pure preparations of the 4D antigen (data not shown); however, since 200- to 400-μg samples of 4D were utilized per assay, it is possible that several moles of any of the sugars tested could exist per mole of 19-kDa 4D and not have been detected under these conditions given the insensitivity of the assays.

DISCUSSION

Several lines of evidence strongly suggest that the 4D antigen we have described is a native surface molecule of *T. pallidum*. Rabbit antisera to 190-kDa 4D immobilizes *T. pallidum* in the *T. pallidum* immobilization test (10). The most plausible model for immobilization requires antibody and complement interaction with a surface-exposed molecule. Using immunoelectron microscopy, we have found that treponemes immobilized by either syphilitic sera or antibody to 4D are covered with an anti-immunoglobulin G immunoferritin label (Radolf et al., submitted for publication), directly demonstrating that the 4D molecule covers the surface of the organism. Furthermore, 17 rabbits immunized with the 190-kDa ordered ring molecule evidenced significant partial protection in the experimental syphilis model as judged by either delays in lesion development ranging from 4 to 14 days or development of atypical lesions or both (Fehniger et al., Fed. Proc. **44**:596, 1985; Borenstein et al., submitted for publication). These observations, together with its surface location, suggest that 4D is a factor in the pathogenesis of experimental syphilis.

The work we have reported here is significant because it represents the first ultrastructural characterization of a recombinant *T. pallidum* surface molecule and, as such, forms the potential basis for study of structure-function relationships in the pathogenesis of syphilis. Our results demonstrate that 4D is a protease-resistant ordered ring. Because proteinase K-digested 4D retains an ordered ring structure, we believe that the proteinase K-sensitive domain must be oriented toward the periphery of the ring to be accessible to proteases. Recently we have learned that monoclonal antibodies specific for four different epitopes of the protease-sensitive domain have potent activity in the *T. pallidum* immobilization test (H. Togashi, J. D. Radolf, T. E. Fehniger, B. L. Johnson, J. N. Miller, and M. A. Lovett, manuscript in preparation). These observations lend further support to our belief that the protease sensitive domains are functionally important and peripherally localized.

An unanswered question concerns the stoichiometric relationship between the two ordered ring structures and the monomeric subunits. The number of monomers composing the 190-kDa ring has not been determined. Proteolysis destroys 25% of the mass of the 19-kDa monomer (based upon amino acid analysis), yet the limit digestion product of

TABLE 1. Amino acid compositions of the recombinant 4D antigen

Amino acid residues	4D (mol%)[a]	
	19 kDa[b]	14 kDa[c]
Hydrophobic	[45.0]	[41.8]
Ile	6.6	5.3
Phe	3.0	3.8
Val	6.6	7.6
Leu	10.8	9.9
Ala	13.2	11.4
Tyr	4.8	3.8
Neutral	[17.4]	[15.2]
Gyl	6.6	6.1
Cys	0.6	0.8
Met	1.8	1.5
Pro	5.4	3.8
His	3.0	3.0
Polar	[11.4]	[12.2]
Thr	6.6	6.1
Ser	4.8	6.1
Charged	[26.5]	[30.5]
Glx	12.6	13.7
Asx	4.8	6.8
Lys	4.2	5.3
Arg	4.8	4.5
Derived from no. of residues:	166	131

[a] Values in brackets indicate total percentage of amino acids in given residues category.
[b] Molecular weight (× 10^3).
[c] Proteinase K-treated 4D.

proteinase K digestion has a molecular weight of 90,000 rather than 140,000. One possibility is that one or both ring structures are migrating anomalously in SDS-PAGE with the relatively mild dissociating conditions (SDS solubilization without boiling or reduction) used for electrophoresis of the intact oligomers. Another possibility is that proteinase K digestion is effecting a greater structural rearrangement of the ordered ring than is immediately apparent from Fig. 1B. Studies to determine the molecular weight of the intact ring structures and their subunits by means other than SDS-PAGE are planned to answer this important question.

Since the treatment of 4D at 100°C with SDS converts the 190-kDa ring to the 19 kDa form, we conclude that covalent bonds do not link the 19-kDa monomers of the ordered ring. Preliminary experiments have indicated that the native form of the 4D molecule in *T. pallidum* is a very high oligomer of 190-kDa ordered rings cross-linked by disulfide bonds (J. D. Radolf and M. A. Lovett, unpublished observations). Although treatment with 4 M urea did not result in dissociation of 190 kDa, we presume that hydrophobic forces are in part responsible for the assembly and maintenance of structural integrity of the ordered ring and that hydrophobic interactions are in part responsible for the high degree of aggregation of the proteinase-treated material (Fig. 1B). Treatment of 4D with other chaotropic agents has not been studied.

The amino acid composition of 190-kDa 4D is noteworthy because the molecule contains a greater percentage of hydrophobic amino acids (45%) than the *E. coli* surface proteins ompF (39%) (6), ompA (39%) (7), and common pili (41%) (9) and the *C. trachomatis* major outer membrane protein (39%) (4). Evidence presented in this report demonstrates that in vitro self-assembly of 19-kDa monomers is the mechanism of ordered ring structure formation and is greatest at physiologic pH, suggesting that self-assembly of 4D occurs in vivo. While we have not examined the 190-kDa molecule assembled in vitro in the reassociation experiments in the electron microscope, we assume that an apparent mobility of 190-kDa is a hallmark of the ordered ring structure. It should be noted that the reassociation experiment presented here was done with a concentration of 4D of only 10 μg/ml. Experiments are planned to ascertain whether more complete reassociation can be achieved by utilizing higher concentrations of 4D monomers and the inclusion of potential cofactors such as divalent cations.

Despite its high content of hydrophobic aminio acids, 190-kDa 4D behaves as a hydrophilic molecule (solubility in dilute aqueous buffers [unpublished observations]). This hydrophilicity is in contrast to the hydrophobicity of certain other well-characterized bacterial membrane proteins (17) (Table 1). The possibility exists that 4D is a cell surface, but not an outer, membrane protein of *T. pallidum*. Experiments are planned to determine whether 190 kDa can be assembled into liposomes and, if so, whether it creates functional channels. Porin activity in vitro would be consistent with an authentic outer membrane location.

The availability of purified 4D in milligram amounts, and monoclonal antibodies to its different domains, has already permitted the initiation of detailed studies directed toward further understanding of the structure-function relationships of this molecule. Such information will be relevant in understanding the role of the 4D molecule in the pathogenesis of syphilis.

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