JOURNAL OF BACTERIOLOGY, Apr. 1987, p. 1365–1371
0021-9193/87/041365-07$02.00/0

Vol. 169, No. 4

Role of Disulfide Bonds in the Oligomeric Structure and Protease Resistance of Recombinant and Native *Treponema pallidum* Surface Antigen 4D

JUSTIN D. RADOLF,[1]†* LEE A. BORENSTEIN,[2] JOON Y. KIM,[3] THOMAS E. FEHNIGER,[2] AND MICHAEL A. LOVETT[1,2,3]

Departments of Medicine[1] and Microbiology and Immunology,[2] School of Medicine, and the Molecular Biology Institute,[3] University of California, Los Angeles, California 90024

Received 28 July 1986/Accepted 20 December 1986

Recombinant *Treponema pallidum* surface antigen 4D isolated from *Escherichia coli* formed a protease-resistant ordered ring structure composed of 19,000-dalton subunits. On gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the higher oligomers of recombinant 4D migrated with molecular masses that were nearly multiples of the 190,000-dalton basic ordered ring. Reduction at room temperature with 2-mercaptoethanol converted the 190,000-dalton ordered ring and the higher oligomers to a 160,000-dalton form and the dissociated monomer. A 190,000-dalton form of 4D was identified in sodium dodecyl sulfate-solubilized *T. pallidum* after reduction at room temperature. Disulfide bonds stabilized both native and recombinant 4D oligomers against dissociation by heating in detergent without a reducing agent. Electron microscopy of recombinant 4D revealed that the characteristic ordered ring structure was maintained after reduction. Reduction of 4D under conditions that preserved the ordered ring structure did not affect the resistance of the molecule to digestion with proteinase K. The properties of 4D suggest that it may fulfill an important structural role in the *T. pallidum* outer membrane.

Detailed study of the surface proteins of *Treponema pallidum* recently has been made possible by the expression of treponemal antigens in *Escherichia coli* (21, 26, 31, 32). We have recently described a recombinant *T. pallidum* protein, designated 4D, which is a protease-resistant, 190,000-dalton ordered ring structure composed of 19,000-dalton monomers (6, 7). Several lines of evidence suggest that the native 4D antigen plays a role in the pathogenesis of syphilis. 4D and immunologically related molecules have been detected only in *T. pallidum* and the other pathogenic treponemes (7, 23; unpublished data). Rabbit antisera to the purified recombinant molecule immobilize *T. pallidum* cells in vitro (7), and 4D antiserum has been used to demonstrate the surface location of the native 4D antigen by immunoelectron microscopy (22). Rabbits immunized intravenously with the recombinant protein develop significant partial protection against subsequent intradermal challenge (L. Borenstein, J. Radolf, T. Fehniger, J. Miller, and M. Lovett, submitted for publication).

Previously we demonstrated that molecules identical to both the 19,000-dalton monomer and the 90,000-dalton proteinase K limit-digestion product of recombinant 4D could be identified in *T. pallidum* immunoblots reacted with recombinant 4D antiserum (7). The native form of 4D, however, was not identified. In this report we demonstrate that disulfide bonds mediate the higher oligomeric structure of both native and recombinant 4D. After reduction with 2-mercaptoethanol, the 190,000-dalton form of native 4D could be identified. We have also found that disulfide bonds stabilize both native and recombinant 4D against dissociating conditions and contribute to the protease resistance of the molecule. The demonstration that native 4D ordered rings are cross-linked by disulfide bonds into large oligomers suggests that 4D may fulfill an important structural role in the *T. pallidum* outer membrane.

(A preliminary report of this work has been presented [Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, D-150, p. 91].)

MATERIALS AND METHODS

T. pallidum Nichols was maintained by intratesticular passage in New Zealand White rabbits without the use of cortisone acetate. *T. pallidum* for immunoblots was obtained from rabbits given daily intramuscular injections of cortisone acetate (5 mg/kg). Ten to twelve days after inoculation, the testes were asceptically removed, and the treponemes were extracted in phosphate-buffered saline on a rotary shaker. *T. pallidum* cells were purified by Percoll (Pharmacia Fine Chemicals, Piscataway, N.J.) density gradient centrifugation (11).

Purification of recombinant 4D antigen. Recombinant 4D was purified to homogeneity by using a modification of our previously described protocol (7). A 30% ammonium sulfate pellet prepared from the cytosolic fraction of 2 liters of *E. coli* RRI(pAW329) cells (7) was exhaustively dialyzed at 4°C against 50 mM Tris hydrochloride, pH 7.4. The Tris hydrochloride-insoluble material, which contained the 4D antigen, was then exhaustively dialyzed at 4°C against 50 mM Tris hydrochloride–0.9% sodium chloride–0.2% sodium azide (pH 7.4) (TSA) to solubilize the 4D antigen. The concentration of 4D in the supernatant fluid was determined by measurement of optical density at 280 nm (extinction coefficient, 1.2) and by protein assay (16). The purity of the 4D antigen was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with the periodate-silver stain (29) and by electron microscopy (6, 30).

Affinity purification of 4D antibodies. Antibodies to recombinant 4D were affinity purified from rabbit anti-4D antisera

* Corresponding author.
† Present address: Division of Infectious Diseases, University of Texas Health Science Center, Dallas, TX 75235.

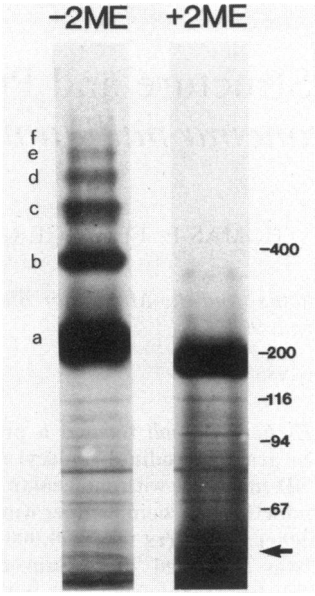

FIG. 1. SDS-PAGE analysis of unreduced and reduced recombinant 4D. Samples (40 μg) of unreduced (−2Me) and reduced (+2Me) 4D were electrophoresed on a 2.5-to-10% polyacrylamide gel and stained with periodate-silver. Unreduced recombinant 4D oligomers are labeled a through f. The arrow near the dye front of reduced 4D indicates the dissociated 19,000-dalton 4D monomer. Numbers at right indicate molecular masses.

by using 5 mg of purified 4D antigen coupled to Reacti-Gel 6X (Pierce Chemical Co., Rockford, Ill.) as previously described (23).

SDS-PAGE and Western blotting. Vertical 1.5-mm 2.5-to-10% gradient and 12.5% slab polyacrylamide resolving gels with 2.6% cross-linking and 10% slab gels with 1.3% cross-linking were run by using the discontinuous buffer system of Laemmli (15). Samples were diluted 1:2 in 2× final sample buffer consisting of 125 mM Tris hydrochloride (pH 6.8), 4% SDS, 20% glycerol, and 10 mg of bromophenol blue per liter, either with or without 10% 2-mercaptoethanol, and were electrophoresed without heating or boiling unless otherwise noted. Samples were loaded into the wells of either 2.5 or 4.5% polyacrylamide stacking gels with 2.6% cross-linking. After electrophoresis, gels were stained with periodate-silver (29) or Coomassie blue or were immunoblotted onto 0.2-μm nitrocellulose sheets (Sartorius, Hayward, Calif.) at 195 mA of constant current for 3 h (28). Nitrocellulose transfers were incubated overnight with 100 to 150 μg of affinity-purified anti-4D antibodies in 50 ml of TSA with 3% ovalbumin (Sigma Chemical Co., St. Louis, Mo.) and probed with staphylococcal protein A (Pharmacia) radiolabeled with ^{125}I (Amersham Corp., Arlington Heights, Ill.) by the lactoperoxidase method (18).

Reduction and carboxymethylation of recombinant 4D. Purified 4D (80 μg in 100 μl of TSA) was placed in 250-μl polypropylene microcentrifuge tubes sealed tightly with Spectra/Por 6 (Spectrum Medical Industries, Los Angeles, Calif.) dialysis tubing with a molecular weight cutoff of 2,000. The inverted tubes were dialyzed overnight at room temperature against a 1-liter volume of TSA containing 10 mM 2-mercaptoethanol or 10 mM dithiothreitol. For carboxymethylation, the reduced 4D was combined with an equal volume of 1.5 M iodoacetic acid (Sigma) in 1 N NaOH plus 1.5 M Tris base (final pH, 8.6), flushed with N_2, and wrapped with aluminum foil (5). After a 30-min incubation at room temperature, the carboxymethylated 4D was dialyzed against 50 mM Tris hydrochloride, pH 7.4.

Reduction of solubilized *T. pallidum*. *T. pallidum* organisms were solubilized and reduced by two different methods which produced indistinguishable results. In our initial experiment, 50-μl samples of Percoll-purified *T. pallidum* containing 1.5×10^9 organisms in phosphate-buffered saline were combined with equal volumes of 1% SDS in 50 mM Tris hydrochloride (pH 7.4) in 250-μl polypropylene microcentrifuge tubes. The solubilized organisms were then dialyzed for 24 h at room temperature against 1-liter volumes of 50 mM Tris hydrochloride (pH 7.4) either with or without 10 mM 2-mercaptoethanol. In later experiments, reduction was accomplished by combining 25-μl samples of purified *T. pallidum* containing 1×10^9 organisms with equal volumes of 2× final sample buffer containing 10% 2-mercaptoethanol or 50 mM dithiothreitol and then incubating at room temperature for 4 h.

Analysis of unreduced *T. pallidum* 4D excised from polyacrylamide gels. Samples containing 1.5×10^9 *T. pallidum* cells were solubilized in 2× final sample buffer without 2-mercaptoethanol for 4 h at room temperature and applied to individual wells of a 4.5% polyacrylamide stacking gel. After the dye front had progressed to within 1 cm of the end of the 10% separating gel, 3-mm slices were excised from the tops of individual stacking gel lanes and either soaked again for 4 h in 1× final sample buffer (62.5 mM Tris hydrochloride, 2% SDS, 10% glycerol, and 10 mg of bromophenol blue per liter, pH 6.8) with or without 5% 2-mercaptoethanol or boiled for 10 min in 1× final sample buffer with 2-mercaptoethanol. The slices were then placed into the wells of a second 4.5% stacking gel, electrophoresed through another 10% separating gel, and transferred to nitrocellulose for immunoblotting.

Proteinase K treatment of recombinant and *T. pallidum* 4D with and without 2-mercaptoethanol. Samples containing either 20 μg of recombinant 4D or 10^9 *T. pallidum* organisms in 25 μl were added to equal volumes of 2× final sample buffer with or without 2-mercaptoethanol. Samples were incubated for 4 h at room temperature or at 55°C in the presence and absence of 10 μg of proteinase K (Sigma). Enzyme was added in 5-μl portions from a freshly prepared stock (2 mg/ml) in 50 mM Tris hydrochloride buffer, pH 7.0.

Electron microscopy. Reduced and unreduced recombinant 4D at a concentration of 25 μg/ml in 50 mM Tris hydrochloride buffer (pH 7.4) were prepared for electron microscopy by using the single carbon layer method (30). Micrographs were analyzed as previously described (6).

RESULTS

Sensitivity of recombinant 4D oligomers to reduction. Samples of unreduced and reduced recombinant 4D were electrophoresed on a 2.5- to 10% gradient gel. The unreduced 4D formed a series of bands (a through f) distributed in a stepladder pattern (Fig. 1). A semilogarithmic plot of their relative mobilities produced a straight line; each oligomer migrated with a molecular mass of approximately 180,000 daltons greater than that of the band immediately below it (data not shown). After reduction with 2-mercaptoethanol at room temperature, the higher oligomers essentially disappeared, while the remaining high-molecular-weight band migrated at a lower molecular mass (160,000 daltons) than the unreduced 190,000-dalton basic oligomer. In addition, monomer could be detected near the dye front of the lane containing reduced 4D (Fig. 1, arrow). Identical results were

produced by reduction with 10 mM dithiothreitol (data not shown).

Identification of 4D oligomers in *T. pallidum*. Initial attempts to identify a high-molecular-weight *T. pallidum* protein corresponding to 190,000-dalton recombinant 4D on polyacrylamide gels were unsuccessful. The finding that disulfide bonds play a major role in recombinant 4D oligomeric structure prompted us to examine the possibility that *T. pallidum* 4D exists in an extensively disulfide-bonded form which is too large to enter a 10% polyacrylamide gel. Samples of SDS-solubilized *T. pallidum* cells were dialyzed at room temperature against Tris hydrochloride buffer with or without 2-mercaptoethanol, electrophoresed, and transferred to nitrocellulose. A faint band that comigrated with the 190,000-dalton oligomer of unreduced recombinant 4D was seen on the nitrocellulose transfer of reduced *T. pallidum*, but not in the lane containing organisms solubilized without a reductant (data not shown). Immunoblotting with affinity-purified antibodies to recombinant 4D confirmed that this band represented *T. pallidum* 4D (Fig. 2). In contrast, *T. pallidum* 4D was identified in cells solubilized without 2-mercaptoethanol as immunoreactive material, the majority of which was unable to enter the separating gel (Fig. 2).

Recombinant and *T. pallidum* 4D are heat dissociable after reduction. The relative contributions of disulfide bonds and noncovalent associations to 4D oligomeric structure were investigated by examining the heat dissociability of the antigens in the presence and absence of a reductant. When recombinant 4D was boiled in final sample buffer without 2-mercaptoethanol, the intensity of the higher oligomers was dramatically reduced, and new oligomeric bands intermediate in molecular mass between 190,000 daltons and the second oligomer appeared (Fig. 3A, lane 2). In addition to the 19,000-dalton monomer, polypeptides with apparent molecular masses of 49,000, 45,000, 35,000, and 31,000 daltons were detected. Lane 3 contains recombinant 4D which was carboxymethylated after reduction so that the 2-mercaptoethanol could be removed completely without reformation of disulfide bonds. Complete dissociation to the monomer occurred when the reduced, carboxymethylated recombinant 4D was boiled without reductant (Fig. 3A, lane 4). The same result, complete dissociation, occurred when recombinant 4D was boiled in final sample buffer containing 2-mercaptoethanol (Fig. 3A, lane 5).

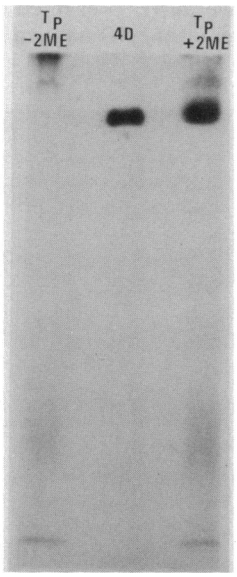

FIG. 2. Identification of native 4D in *T. pallidum*. Samples containing 10^9 *T. pallidum* cells were solubilized in 1% SDS in the presence (Tp+2Me) or absence (Tp−2Me) of reducing agent and immunoblotted with affinity-purified antibodies to recombinant 4D. The middle lane contains approximately 2 μg of unreduced recombinant 4D.

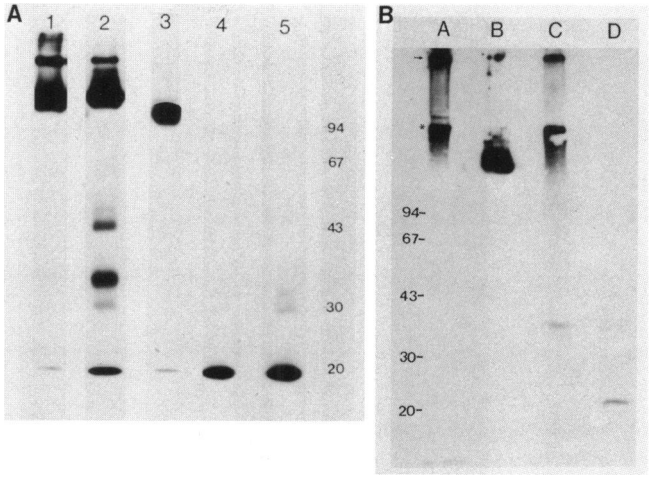

FIG. 3. Role of disulfide bonds in thermal stability of 4D. (A) Samples (20 μg) of unreduced and reduced recombinant 4D were boiled in final sample buffer with or without 2-mercaptoethanol before SDS-PAGE and immunoblotting with anti-4D affinity-purified antibodies. Lanes: 1, unreduced 4D incubated at room temperature in final sample buffer without 2-mercaptoethanol; 2, unreduced 4D boiled for 10 min in final sample buffer without 2-mercaptoethanol; 3, reduced and carboxymethylated 4D; 4, reduced and carboxymethylated material shown in lane 3, after boiling for 10 min in final sample buffer without 2-mercaptoethanol; 5, 4D boiled for 10 min in final sample buffer with 2-mercaptoethanol. Molecular masses (in kilodaltons) are listed to the right. (B) Samples containing 10^9 *T. pallidum* cells were incubated at room temperature or boiled for 10 min in final sample buffer with and without 2-mercaptoethanol before immunoblotting with affinity-purified anti-4D antibodies. Lanes: A, cells solubilized in final sample buffer without 2-mercaptoethanol; B, cells incubated for 4 h at room temperature in final sample buffer containing 2-mercaptoethanol; C, cells boiled for 10 min in final sample buffer without 2-mercaptoethanol; D, cells boiled for 10 min in final sample buffer containing 2-mercaptoethanol. Molecular masses (in kilodaltons) are listed to the left. The arrow indicates the top of the stacking well; the asterisk indicates the interface between the stacking and separating gels.

Similar results were obtained for *T. pallidum* 4D by using organisms solubilized in final sample buffer. In these experiments both the stacking and separating gels were immunoblotted with the affinity-purified anti-4D antibodies. After solubilization at room temperature without 2-mercaptoethanol, more than half of the immunoreactive material was caught at the top of the stacking gel (Fig. 3B, lane A). Solubilization in the presence of 2-mercaptoethanol dramatically increased the ability of the *T. pallidum* 4D to enter the separating gel (Fig. 3B, lane B). The amount of *T. pallidum* 4D trapped in the stacking and separating gels decreased somewhat after boiling without 2-mercaptoethanol; as with recombinant 4D, this treatment produced a 35,000-dalton breakdown product (Fig. 3B, lane C). Complete dissociation occurred after boiling in final sample buffer containing 2-mercaptoethanol (Fig. 3B, lane D). Identical results were produced if 50 mM dithiothreitol was substituted for 2-mercaptoethanol (data not shown). It is also

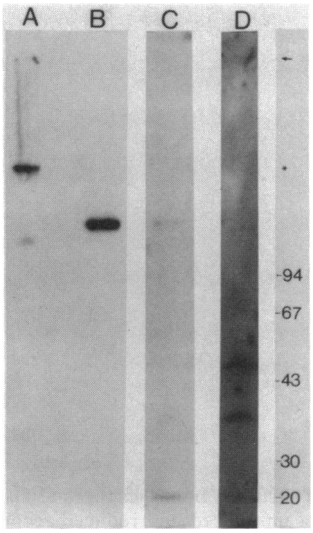

FIG. 4. Analysis of high-molecular-weight *T. pallidum* material caught in wells of a 4.5% stacking gel. Strips (4 mm) were excised from the tops of stacking gel lanes after SDS-PAGE of material from *T. pallidum* cells and electrophoresed after the following treatments. Lanes: A, 4 h of incubation in final sample buffer without 2-mercaptoethanol; B, 4 h of incubation in final sample buffer containing 2-mercaptoethanol; C and D, 10 min of boiling in the final sample buffer containing 2-mercaptoethanol. Lanes were immunoblotted with either affinity-purified 4D antibodies (lanes A through C) or pooled rabbit immune syphilitic serum at a 1:100 dilution (lane D). Numbers at the right are molecular masses in kilodaltons.

apparent that a significant degree of *T. pallidum* 4D immunoreactivity was lost after dissociation to the 19,000-dalton monomer as previously described (6, 7).

The unreduced high-molecular-weight material which became trapped in the stacking gel was further investigated. Narrow strips were excised from the tops of individual stacking gel lanes after SDS-PAGE of material solubilized in final sample buffer without 2-mercaptoethanol, treated as described in Materials and Methods, and immunoblotted with the anti-4D antibodies or pooled immune rabbit syphilitic serum. After an additional 4-h incubation at room temperature in final sample buffer without 2-mercaptoethanol, most of the *T. pallidum* 4D was able to traverse the stacking gel before being stopped at the interface between the stacking and separating gels (Fig. 4, lane A). If, on the other hand, the strip was incubated in final sample buffer with 2-mercaptoethanol, then all of the material was detected in the separating gel as reduced *T. pallidum* 4D (Fig. 4, lane B). Nearly complete dissociation to the 19,000-dalton monomer was produced by boiling with 2-mercaptoethanol (Fig. 4, lane C). An additional stacking gel strip was boiled with 2-mercaptoethanol and immunoblotted with pooled immune rabbit syphilitic serum to detect polypeptides other than *T. pallidum* 4D also caught in the stacking gel. Of note, proteins of 38,000 and 48,000 daltons were also detected by immunoblotting with pooled immune rabbit syphilitic serum (Fig. 4, lane D).

Electron microscopy of unreduced and reduced recombinant 4D. Unreduced and reduced recombinant 4D were examined by electron microscopy. Unreduced recombinant 4D possesses an ordered ring structure which we have previously described (6). After reduction, recombinant 4D retained the ordered ring structure; however, subtle differences between the unreduced and reduced ring structures were observed. The reduced 4D rings (Fig. 5) were slightly larger, with a mean diameter of 11 nm compared with 10 nm for unreduced recombinant 4D (data not shown). The subunits of the reduced rings also appeared to be more regularly arranged than those of the unreduced molecules. A greater tendency for the reduced material to form large aggregates was also consistently observed.

Effect of temperature and reducing agents on proteinase K susceptibility of recombinant and *T. pallidum* 4D. Samples of recombinant 4D and *T. pallidum* were treated with proteinase K at room temperature and at 55°C in the presence and absence of 2-mercaptoethanol. Recombinant 4D was resistant to proteolysis at both 55°C (Fig. 6A, lane 2) and room temperature (data not shown) in the absence of 2-mercaptoethanol. After reduction at room temperature, the 160,000-dalton reduced ordered ring remained resistant to proteinase K, whereas the dissociated monomer was completely degraded (Fig. 6A, lane 4). A 4-h incubation in final sample buffer with 2-mercaptoethanol at 55°C resulted in complete dissociation of recombinant 4D (Fig. 6A, lane 5); under these conditions, recombinant 4D was completely degraded by proteinase K (Fig. 6A, lane 6).

Similar results were obtained for *T. pallidum* 4D. *T. pallidum* 4D was dissociated by reduction and incubation at 55°C (Fig. 6B, lane 2) but not at room temperature (Fig. 6B, lane 1). A 90,000-dalton proteinase K-resistant band was detected in organisms proteolyzed at room temperature in the presence and absence of reductant (Fig. 6B, lanes 3 and 4) as well as in organisms proteolyzed at 55°C without 2-mercaptoethanol (Fig. 6B, lane 5). In contrast, *T. pallidum* 4D was not detected after proteolysis of cells at 55°C in final sample buffer containing 2-mercaptoethanol (Fig. 6, lane 6), indicating that the dissociated 4D monomer had been completely degraded.

DISCUSSION

In this report we have presented evidence that the oligomeric structures of the 4D antigen synthesized in both

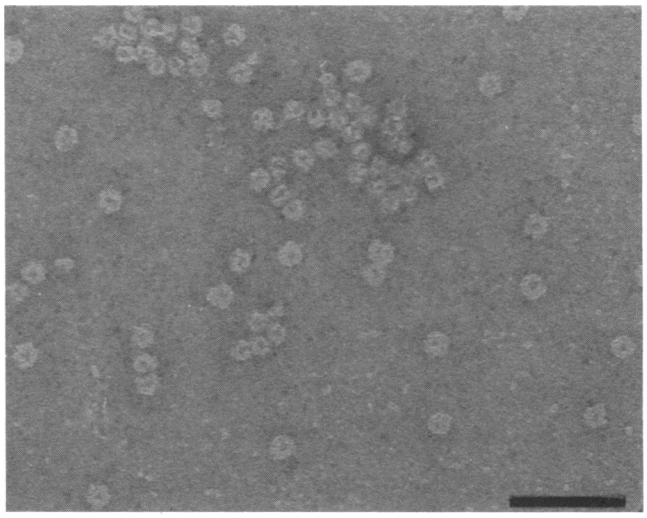

FIG. 5. Electron micrograph of purified recombinant 4D after reduction by dialysis with 10 mM 2-mercaptoethanol. The ordered ring structure of recombinant 4D is retained after reduction. Bar, 50 nm.

T. pallidum and *E. coli* are stabilized by disulfide bonds. Our studies also demonstrate that despite strong overall structural similarities between the recombinant and native molecules, significant differences in higher-order structure also exist.

Unreduced recombinant 4D formed a ladderlike banding pattern when examined on a 2.5-to-10% gradient polyacrylamide gel (Fig. 1). Each higher oligomer migrated with a molecular mass 180,000 daltons greater than that of the band below it. Because the basic recombinant 4D oligomer has a molecular mass of 190,000 daltons, the banding pattern strongly indicates that higher oligomers are formed by joining basic ordered rings. The disappearance of the higher oligomers after reduction with either 2-mercaptoethanol or dithiothreitol indicates that disulfide bonds provide covalent linkage between the unit ordered rings. After reduction, the remaining band migrated with an apparent lower molecular mass (160,000 daltons) than the unreduced basic oligomer (190,000 daltons), suggesting that disulfide bonds also occur within the 190,000-dalton ordered ring, most likely between adjacent subunits. The more regular appearance of reduced recombinant 4D (160,000 daltons) examined by electron microscopy (Fig. 5) suggests that its lower molecular mass on SDS-PAGE reflects conformational changes rather than an actual loss of subunits from the remaining undissociated ordered rings.

The discovery that disulfide bonds mediate the oligomeric structure of recombinant 4D provided a means for identifying the native form of 4D in *T. pallidum*. Unreduced *T. pallidum* 4D exists as high-molecular-weight material which barely entered the 10% polyacrylamide gel (Fig. 2). After reduction, however, most of the *T. pallidum* 4D was detected comigrating with the basic oligomer of unreduced recombinant 4D. When both stacking and separating gels were immunoblotted, immunoreactive material of even higher molecular weight was detected which failed to penetrate the 4.5% stacking gel (Fig. 3B, lane A). The inability to enter a polyacrylamide gel cannot be explained on the basis of insolubility of unreduced *T. pallidum* 4D. Centrifugation for 1 h at 130,000 × *g* failed to pellet unreduced 4D from SDS-solubilized *T. pallidum* cells (data not shown). These findings suggest that *T. pallidum* 4D consists of large complexes of extensively disulfide-bonded ordered rings. Based on their inability to enter the stacking gel, the molecular masses of these higher oligomers must be at least several million daltons (2).

In addition to mediating the oligomeric structure of 4D, disulfide bonds confer upon the antigen the ability to resist powerful dissociating conditions, namely, boiling or prolonged incubation at 55°C in high concentrations of SDS. Boiling in SDS, however, did produce changes in both recombinant and *T. pallidum* 4D structure. Of greatest importance was the appearance of lower-molecular-mass bands, which most likely represent disulfide-bonded dimers, and possibly trimers, of the 19,000-dalton subunit (Fig. 3A, lane 2; Fig. 3B, lane C). These disulfide-bonded intermediates appear to be noncovalently associated within the 4D unit ordered ring and higher oligomers. Noncovalent associations also appear to contribute to the higher-order structure of *T. pallidum* 4D. Unreduced *T. pallidum* 4D, which had been unable to penetrate a 4.5% stacking gel, was able to do so after further prolonged incubation at room temperature in high concentrations of SDS (Fig. 5, lane A). Similarly, immunoblots of organisms solubilized for several hours at 55°C in the final sample buffer without 2-mercaptoethanol also showed a variable tendency for the material caught in the stacking and separating gels to disaggregate (data not shown).

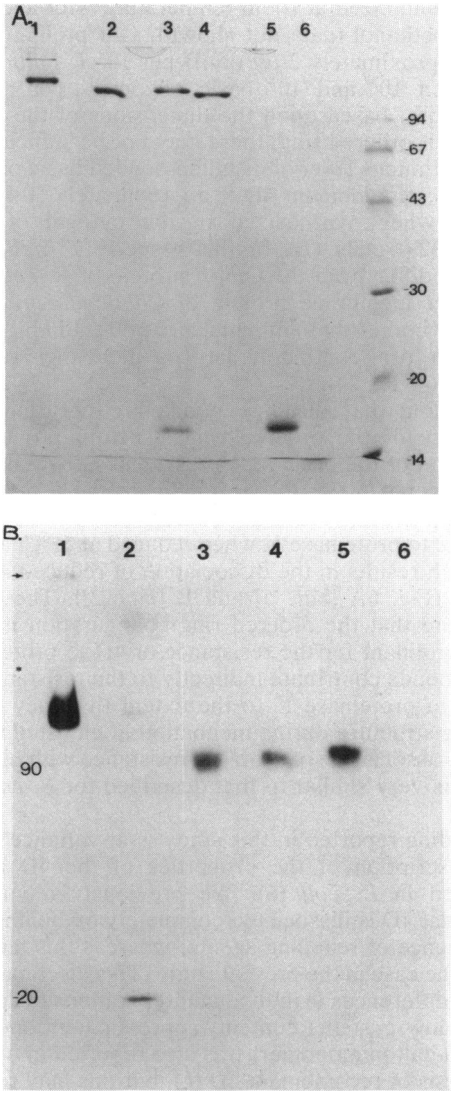

FIG. 6. Role of disulfide bonds in resistance to proteinase K of 4D. Samples containing 20 μg of recombinant 4D (A) and 10^9 *T. pallidum* cells (B) were incubated in the final sample buffer with and without 2-mercaptoethanol and proteinase K as described in Materials and Methods. (A) Coomassie blue-stained 12.5% gel. Lanes: 1, 4D incubated at 55°C in final sample buffer without 2-mercaptoethanol; 2, 4D treated with proteinase K at 55°C without 2-mercaptoethanol; 3, 4D reduced at room temperature; 4, 4D reduced and treated with proteinase K at room temperature; 5, 4D reduced at 55°C; 6, 4D reduced and treated with proteinase K at 55°C. Molecular masses (in kilodaltons) are listed to the right. (B) *T. pallidum* immunoblots reacted with anti-4D affinity-purified antibodies. Organisms solubilized in final sample buffer were reduced without proteinase K at room temperature (lane 1) and at 55°C (lane 2) or were treated with proteinase K at room temperature without (lane 3) and with (lane 4) 2-mercaptoethanol and at 55°C without (lane 5) and with (lane 6) 2-mercaptoethanol. Molecular masses (in kilodaltons) are listed to the left. The arrow indicates the top of the stacking gel; the asterisk indicates the interface between the stacking and separating gels.

Recently, we determined the abundance of 4D in *T. pallidum* by comparing densitometric tracings on SDS-PAGE gels of known quantities of recombinant 4D and *T.*

pallidum solubilized at room temperature with and without 2-mercaptoethanol (data not shown). Our preliminary estimate of approximately 2 μg of 4D per 10^9 *T. pallidum* cells (or between 10^3 and 10^4 basic oligomers per organism) indicates that, based upon the dimensions of the recombinant 4D unit ordered ring, there may not be sufficient 4D to form a continuous layer of disulfide-bonded basic oligomers. In contrast, recombinant 4D is approximately 50-fold more abundant when synthesized in the cytosol of *E. coli* RR1(pAW329) cells (7). In this respect, *T. pallidum* 4D appears to differ from the OmpF protein of *E. coli* and the major outer membrane protein of *Chlamydia trachomatis* elementary bodies, both of which are present in high enough numbers to form continuous lattices about their respective organisms (4, 17, 24).

Our finding that disulfide bonds are not required for maintenance of the ordered ring structure prompted our investigation of the basis for the proteinase K resistance of the 4D molecule (6, 7). Both recombinant and *T. pallidum* 4D were resistant to proteinase K when reduced at 25°C but susceptible to proteinase K when reduced at 55°C, a temperature which results in the dissociation of reduced 4D to the monomer (Fig. 6A [lane 5] and B [lane 2]). These results demonstrate that the ordered ring configuration is the primary determinant for the resistance of 4D to proteinase K. Disulfide bonds contribute indirectly to the resistance of the molecule to proteinase K to the extent that they maintain oligomeric structure during incubation at elevated temperatures. The association of protease resistance with oligomeric structure is very similar to that described for *E. coli* porins (17, 24).

One finding reported in this study is at variance with our recent description of the properties of the 4D molecule synthesized in *E. coli* (6). We previously reported that recombinant 4D is dissociated completely by boiling in SDS in the absence of reducing agents, whereas this has clearly not been the case in the present study. This discrepancy may represent differences in individual preparations of antigen. A relatively low cysteine content, approximately one residue per 19,000-dalton monomer, was also reported in our amino acid analysis of recombinant 4D (6), but this may reflect the recognized difficulty in accurately determining cysteine content by conventional amino acid analysis (14).

Disulfide-bonded proteins have been demonstrated in the outer membranes of a number of bacterial pathogens, including *Legionella pneumophila* (3, 8), *Chlamydia psittaci* (12), *Chlamydia trachomatis* (1, 9, 12, 19), *Pseudomonas aeruginosa* (10), and *Neisseria gonorrhoeae* (20). The unreduced major outer membrane proteins of both *L. pneumophila* (3, 8) and *C. trachomatis* (19), like unreduced *T. pallidum* 4D, are also extraordinarily resistant to dissociation by heating. In the case of *Chlamydia* species, the lattice structure formed by the major outer membrane protein (4) imparts rigidity during the elementary-body phase of the *Chlamydia* life cycle (9, 12). Of particular interest was the finding that proteins of 38,000 and 48,000 daltons, in addition to *T. pallidum* 4D, appear to exist as high-molecular-weight complexes in *T. pallidum* (Fig. 4, lane D). It is tempting, therefore, to speculate that *T. pallidum* 4D, perhaps in association with these other components, fulfills a structural role in the *T. pallidum* outer membrane similar to that of the major outer membrane protein of *Chlamydia* species. This could be especially important given the greater fragility of the *T. pallidum* outer membrane (11, 27) compared with that of typical gram-negative bacteria, the absence of an external surface layer such as that observed in many nonpathogenic treponemes (13, 25), and the fact that peptidoglycan has not been unequivocably demonstrated in pathogenic treponemes. Experiments to clarify the precise ultrastructural relationship between the *T. pallidum* outer membrane and the 4D antigen are currently under way in our laboratory.

ACKNOWLEDGMENTS

This work was supported by Public Health Service research grant AI-21352 from the National Institute of Allergy and Infectious Diseases. J.D.R. is a postdoctoral research fellow of the American Social Health Association.

We thank Barbara Mueller for excellent assistance with the electron microscopy. We thank James N. Miller for his careful review of the manuscript.

LITERATURE CITED

1. **Bavoil, P., A. Ohlin, and J. Schachter.** 1984. Role of disulfide bonding in outer membrane structure and permeability in *Chlamydia trachomatis*. Infect. Immun. **44**:479–485.
2. **Blatter, D. P., and F. J. Reithel.** 1970. Molecular weight determinations and the influence of gel density, protein charges, and protein shape in polyacrylamide electrophoresis. J. Chromatogr. **46**:286–292.
3. **Butler, C. A., E. D. Street, T. P. Hatch, and P. S. Hoffman.** 1985. Disulfide-bonded outer membrane proteins in the genus *Legionella*. Infect. Immun. **48**:14–18.
4. **Chang, J. J., K. Leonard, T. Arad, T. Pitt, Y. N. Zhang, and L. H. Zhang.** 1982. Structural studies of the outer envelope of *Chlamydia trachomatis* by electron microscopy. J. Mol. Biol. **161**:579–590.
5. **Crestfield, A. M., and W. H. Stein.** 1963. The preparation and enzymatic hydrolysis of reduced and s-carboxymethylated proteins. J. Biol. Chem. **138**:622–627.
6. **Fehniger, T. E., J. D. Radolf, and M. A. Lovett.** 1986. Properties of an ordered ring structure formed by recombinant *Treponema pallidum* surface antigen 4D. J. Bacteriol. **165**:732–739.
7. **Fehniger, T. E., A. M. Walfield, T. M. Cunningham, J. D. Radolf, J. N. Miller, and M. A. Lovett.** 1984. Purification and characterization of a cloned protease-resistant *Treponema pallidum*-specific antigen. Infect. Immun. **46**:598–607.
8. **Gabay, J. E., M. Blake, W. D. Niles, and M. A. Horwitz.** 1985. Purification of *Legionella pneumophila* major outer membrane protein and demonstration that it is a porin. J. Bacteriol. **162**: 85–91.
9. **Hackstadt, T., W. J. Todd, and H. D. Caldwell.** 1985. Disulfide-mediated interactions of the chlamydial major outer membrane protein: role in the differentiation of chlamydiae? J. Bacteriol. **161**:25–31.
10. **Hancock, R. E. W., and A. M. Carey.** 1979. Outer membrane of *Pseudomonas aeruginosa*: Heat- and 2-mercaptoethanol-modifiable proteins. J. Bacteriol. **140**:902–910.
11. **Hanff, P. A., S. J. Norris, M. A. Lovett, and J. N. Miller.** 1984. Purification of *Treponema pallidum*, Nichols strain, by Percoll density gradient centrifugation. Sex. Transm. Dis. **11**:275–286.
12. **Hatch, T. P., I. Allan, and J. H. Pearce.** 1984. Structural and polypeptide differences between envelopes of infective and reproductive life cycle forms of *Chlamydia* spp. J. Bacteriol. **157**:13–20.
13. **Hovind-Hougen, K.** 1975. The ultrastructure of cultivable treponemes. 3. *Treponema genitalis*. Acta Pathol. Microbiol. Scand. Sect. B **83**:91–99.
14. **Hunt, S.** 1985. Degradation of amino acids accompanying *in vitro* protein hydrolysis, p. 376–398. *In* G. C. Barrett (ed.), Chemistry and biochemistry of the amino acids. Chapman & Hall Ltd., London.
15. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
16. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
17. **Lugtenberg, B., and L. van Alphen.** 1983. Molecular architec-

ture and functioning of the outer membrane of *Escherichia coli* and other gram-negative bacteria. Biochim. Biophys. Acta **737:** 51–115.

18. **Morrison, M.** 1980. Lactoperoxidase-catalyzed iodination as a tool for investigation of proteins. Methods Enzymol. **70:**214–220.
19. **Newhall, W. J., V, and R. B. Jones.** 1983. Disulfide-linked oligomers of the major outer membrane protein of chlamydiae. J. Bacteriol. **154:**998–1001.
20. **Newhall, W. J., V, C. E. Wilde III, W. D. Sawyer, and R. A. Haak.** 1980. High-molecular-weight antigenic protein complex in the outer membrane of *Neisseria gonorrhoeae*. Infect. Immun. **27:**475–482.
21. **Norgard, M. V., and J. N. Miller.** 1983. Cloning and expression of *Treponema pallidum* (Nichols) antigen genes in *Escherichia coli*. Infect. Immun. **42:**435–445.
22. **Radolf, J. D., T. E. Fehniger, F. J. Silverblatt, J. N. Miller, and M. A. Lovett.** 1986. The surface of virulent *Treponema pallidum*: resistance to antibody binding in the absence of complement and surface association of recombinant antigen 4D. Infect. Immun. **52:**579–585.
23. **Radolf, J. D., E. B. Lernhardt, T. E. Fehniger, and M. A. Lovett.** 1986. Serodiagnosis of syphilis by ELISA using purified recombinant *Treponema pallidum* antigen 4D. J. Infect. Dis. **153:**1023–1027.
24. **Rosenbusch, J. P.** 1974. Characterization of the major envelope protein from *Escherichia coli*: regular arrangement on the peptidoglycan and unusual dodecyl sulfate binding. J. Biol. Chem. **249:**8019–8029.
25. **Sleytr, U. B.** 1978. Regular arrays of macromolecules on bacterial cell walls: structure, chemistry, assembly, and function. Int. Rev. Cytol. **53:**1–64.
26. **Stamm, L. V., J. D. Folds, and P. J. Bassford, Jr.** 1982. Expression of *Treponema pallidum* antigens in *Escherichia coli* K-12. Infect. Immun. **36:**1238–1241.
27. **Sykes, J. A., and J. N. Miller.** 1973. Ultrastructural studies of treponemes: location of axial filaments and some dimensions of *Treponema pallidum* (Nichols strain), *Treponema denticola*, and *Treponema reiteri*. Infect. Immun. **7:**100–110.
28. **Towbin, H. T., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins. Proc. Natl. Acad. Sci. USA **76:**4350–4354.
29. **Tsai, C. M., and C. M. Frasch.** 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Anal. Biochem. **119:**115–119.
30. **Valentine, R. C., B. M. Shapiro, and E. R. Stadtman.** 1968. Regulation of glutamine synthetase. XII. Electron microscopy of the enzyme from *E. coli*. Biochemistry **7:**2143–2152.
31. **van Embden, J. D., H. J. van der Donk, R. V. van Eijk, H. G. van der Heide, J. A. de Jong, M. F. van Olderen, A. D. Osterhaus, and L. M. Schouls.** 1983. Molecular cloning and expression of *Treponema pallidum* DNA in *Escherichia coli* K-12. Infect. Immun. **42:**187–196.
32. **Walfield, A. M., P. A. Hanff, and M. A. Lovett.** 1982. Expression of *Treponema pallidum* antigens in *Escherichia coli*. Science **216:**522–523.