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Immunological Studies of the Disulfide Bridge Region of *Pseudomonas aeruginosa* PAK and PAO Pilins, Using Anti-PAK Pilus and Antipeptide Antibodies

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***Pseudomonas aeruginosa* is an opportunistic pathogen that attaches to host cells via their pili. The pilus of *P. aeruginosa* PAK consists of a polymer of a single subunit, pilin, which is a 144-residue polypeptide. The C-terminal end of this protein is semiconserved in a number of strains and contains a disulfide bridge. We have synthesized the C-terminal peptide PAK (128-144)-OH in both its reduced and oxidized forms and the analog PAK(A-129) (128-144)-OH, in which cysteine-129 was substituted by alanine. These three peptides were used to immunize rabbits and prepare antipeptide antisera. It was found that antipeptide antisera to reduced peptide (17-R) and to oxidized peptide (17-O) bound to native PAK pili and cross-reacted with strain PAO pili in direct enzyme-linked immunosorbent assay (ELISA) and immunoblot experiments. However, the antiserum to the peptide immunogen PAK(A-129)(128-144)-OH, which does not have the ability to form the disulfide bridge, did not bind to either PAK or PAO pili. Competitive ELISA experiments with reduced and oxidized peptides of Ac-PAK(128-144)-OH showed that there was no difference in binding between the two peptides for 17-R or 17-O immunoglobulin G. When immunoglobulin G from native PAK antipilus antiserum was used in competitive or direct ELISA experiments, there was also no preference in binding to reduced or oxidized Ac-PAK(128-144)-OH or to PAK(A-129)(128-144)-OH. This result showed that the disulfide bridge in *Pseudomonas* pili is not critical to the immunogenicity of this region. However, the disulfide bridge is important in the immunogenicity of the C-terminal peptide when preparing antipeptide antisera that are cross-reactive with pili from different strains, since only the disulfide bridge peptide antisera cross-reacted well with the PAO pili as shown by competitive ELISA, suggesting that this region could be an important candidate for development of a synthetic vaccine.**

Pseudomonas aeruginosa is an opportunistic pathogen which infects patients who have cystic fibrosis, leukemia, or burn injury. This organism attaches to host cells via their polar pili (15, 25). The pilus is made up of a polymer of a single subunit called pilin (~15,000 daltons). The amino acid sequences of pilins from a number of strains, including the two strains PAK and PAO, have been determined by Sastry et al. (18). From the sequence data available, the pilin molecule consists of a highly conserved hydrophobic N-terminal region of about 35 amino acid residues, a highly variable internal region, and a semiconserved C-terminal region which contains a disulfide bridge.

Since pilus-mediated adhesion by *P. aeruginosa* is believed to be an important step to infection, blocking the attachment process with pilus-directed antibodies would prevent colonization and infection with these organisms. Woods et al. (25) have shown that antiserum to pili decreased adherence to mammalian buccal epithelial cells. No other pilus-associated protein has been found which might mediate attachment, unlike the case of *Escherichia coli*, in which the PapG protein, found at the tip of the pilus, was responsible for adhesion (8). To develop a synthetic vaccine against *Pseudomonas* infection based on pilus-mediated attachment, studies have been carried out to map the antigenic determinants of *Pseudomonas* pili by using antipeptide antibodies. Similar experiments to map the antigenic determinants on *E. coli* pili (6) and gonococcal pili (16) have been reported. The gonococcal pilus which is involved in bacterial attachment to host cells (21) is also an assembled filamentous structure consisting of identical pilin subunits of molecular weights which vary between 17,500 and 21,000, depending on the strain (1). The gonococcal pilin molecule has a *N*-methylphenylalanine at its N terminus followed by a stretch of highly conserved hydrophobic sequences homologous to the *Pseudomonas* pilin sequence and a disulfide bridge at the variable C-terminal end (19). Cyanogen bromide cleavage of pilin from gonococcal strain MS11 gave rise to a C-terminal disulfide bridge fragment, CNBr-3, which contained the immunodominant, type-specific antigenic determinant (20). Two strain-specific epitopes in the regions of amino acid residues 121 to 134 and 135 to 151 were located within the disulfide loop (16). Reduction and subsequent alkylation of the intrachain disulfide bond in the CNBr-3 fragment destroyed the antigenicity of this region (20). Watts et al. (24) have reacted enzymatically cleaved *Pseudomonas* pilin fragments with native PAK antipilus antiserum and have found that a fragment corresponding to the C-terminal end (residues 121 to 144) reacted positively in direct enzyme-linked immunosorbent assay (ELISA) experiments. In contrast to the gonococcal pilin, modification of the two cysteine residues in the fragment by reduction and carboxymethylation of the disulfide bridge did not abolish the antigenicity of this determinant. It has also been demonstrated in the PAK pilin that the C-terminal region is part of the pilus-binding domain and that the C-terminal disulfide bridge is important in maintaining the functionality of this binding domain (2). We have now investigated the immunogenicity of this region in the synthetic peptides [reduced and oxidized PAK(128-

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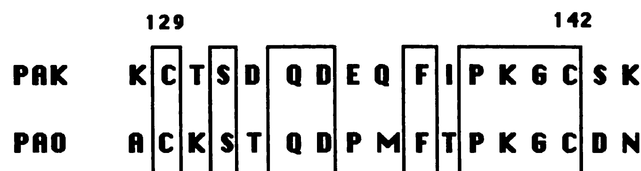

FIG. 1. Amino acid sequence of the C-terminal region of PAK and PAO pilins. Boxed regions represent homologous sequences in the two proteins.

144-OH) and PAK(A-129)(128-144)-OH] to produce antipeptide antibodies which bind to native PAK pili. The synthesized peptide sequence of the PAK pilin and the corresponding sequence of the disulfide bridge region of PAO pilin are shown in Fig. 1. The importance of the disulfide bridge to the antigenicity of this region was further investigated by using competition ELISA experiments with both native antiserum and antisera raised against the synthetic peptides. These studies are important in understanding the role of the disulfide bridge and the significance it might have in developing vaccines that would block the attachment process.

MATERIALS AND METHODS

Peptide nomenclature. Ac-PAK(128-144)-OH is the synthetic NH_2-terminal-acetylated *P. aeruginosa* K fragment comprising residues 128 to 144 with a COOH-terminal carboxyl group; 17-R1 and 17-R2 are antipeptide antisera raised against the 17-residue reduced form of PAK(128-144)-OH coupled to KLH as described below; 1 and 2 denote different rabbits; 17-01 and 17-02 are antipeptide antisera raised against the 17-residue intrachain disulfide-bridged form of PAK (128-144)-OH coupled to KLH; 1 and 2 denote different rabbits. PAK(A-129)(128-144)-OH is the peptide with an alanine substitution in place of cysteine at position 129.

Bacteria and pilus purification. The *P. aeruginosa* strains used were PAK/2Pfs and PAO/DB2. PAK and PAO pili were purified as described previously (11).

Peptide synthesis. The 17-residue peptides were synthesized by using the Merrifield solid-phase procedure on a peptide synthesizer (model 990; Beckman Instruments, Fullerton, Calif.). The carboxy-terminal lysine was coupled to a 1% cross-linked Merrifield resin (0.9 meq of Cl per g substitution; Pierce Chemical Co., Rockford, Ill.) via the cesium salt of t-butyloxycarbonyl lysine (2-chlorobenzoxycarbonyl) (3). The addition of subsequent protected amino acids and deprotection and cleavage of the peptides from the resins by using hydrogen fluoride was carried out by previously described procedures (13). The peptides were purified by reversed-phase high-performance liquid chromatography (HPLC) on a SynChropak C18 column (Synchrom, Linden, Ind.; dimensions, 250 by 10 mm [inside diameter]; flow, 2.0 ml/min) using a linear gradient in which solvent A was 0.1% trifluoroacetic acid–H_2O and solvent B was 0.1% trifluoroacetic acid–acetonitrile and a gradient rate of 1.0% solvent B/min. The intrachain disulfide bridge was formed in the peptide by air oxidation. The peptide was dissolved at a concentration of 0.1 mg/ml in an aqueous buffer (0.05 M NaH_2PO_4, pH 7.5, containing 0.5 M NaCl and 0.02 mM $CuCl_2$) and stirred overnight at room temperature (5). The oxidative process was checked by circular dichroism, in which the spectrum of the disulfide bridge was distinctively different from the reduced state (9). Using an Aquapore RP-300, C-8 reversed-phase column (220 by 4.6 mm [inside diameter]) the oxidation process was followed by using a 1% solvent B/min gradient rate and flow rate of 1 ml/min in the solvents described above.

Peptide conjugation to protein carriers. The peptides were conjugated to keyhole limpet hemocyanin or bovine serum albumin (BSA) via a linker consisting of a [^{14}C]glycine and a benzophenone cross-linking group (benzoyl benzoic acid), which was added to the peptide during synthesis while the peptide was still on a solid matrix (13). The protein (~3 mg) was first dissolved in 10 to 20 μl of water in a test tube. The protein carriers (10 mg/100 μl) were then added and mixed. Covalent attachment of the peptide to the carrier occurred following activation of the benzoylbenzoyl group by UV irradiation at 4°C for 1 h in a RPR 208 preparative reactor (Rayonet, The Southern New England Ultraviolet Co., Middletown, Conn.) equipped with RPR 350-nm lamps. Unconjugated haptens were removed by successive dialysis against 8 M urea, 100 mM ammonium bicarbonate, and 25 mM ammonium bicarbonate. The product was freeze-dried, and the peptide incorporation was determined by measuring the radioactivity incorporated per mole of carrier. Peptide/protein ratios of about 4:1 and 10:1 were obtained for the oxidized and reduced peptides, respectively, and a ratio of 5:1 was obtained for PAK(A-129)(128-144)-OH.

Immunization. Eight-week-old female Flemish rabbits were immunized with 500 μg of peptide-keyhole limpet hemocyanin conjugates dissolved in 700 μl of a 1:1 mixture of Freund complete adjuvant and phosphate-buffered saline. The injections were given subscapularly (2 × 200 μl) and intramuscularly (1 × 300 μl). Two rabbits were used for each peptide conjugate. The rabbits were boosted after 2 weeks with the same amount of peptide conjugate in incomplete Freund adjuvant. Rabbits were bled 2 weeks later, and the antibody titers were checked by ELISA.

Enzyme immunoassays. The ELISA was carried out as described previously (27) according to the principles of Voller et al. (23). The immunoglobulin G (IgG) used in competitive ELISA was purified by using a Protein A-Sepharose column (Pharmacia, Inc., Piscataway, N.J.), and the preparation of papain-digested IgG to get Fab fragments was carried out as described previously by Worobec et al. (26). The ELISA wells were coated with either a 5-μg/ml solution of pili or peptide-BSA conjugate. In these assays, 1-nM IgG or 100-nM Fab fragments were used. Readings of the alkaline phosphatase reaction on *p*-nitrophenylphosphate were taken at 405 nm.

Immunoblot assays. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out according to the Laemmli method (7) by using 15% polyacrylamide running gels. Purified pili (5 μg) and whole-cell lysate (3 × 10^7 bacteria) were used. The whole-cell lysate was prepared by boiling the PAK cells for 2 to 3 min in 100 μl of sample buffer (2.5% sodium dodecyl sulfate in 0.25 M Tris, pH 6.8) used to dissolve proteins to be loaded onto the sodium dodecyl sulfate-polyacrylamide gel. The mixture was then centrifuged on a bench-top Microfuge; 10 μl of the supernatant was removed and diluted with 15 μl of sample buffer before being loaded onto the gel. After separation, the proteins were transblotted at 0.2 A for 2 to 6 h onto nitrocellulose paper according to the method of Towbin et al. (22). Excess protein-binding capacity of the nitrocellulose sheet was blocked with a 1% gelatin solution. The sheet was then reacted with antipeptide antisera (1:250 dilution). The pilin bands were detected with an Immunoassay Kit (Bio-Rad Laboratories, Richmond, Calif.) by using a goat anti-rabbit IgG alkaline phosphatase conjugate which gave rise to

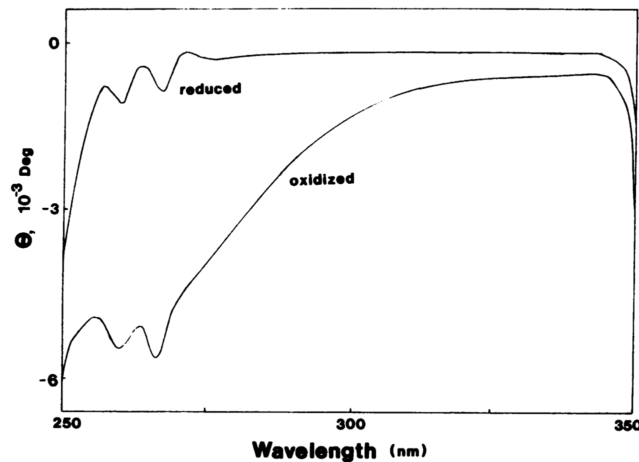

FIG. 2. Circular dichroism spectra of reduced and oxidized N_α-acetylated 17-residue peptides corresponding to the C-terminal end of PAK pilin. A 1.0 mg/ml concentration of the peptide was used in each case, and the buffers used contained 50 mM phosphate, pH 7.0. The relative ellipticity values (Θ) are expressed in millidegrees.

purple-red bands due to the reagents, *p*-Nitro Blue Tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate-toluidine salt.

RESULTS AND DISCUSSION

Peptide synthesis. The PAK benzoylbenzoyl peptide following cleavage from the resin was purified by reversed-phase high-performance liquid chromatography and air oxidized to form the intradisulfide bridge. A low peptide concentration (0.1 mg/ml) was used in the oxidation process to prevent interchain disulfide bridge formation. The air oxidation to form the disulfide bridge in the synthetic peptides could be easily monitored by reversed-phase chromatography, since the oxidized peptide eluted from the column approximately 1 min prior to the reduced form. No interchain dimer was observed by high-performance liquid chromatography after the oxidation procedure. This dimer would be expected to elute later than the reduced peptide due to the doubling of its hydrophobicity. The extent of the oxidation was also checked by circular dichroism by using the acetylated peptides Ac-PAK(128-144)-OH, which had been oxidized under the same conditions. The profiles of reduced and oxidized peptides were easily distinguishable between 250 and 350 nm (Fig. 2). Reduction of the air-oxidized peptide with 1.0 mM dithiothreitol gave rise to a profile similar to reduced peptide by circular dichroism or reversed-phase chromatography (data not shown).

Antipeptide antisera reactivities with PAK pili. The antipeptide antisera raised against reduced or oxidized PAK(128-144)-OH peptide bound to native PAK pili in direct ELISA experiments. The antipeptide antiserum raised against the reduced peptide was designated 17-R and that raised against oxidized peptide was designated 17-O. The antibody titers for each of the antisera are tabulated in Table 1. High antibody titers in the range of a 10^{-6}-fold dilution were engendered by the peptide-keyhole limpet hemocyanin immunogens. The antipeptide antibody titers were similar for both the reduced and oxidized peptides when tested against the corresponding peptide BSA conjugates. The antipeptide antisera were able to bind to native PAK pili, and titers in the range of 1.5×10^{-5} were recorded. Thus, the synthetic 17-amino-acid peptide in its native or reduced form was able to stimulate the production of rabbit polyclonal antibodies which bound to whole pilin molecules. The titers for the antisera raised against reduced or oxidized peptides were similar when assayed against native PAK pili. Thus, both peptides were equally immunogenic and antigenic.

One concern that must be addressed is the possibility that the reduced peptide coupled to the carrier protein did not remain reduced after immunization. One approach would be to carboxamidomethylate the cysteines, which would prevent formation of the disulfide bond. However, the presence of the carboxamidomethyl groups would be expected to generate antipeptide antibodies that would also recognize these groups in the peptide, and thus the antipeptide antibodies would not recognize the native protein. This is strongly supported by the antigen-antibody studies of Hodges et al. (4), who clearly demonstrated that the removal of a single methyl group can result in loss of antibody binding. We felt that the only option available was to substitute cysteine 129 at the N terminus of the peptide near the site of attachment to the carrier protein with an alanine residue. The disulfide bridge could not be formed, and the C-terminal 15 residues were identical to the native protein. Interestingly, the antipeptide antibodies to this sequence did not recognize the native PAK or PAO pili, suggesting the importance of the disulfide bond in generating antipeptide antibodies that bind the native proteins or the importance of the side chain of cysteine 129 in antibody binding.

Cross-reactivity of anti-PAK(128-144) antisera with PAO pili. When the ELISA plates were coated with PAO pili, it was found that the 17-R and 17-O antisera were able to bind and cross-react with these heterologous pili. However, only antiserum 17-O1 (Table 1) gave an endpoint titer with PAO pili similar to that obtained with PAK pili. The other three antisera titers with PAO pili were at least 10-fold lower compared with those of PAK pili. From the sequence data (Fig. 1), it can be seen that the amino acid residues in the C-terminal end of the PAK and PAO pilin proteins are only

TABLE 1. Endpoint titers of antisera raised against reduced and oxidized PAK(128–144)-OH and PAK(A-129)(128-144)-OH peptides

Antiserum[a]	Peptide-conjugate titer	Endpoint titer (±SD) (n = 3)[b]	
		PAK pili	PAO pili
17-R1	3.5×10^{-6}	1.0×10^{-5} (1.2×10^{-5})	4.15×10^{-4} (3.7×10^{-4})
17-R2	7.1×10^{-6}	1.6×10^{-5} (0.4×10^{-5})	4.50×10^{-4} (0.3×10^{-4})
17-O1	4.5×10^{-6}	1.5×10^{-5} (0.7×10^{-5})	2.0×10^{-5} (0.8×10^{-5})
17-O2	2.1×10^{-6}	2.0×10^{-5} (1.2×10^{-5})	1.3×10^{-4} (1.6×10^{-4})
PAK(A-129)	9.8×10^{-6}		

[a] R1 and R2 antisera were raised against the reduced PAK(128-144)-OH peptide, and O1 and O2 antisera were raised against the oxidized PAK(128-144)-OH peptide.
[b] The endpoint was determined as the cutoff at an A_{405} of 0.05 U.

semiconserved. From the primary amino acid sequences, surface profile predictions based on the combination of hydrophilicity, mobility, and accessibility parameters (12) were carried out, and it was shown that the C-terminal regions of both PAK and PAO pilin are surface-exposed regions. This C-terminal region of PAO pilin also contains two cysteine residues, which can form a disulfide bridge in the native molecule. The results of the direct ELISA experiments showed that the antipeptide antisera raised against the oxidized peptide were able to bind better to PAO pili than were those raised against the reduced peptide, PAK(128-144)OH. This was also shown by the competitive ELISA experiments (see below). This was not the case with PAK pili, for which antisera to reduced or oxidized peptide had similar binding activities.

Immunoblot assays. The binding of antipeptide antibodies to pili was checked by using immunoblot assays. Purified PAK and PAO pili and pili from the lysate fraction (see Materials and Methods) were used. It was found that the native antipilus antisera bound to its homologous pilus strain and also cross-reacted with its heterologous counterpart. Both antipeptide antisera raised against reduced and oxidized peptides reacted with purified pili and pili from a whole-cell preparation. It was also seen that the two different antisera, 17-R and 17-O, cross-reacted with purified PAO pili. This interaction was not as strong as that with the PAK pili, even though the same amount of material was loaded on the gel. However, antiserum 17-O1 showed equally intense pilin bands with PAK or PAO pili, consistent with the ELISA data of Table 1. Interaction of the antipeptide antisera with PAO pili from whole cells only gave faint bands. This could be due to smaller quantities of pili produced per PAO cell or a combination of reduced amount of pili and the weaker interactions between antisera and pili.

Significance of the disulfide bridge. Watts et al. (24) showed that carboxymethylated PAK(128-144) peptide reacted as well as a chymotryptic PAK(120-144) fragment (intact intrachain disulfide bridge) in a direct ELISA with native PAK antiserum. This suggested that the disulfide bridge is not important in the immunogeneity of the C-terminal region of the pilin protein. This observation was in contrast to those made for the gonococcal pilin. Schoolnik et al. (20) found that the reduction and carboxymethylation of free sulfhydryl groups resulted in the loss of antigenicity of this disulfide loop region in the native gonococcal pilin. The disulfide bridge of gonococcal pilin (MS 11) encompassing a longer stretch of amino acid residues (residues 121 to 151) contains two highly immunogenic, strain-specific epitopes of the intact protein, 121 to 134 and 135 to 151 (16). When these peptides were used to raise antipeptide antibodies, it was found that only anti-121-134 was strain specific. However, anti-135-151 bound to heterologous pili from three other gonococcal strains (17). The immune response against the peptide is distinct from that against the intact protein in the specificity of the antibodies generated against residues 135 to 151. Reactivities of monoclonal antibodies to highly variable regions in the disulfide loop blocked pilus binding to epithelial cells, suggesting that the loop region may contribute to pilus-host cell adhesion (10). Thus, an intact disulfide loop is

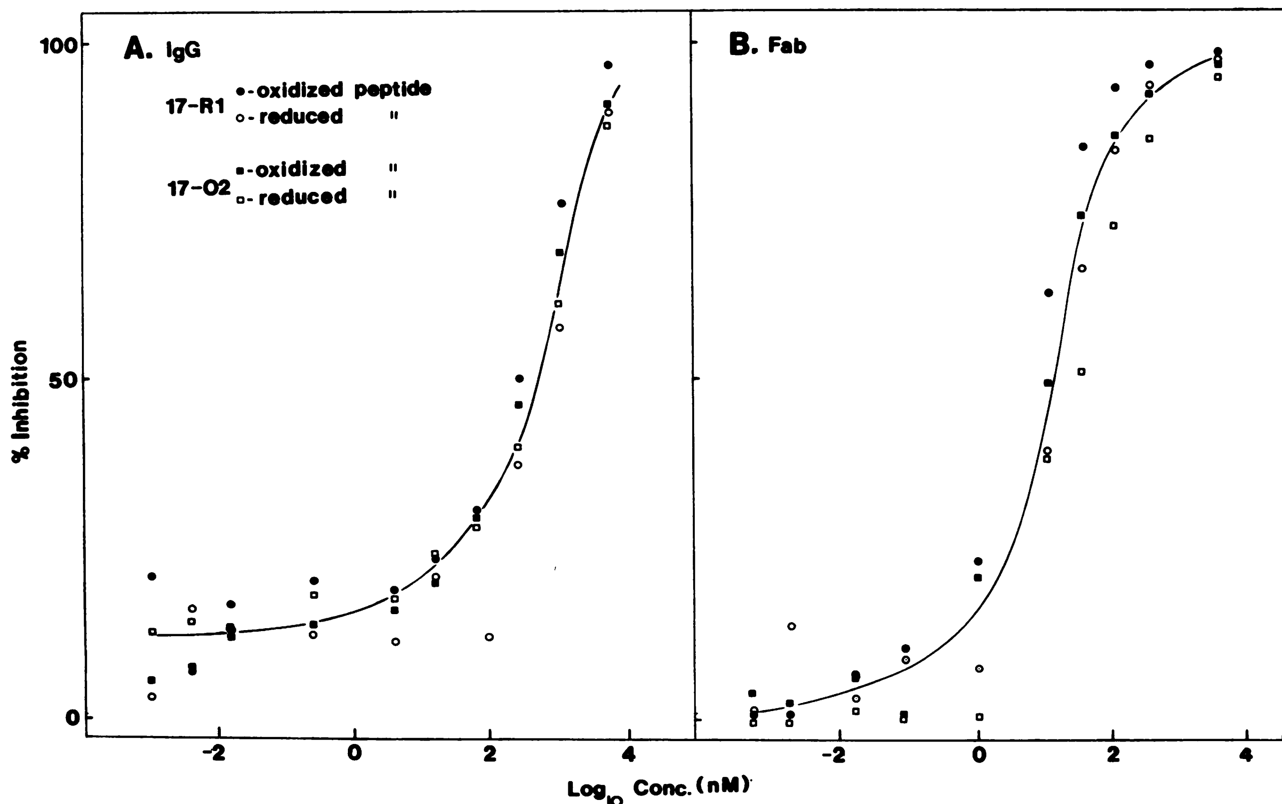

FIG. 3. Competitive ELISA experiments with IgG (1.0 nM; panel A) and Fab fragments (100 nM; panel B) of 17-R1 and 17-O2. The wells on the ELISA plate were coated with a 5-μg/ml PAK pili solution. The competing antigens used in these assays were Ac-PAK(128-144)-OH oxidized peptide (● or ■) and reduced peptide (○ or □). In both instances, a single line was fitted to the data to illustrate the similar interactions of the two different antibodies for reduced and oxidized peptides.

important for the conformational epitopes of gonococcal pili and perhaps for pilus binding to host cells.

The relative affinities of the antibodies raised against reduced and oxidized peptides for the PAK pili were determined by using indirect ELISA experiments. The antipeptide IgGs from each serum (17-R1, 17-R2, 17-O1, and 17-O2) were purified on a Protein-A-Sepharose affinity column. The wells on the ELISA plates were coated with a 5-μg/ml pilus solution, and competition assays were carried out by incubating a 1.0 nM IgG solution (final concentration) with serial dilutions of acetylated PAK(128-144)-OH reduced and oxidized peptides and also with whole PAK pili. The results are shown in Fig. 3 and 4. In each instance, when either reduced or oxidized peptide was used to compete for binding to IgG (Fig. 3A), there was no significant difference in the inhibitory dose which gave a 50% reduction in binding of the peptides for either IgG raised against the reduced or oxidized 17-residue peptide. In the case of reduced peptides, incubations were carried out in the presence of β-mercaptoethanol to keep them in a reduced state. The antibodies that were raised against the oxidized peptide were also able to recognize the reduced peptide and vice versa. This suggested that the native conformation with the disulfide chain was not critical in the antigenicity of this region. This was illustrated again when whole native pili was used as the competing antigen with IgG raised against either the reduced or oxidized peptide as shown in Fig. 4A. When the 17-R or 17-O IgG was preincubated with native PAK pili, the indirect ELISA experiments showed that all four sets of data points could be fitted by a single line, illustrating the similarities in the antibody interactions of the two different antibodies with PAK pili. Another set of experiments was carried out with papain-digested IgG, i.e., the Fab fragments, to show that the bivalency of IgG did not affect the results (Fig. 3B). The inhibition patterns of all four different Fab fragments gave similar results, and these patterns corresponded to those of the IgG molecules.

The binding of native PAK antipilus antiserum to the C-terminal 17-residue peptide was also studied by using competition ELISA experiments. In this case, the wells of the ELISA plates were coated with the reduced or oxidized peptide conjugate. The peptide conjugate was used instead of native PAK pili because the C-terminal region is not the immunodominant region and the percentage of antibodies in the native antipilus antiserum which bind to this region is less than 10%. Any changes in the absorbance readings due to the inhibition by the peptides would be small and hence, if pili were used to coat the wells, these small changes would be difficult to detect in the presence of all the other epitopes on the pili which would be bound by other antibodies in the polyclonal serum. Native antiserum (final concentration, 1:8,000) was preincubated with oxidized or reduced peptide before being added to the peptide conjugates. The incubation with the reduced peptide and coating of the wells with reduced peptide conjugate were carried out in the presence of β-mercaptoethanol. The results are shown in Fig. 5A and 5B. When the wells were coated with oxidized peptide conjugate, competition with reduced or oxidized peptide showed similar inhibition patterns with almost identical 50% inhibitory dose values. The converse experiment with wells coated with reduced peptide conjugates gave similar results, with reduced and oxidized peptides competing equally well for binding to antibodies from native PAK antipilus antiserum.

An interesting result was obtained when PAO pili were used as the competing antigen in these competitive ELISA

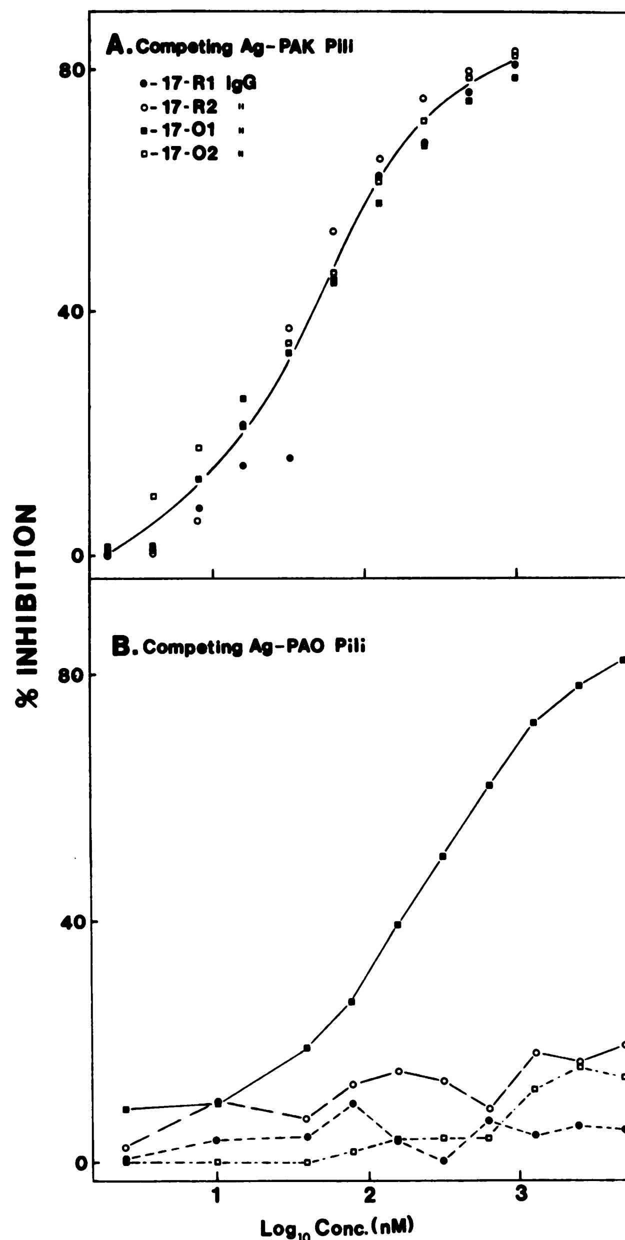

FIG. 4. Competitive ELISA experiments with native PAK (A) and PAO (B) pili. The wells were coated with a PAK pili solution (~5 μg/ml), and all four antipeptide antibodies (1.0 nM) were assayed. ●, 17-R1; ○, 17-R2; ■, 17-O1; □, 17-O2. In panel A, a single line was able to fit all four sets of data points, illustrating the similarities in their interactions with PAK pili.

experiments with IgG molecules and with the wells coated with PAK pili. Earlier results with the direct ELISA and immunoblot experiments have shown that all four of the antipeptide antisera raised against either reduced or oxidized peptide were able to bind to PAO pili. Only one of the IgG raised against the oxidized peptide, 17-O1, was saturated and competed out by the PAO pili (Fig. 4B). The other three antipeptide antisera showed little or no preference for binding to PAO pili. With the 17-01 IgG, the 50% inhibitory dose when PAO pili were used as the competitor was 4.36-fold less compared with when PAK pili were used as the com-

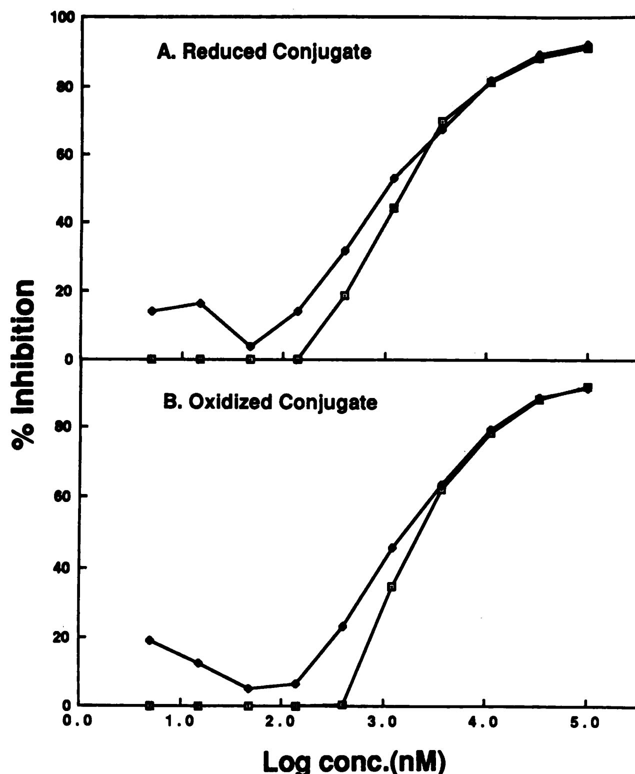

FIG. 5. Competitive ELISA with anti-PAK pilus antiserum and with the wells coated with a 5-μg/ml solution of BSA-PAK(128-144)-OH reduced (A) and BSA-PAK(128-144)-OH oxidized (B) peptides. The concentration of native antiserum used was a 1: 8,000-diluted solution. The conjugates were competed with reduced (⊡) and oxidized (◆) Ac-PAK(128-144)-OH peptides.

petitor. From the amino acid sequence of the disulfide bridge region, both PAK and PAO showed some sequence homology (9 of the 17 residues). There are five of six residues which are conserved in one region. It is possible that on binding to the ELISA wells and to the nitrocellulose paper, denaturation of the pilin subunits may enable all four antipeptide antisera to recognize and bind to this conserved region with high affinities in the ELISA and immunoblot experiments. In a competitive ELISA test, in which the PAO pili were in solution, the pili were in their native state and only the 17-O1 IgG which recognizes this native conformation would bind to cause the inhibition that was observed. That only one of the two antisera raised against oxidized peptide (17-O1 and not 17-O2) bound to PAO pili could have been attributed to different specificities of those antibodies prepared in different rabbits. Although all four antisera reacted equally well with the PAK pili in the competitive ELISA (Fig. 4A), it was interesting that only the 17-O1 IgG bound well enough to PAO pili to give a binding curve. The kinds of interactions observed in the binding to the C-terminal region of the PAK pili were equally strong, as shown by the similarities in the binding curves in the competitive ELISA, but the abilities to bind to PAO pili were different. Even though both the 17-O1 and 17-O2 antisera were raised against the same oxidized peptide, only one of them recognized and bound PAO pili. As noted earlier, there are five of six amino acids which are conserved in both pilins. The 17-O1 IgG may recognize this epitope, and the 17-O2 may recognize a separate epitope.

Hence, the antibodies in the polyclonal sera raised against PAK pili are able to recognize and bind to the C-terminal 17-mer peptide in the presence or absence of an intrachain disulfide bridge. This suggests that the antigenic determinants in this region of the pilin molecule are mostly linear epitopes when native pili are used as the immunogen. Thus, this disulfide bridge is not critical in the antigenicity of this region, unlike in the case of the gonococcal pilin. Although the presence of an intact disulfide bridge was not important in the antigenicity of the *Pseudomonas* pilin, it was found that only one of the antipeptide antisera which was raised against the oxidized peptide was able to bind and cross-react well enough with PAO pili to give good inhibition curves in an indirect ELISA experiment. Hence, there may be other structural features in the native oxidized state which allowed us to raise antibodies that cross-reacted well with other *Pseudomonas* strains. This cross-reactivity would be important in looking for a suitable vaccine candidate for *P. aeruginosa*. We are still screening a number of peptides which have high surface potential and raising antipeptide antisera to look at the binding to *Pseudomonas* pili and cross-reactivity with pili from other strains. The sera in this study and subsequent sera will be tested for their abilities to block *Pseudomonas* pilus-mediated attachment.

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