PURIFICATION AND PROPERTIES OF M PROTEIN EXTRACTED FROM GROUP A STREPTOCOCCI WITH PEPsin: COVALENT STRUCTURE OF THE AMINO TERMINAL REGION OF TYPE 24 M ANTIGEN*

By EDWIN H. BEACHEY, GENE H. STOLLERMAN, EDNA Y. CHIANG, THOMAS M. CHIANG, JEROME M. SEYER, AND ANDREW H. KANG

(From the Veterans Administration Hospital and the Departments of Medicine and Biochemistry, University of Tennessee Center for the Health Sciences, Memphis, Tennessee 38104)

The type-specific M protein on the surface of group A streptococci renders the organisms resistant to the phagocytic defenses of the nonimmune host. In the immune host, antibodies which are opsonic for streptococci in vitro and protective against streptococcal challenge infections in vivo are directed toward the M protein antigen located on the surface of virulent organisms. Efforts to immunize humans against streptococcal infections have been frustrated by toxic reactions to almost any streptococcal product introduced into the human host (1). "Purified" M protein vaccines have apparently contained tenaciously associated nontype-specific antigens which have accounted, at least in part, for the toxic effects of various M protein preparations (2, 3).

Since conventional methods have failed to separate the nontype-specific antigens from the type-specific M antigen, various new methods for the extraction and purification of M protein have been tried by a number of different investigators (4-10). Fox and Wittner (4) used a treatment with caustic soda or hydroxylamine to extract a molecular species of M protein that was larger and apparently more immunogenic than the conventional HCl-extracted M protein. In our hands (5), a purified preparation of an alkaline extract of type 24 streptococci was found to be free of immunotoxic or cross-reactive properties while retaining the type-specific M determinant as judged by its anti-opsonic effect in blocking the homologous M-anti-M opsonic system. The purified antigen, however, lacked immunogenicity in rabbits even though the mol wt was 36,500 daltons. Fischetti et al. (6) extracted an immunogenic M protein from type 6 streptococci and found that the purified protein appeared to be composed of subunits less than 10,000 daltons in size; the subunits precipitated type-specific M antiserum but lacked anti-opsonic activity. Radiolabeling studies of viable streptococci suggested that the subunits constitute the structural elements of a larger, 33,000 dalton M protein molecule(s) which possessed type-specific anti-opsonic activity. Russell and Facklam (7) extracted M protein with 6.0 M guanidine and further purified it by hydroxyapatite column chromatography. Whether or not the guanidine-extracted material contained cross-reactive antigens, however, was not reported. Vosti and Williams (8) extracted whole type 12 group A streptococci with cyanogen bromide. After further purification they obtained a relatively homogenous fraction.

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PROPERTIES OF STREPTOCOCCAL M PROTEIN

which, however, contained both type-specific and nontype-specific antigens. Havlicek (9) extracted M protein by sonic disruption of whole streptococci followed by ammonium sulfate precipitation and isoelectric focusing. The purified material contained two precipitating moieties but produced only traces of nontype-specific reactions. Widdowson (10) compared the isoelectric-focusing patterns of opacity factor (OF)-negative and OF-positive streptococci. They found that the ability to separate the non-type-specific antigens from the type-specific M antigen varied among the different serotypes tested.

Recent studies in our laboratories (11) have demonstrated that purified M proteins lose much of their toxic properties without losing immunogenicity after they have been digested under suboptimal conditions with pepsin. We took advantage of the finding and have subsequently prepared immunogenic protein from mild pepsin digests of whole group A streptococci (12). In the present study we further purified the pepsin-extracted M protein (pep M) to homogeneity by ion-exchange chromatography and isoelectric focusing. We present data to indicate that the type-specific M (TSM) antigen which was obtained in high yields, was readily separable from cross-reactive, toxic material, and that the purified material remained immunogenic producing high titers of type-specific opsonic and precipitating antibodies in immunized rabbits.

We also present the amino acid sequence of the first 29 amino acids from the amino-terminus. Only one new amino acid at each step of Edman degradation confirmed the homogeneity of the purified pep M24. The NH₂-terminal amino acid was found to be valine.

Materials and Methods

Culturing and Extraction of Group A Streptococci. Group A streptococci were cultured in Todd-Hewitt broth (THB), intermittently passaged through mice or fresh human blood (13) to stimulate optimal M protein production, and stored lyophilized or frozen in 20% serum broth at -70°C as previously described (2). The organisms were grown in THB for 16 h in 60-liter batches, sedimented by centrifugation, washed twice in 0.02 M phosphate-0.15 M NaCl, pH 7.4 (PBS) and once in 0.067 M phosphate, pH 5.8. The organisms were finally resuspended in three volumes of the latter buffer containing 50 μg/ml of pepsin (Worthington Biochemical Corp., Freehold, N. J.) and incubated for 1 h at 37°C. Digestion was stopped by adding sufficient 7.5% NaHCO₃ to raise the pH to 7.5. The streptococci were sedimented by centrifugation at 10,000 g (DuPont Instruments, Sorvall Operations, Newtown, Conn.) for 20 min and the cells were extracted a second time with pepsin under identical conditions. The supernates were combined, filtered through a 0.45 μm membrane filter (Millipore), dialyzed against PBS, lyophilized, redissolved in 10 ml distilled water, further dialyzed against distilled water, and relyophilized. The lyophilized material was redissolved in PBS at a concentration of 10 mg/ml and precipitated with 30% saturated and 60% saturated ammonium sulfate. The ammonium sulfate precipitates were redissolved and digested with ribonuclease and lyophilized as previously described (12). M protein was also prepared by the conventional method of extracting washed streptococci with HCl at 95°C and was partially purified by ribonuclease digestion and ammonium sulfate precipitation by the method of Lancefield and Perlmann (14) as previously described (15).

Ion-Exchange Chromatography. The ribonuclease-digested, pep M was redissolved in 0.05 M Tris-HCl buffer at pH 7.5 and applied to a 1.6 × 32 cm column of quaternary amino ethyl (QAE)-Sephadex A50 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) equilibrated with the same

1 Abbreviations used in this paper: CF, complement fixation; LTA, lipoteichoic acid; NTSM, nontype-specific M-associated; OI, opsonization inhibition; OF, opacity factor; PBS, 0.02 M phosphate-0.15 M NaCl, pH 7.4; pep M, pepsin-extracted M protein; PHA, passive hemagglutination; ppt'n, precipitinogen; QAE, quaternary amino ethyl; SDS, sodium dodecyl sulfate; THB, Todd-Hewitt broth; TSM, type-specific M.
buffer. The protein was eluted in a stepwise fashion with increasing concentrations of NaCl. The immunoreactive protein peaks were pooled, lyophilized, redissolved and dialyzed against distilled water, and relyophilized.

 Isoelectric Focusing. 10- and 50-mg samples of the pep M purified by QAE-Sephadex chromatography were dissolved in a 5% Ampholyte solution (LKB Produkt AB, Bromma, Sweden) and pumped into 110-ml or 440-ml isoelectric-focusing columns (LKB Instruments, Inc., Rockville, Md.) (5), and electrofocused at 450 V until the flow of current approached zero. Usually 48 h for the 110-ml column or 72 h for the 440-ml column was required. Fractions were collected and analyzed for pH, absorbance at 280 nm, and immunoreactivity with M24 antiserum. Active peaks were pooled, exhaustively dialyzed against distilled water, and lyophilized.

 Analytic Methods. Protein content was estimated by the method of Lowry et al. (16). Hexose was determined by the method of Dubois et al. (17) and methyl pentose by the cysteine-sulfuric acid method of Dische and Shettles (18).

 Polyacrylamide gel electrophoresis was performed in 7% gel according to the method of Davis (19) as previously described (11). Molecular weights of the purified pep M were estimated by electrophoresis in 10% polyacrylamide gels containing sodium dodecyl sulfate (SDS) according to the method of Weber and Osborn (20).

 Amino acid analyses were performed as previously described (21). 1-mg samples were hydrolyzed in constant boiling 6 N HCl in an atmosphere of nitrogen for 24 h at 108°C. The samples were then analyzed with a Beckman 121 automatic amino acid analyzer (Beckman Instruments, Inc., Fullerton, Calif.) by a single column technique (22).

 Automatic Edman degradations were performed with a Beckman Sequencer (Model 890C; Beckman Instruments, Inc.) according to the principles first described by Edman and Bevans (23). The protein-Quadrol (0.01 M) program (122974) of Beckman Instruments was employed (24). The phenylthiohydantoin-amino acids were identified by high pressure liquid chromatography (25). Arginine derivatives were identified as their parent amino acid by amino acid analysis after hydrolysis with 65% HI (26). Repetitive yields of 97% were obtained during the automated Edman degradation.

 Streptococcal Antisera. Antisera to whole group A streptococci were prepared by immunizing rabbits with heat-killed or ultraviolet-irradiated streptococci as previously described (5). Standard, M type-specific antisera were obtained from the Center for Disease Control, Atlanta, Ga.

 The immunogenicity of the purified pep M was studied by injecting rabbits intracutaneously with a 100 µg (dry weight) dose of M protein emulsified in complete Freund's adjuvant (27) as previously described (5). Rabbits were bled at 2-wk intervals for 8 wk after immunization. Sera were collected and stored at 4°C. Rabbits were also immunized with purified lipoteichoic acid (LTA) prepared from a strain (1RP41) of group A streptococci, and precipitated with methylated bovine serum albumin according to the method of Fiedel and Jackson (28) as previously described (29).

 Streptococcal Opsonization Tests. In vitro timed phagocytosis tests for type-specific opsonic M antibody were performed as previously described (5, 30). The test mixtures consisted of 0.4 ml fresh heparinized (10 U/ml) human blood, 0.05 ml of a standard suspension of phagocytosis-resistant streptococci, and 0.05 ml of test serum. The ratio of streptococcal units per leukocyte was approximately 10:1. The percentage of neutrophilic leukocytes which had ingested streptococci (percent phagocytosis) was estimated by microscopic examination of stained smears prepared from a drop of test mixture at 15 and 30 min of incubation. The opsonic antibody titer is expressed as the reciprocal of the highest twofold dilution of test serum that in three separate tests of each serum produced phagocytosis of 10% or greater at 30 min of incubation at 37°C while phagocytosis of the same organisms in the presence of nonimmune rabbit serum was less than 2% in each test. M antisera exhibiting titers greater than 1:4 all produced phagocytosis in the range of 40-70% when tested undiluted. The results of phagocytosis tests were confirmed by indirect bactericidal tests performed as previously described (2). Type specificity was assured by failure of antisera to opsonize heterologous M serotypes of streptococci.

 Titration of TSM Antigen. Capillary precipitin tests of serial twofold dilutions of M protein in PBS were performed by the method of Swift et al. (31) as previously described (2). The reciprocal of the highest dilution of a 1 mg/ml solution of M protein producing a type-specific precipitate was designated the TSM precipitinogen (ppt'n) titer.

 Tests of the inhibition of type-specific opsonization were performed as previously described in
SAMPLES OF OPSONIC ANTISERUM DILUTED IN PBS TO THE HIGHEST DILUTION PRODUCING 40-60% OXONIZATION WERE ABSORBED WITH SERIAL TOWFOLD DILUTIONS OF M PROTEIN SOLUTIONS. AFTER REMOVING PRECIPITATES BY CENTRIFUGATION THE ABSORBED AND UNABSORBED ANTISERUM SAMPLES WERE USED TO OXONIZE HOMOLOGOUS TYPE STREPTOCOCCI THAT WERE RESISTANT TO PHAGOCYTOSIS. IT MUST BE STRESSED THAT IN THIS SYSTEM THE OXONIZED STREPTOCOCCI ARE THOROUGHLY WASHED BEFORE ADDING THEM TO FRESH HUMAN BLOOD SAMPLES. WASHING REMOVES EXCESS ANTIGEN AND ANTIGEN-ANTIBODY COMPLEXES THAT MIGHT INHIBIT PHAGOCYTOSIS NONSPECIFICALLY EITHER BY DEPLETING THE FRESH HUMAN BLOOD OF COMPLEMENT OR BY MEDIATING CYTOTOXIC EFFECTS UPON POLYMORPHONUCLEAR LEUKOCYTES AS PREVIOUSLY REPORTED (2). THE RECIPROCAL OF THE HIGHEST DILUTION OF A 1 MG/ML SOLUTION OF THE ABSORBING ANTIGEN THAT PREVENTED OXONIZATION WAS DESIGNATED THE TSM OXONIC INHIBITORY (OI) TITER. THE TYPE SPECIFICITY OF PREOPSONIC INHIBITION WAS ASSURED BY ABSORBING HETERLOGOUS ANTISERA WITH THE M PREPARATIONS. IN NO CASE WERE THE PREOPSONIC EFFECTS OF THE ABSORBED HETERLOGOUS ANTISERA REDUCED AGAINST THEIR RESPECTIVE SEROTYPES OF STREPTOCOCCI.

Tests for Nontype-Specific M(NTSM)-Associated Antigens. Complement fixation (CF) tests were performed by a microtechnique as previously described (21). NTSM antigen titers were expressed as the reciprocal of the highest dilution of a 1 mg/ml solution of M protein preparation that produced 50% lysis of antibody-coated sheep erythrocytes in the presence of rabbit antiserum against whole type 30 streptococci. In addition to M30-specific antibody the serum had a known high titer of NTSM antibody. The type 30 antiserum was analyzed for oposnic activity against type 24 streptococci to confirm the absence of M24-specific antibody which might have interfered with the test for NTSM.

Tests for the presence of LTA antigen were performed as previously described (21, 32). Human erythrocytes were incubated with serial dilution of a 1 mg/ml solution of the M protein preparations, washed, and mixed with a dilution of antiserum containing high agglutinating titers of anti-LTA. Control erythrocytes were incubated with serial dilutions of a 1 mg/ml solution of purified LTA or with PBS. The reciprocal of the highest dilution of antigen capable of modifying erythrocytes was expressed as the passive hemagglutination (PHA) titer of LTA antigen. To assure the absence of teichoic acid antigen that may have lost its erythrocyte-sensitizing activity, the antigen preparations were tested for their ability to inhibit the PHA activity of anti-LTA against LTA-modified erythrocytes as previously described (32).

Delayed skin reactivity of the various M protein fractions were tested in adult guinea pigs as previously described (5, 15). 100-μg doses of the antigen preparations were dissolved in 0.1 ml PBS and injected intradermally at a shaved area of the back. Diameters of induration were recorded at 24 h.

Results

The crude pepsin extracts of type 24 M protein were fractionated first by ammonium sulfate precipitation. Most of the immunoreactive material precipitated at 30% saturation (Table 1). Although immunoreactive material was precipitated also at 60% the fraction was more heterogeneous (see below). Therefore, the 30% fraction was employed in the following studies.

Ion-Exchange Chromatography and Isoelectric Focusing of Pep M24. After ribonuclease digestion, the ammonium sulfate (30% saturation) precipitated material was applied to a column of QAE-Sephadex equilibrated with 0.05 M Tris-HCl and was eluted in step-wise fashion with increasing concentrations of NaCl (Fig. 1). The type-specific immunoreactive material eluted with 0.3 M NaCl.

The protein in the 0.3 M NaCl eluate was pooled, dialyzed against distilled water, and lyophilized. It was then redissolved in the appropriate ampholine-sucrose solution and loaded onto an isoelectric-focusing column (see Materials and Methods). A typical elution pattern is depicted in Fig. 2. Most of the type-specific immunoreactive material was located in a broad ultraviolet (280 nm) absorbing area with a peak pH of 7.15. The opsonic inhibitory activity was
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TABLE I
Immunoreactivity of Ammonium Sulfate Fractions of Crude pep M24

<table>
<thead>
<tr>
<th>Ammonium Sulfate Fraction</th>
<th>TSM antigen titers*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Precipitinogen</td>
</tr>
<tr>
<td>30% Saturation</td>
<td>1:256</td>
</tr>
<tr>
<td>60% Saturation</td>
<td>1:128</td>
</tr>
<tr>
<td>Supernate</td>
<td>&lt;1:2</td>
</tr>
</tbody>
</table>

* Antigen titers are expressed as the highest dilution of a 1 mg/ml solution producing a positive reaction.

![Graph](https://via.placeholder.com/150)

**Fig. 1.** Stepwise elution of pep M from a QAE-Sephadex column. 50 mg of pep M24 was applied to a 1.6 x 32 cm column equilibrated with 0.05 M Tris-HCl, pH 7.0 (buffer). Samples were eluted with stepwise increases in NaCl concentrations as indicated. Samples were pooled into fractions I, II, and III as indicated. Most of the immunoreactive material was located in fraction I. Ppt'n, precipitation with type-specific M24 antiserum; +, positive reaction; -, negative reaction.

located in the same peak indicating that the type-specific opsonogen and ppt'n are closely associated in pep M24. The final yield of electrofocused M protein was 84 mg/60 liters of streptococcal culture (Table II).

**Immunoochemical Purity and Properties of Pep M24.** Methylpentose, which is associated with cell walls, decreased from 1.4 to less than 0.5%, and NTSM antigens, estimated by CF in the presence of antiserum to type 30 streptococci, decreased from a titer of 1:256 to less than 1:2 while the type-specific antigenic activity increased from a ppt'n titer of 1:128 to 1:1,024 and an anti-opsonic titer of 1:4 to 1:64 (Table II). The purified material in concentrations of 1 mg/ml was unable to spontaneously modify group O erythrocytes to enable them to agglutinate in the presence of antiserum against purified LTA; it also was unable to inhibit anti-LTA in passive hemagglutination tests of LTA-modified erythrocytes, indicating the absence of LTA or teichoic acid antigens in the purified M protein.
Fig. 2. Isoelectrofocusing of pep M24. 50 mg of M protein was applied to a 440 ml electrofocusing column containing 5% ampholyte solution composed of 1 part pH 3-10 to 9 parts pH 4-6 ampholines in a sucrose gradient. The tubes in the first peak were pooled as indicated (FI). Ppt’n, precipitation with type-specific M24 antiserum; +, positive reaction; −, negative reaction.

Table II

<table>
<thead>
<tr>
<th>M protein preparation</th>
<th>Yield per 60-liter batch</th>
<th>Methyl-pentose</th>
<th>NTSM antigen titers*</th>
<th>TSM Antigen titers*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
<td>%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude pepsin extract</td>
<td>615</td>
<td>1.4</td>
<td>1:256</td>
<td>1:128</td>
</tr>
<tr>
<td>Ammonium sulfate fraction, 30% saturation</td>
<td>150</td>
<td>1.2</td>
<td>ND</td>
<td>1:256</td>
</tr>
<tr>
<td>QAE-Sephadex fraction, 0.3 M NaCl eluate</td>
<td>120</td>
<td>&lt;0.5</td>
<td>ND</td>
<td>1:1,024</td>
</tr>
<tr>
<td>Isoelectric fraction, pH 7.15</td>
<td>84</td>
<td>&lt;0.5</td>
<td>&lt;1:2</td>
<td>1:1,024</td>
</tr>
</tbody>
</table>

* See footnote in Table I.

Previous studies in our laboratories have indicated that guinea pigs serve as a model for toxic skin reactions to M protein preparations in man (15). Guinea pigs were injected intradermally with pep M preparations obtained at various steps of purification. Five of five guinea pigs injected with the crude pepsin extract developed strong (average diameter of 14 mm of induration) delayed-type skin reactions (Table III). With increasing purification however, the frequency and degree of reactivity decreased; the electrofocused pep M was unreactive in the five guinea pigs tested.

The immunogenicity of the highly purified pep M was studied in rabbits. 100-μg doses of purified pep M were emulsified in complete Freund’s adjuvant and injected intracutaneously into rabbits. Sera obtained at 2-wk intervals demon-
### Table III

**Delayed-Type Skin Reactions in Unimmunized Adult Guinea Pigs to Intradermal Injections of Pep M24 Preparations**

<table>
<thead>
<tr>
<th>Pep M24 preparation</th>
<th>No. of skin reactions/no. of guinea pigs tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>5/5 (14)*</td>
</tr>
<tr>
<td>Ammonium sulfate (30% saturation)</td>
<td>5/5 (9)</td>
</tr>
<tr>
<td>QAE-Sephadex (0.3 M NaCl eluate)</td>
<td>3/5 (9)</td>
</tr>
<tr>
<td>Electrofocused pep M24</td>
<td>0/5 (0)</td>
</tr>
</tbody>
</table>

* Numbers in parentheses indicate average diameter of induration among guinea pigs showing positive skin reactions.

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**Fig. 3.** Opsonic antibody response in three rabbits to a single injection of 100 µg of purified pep M24 emulsified in complete Freund's adjuvant. O, rabbit no. 1; Δ, rabbit no. 2; ●, rabbit no. 3. None of the sera were able to opsonize heterologous type 30 streptococci.

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**Fig. 4.** Immunodiffusion analysis of the sera at 6 and 8 wk revealed a single precipitin arc of identity between a conventional crude acid extract of M24 and the highly purified pep M24, indicating that the immunizing pep M antigen lacked the contaminating precipitating antigen(s) often present in crude acid extracts of M protein. The lack of cross-reactive antibodies was further confirmed by the inability of the sera to passively hemagglutinate LTA-modified erythrocytes or to fix complement using a partially purified HCl extract of type 30 M protein as antigen. These results indicated that the electrofocused pep M24 was immunogenic and immunochemically homogeneous.

**Physicochemical Properties of Pep M24.** Electrophoresis in 7% polyacrylamide gels demonstrated a single Comassie blue staining band in the electrofocused fraction as compared to the multiple bands present in the fractions obtained during earlier steps of purification (Fig. 5). Electrophoresis in SDS
TABLE IV

Indirect Bactericidal Tests of Sera of Rabbits Immunized with pep M24

| Rabbit serum*: collected at 2-wk intervals after immunization with pep M24 | No. of colonies of type 24 streptococci after 3 h growth in text mixture |
|---|---|---|
| Preimmune | 2,415 | 1,115 | 245 |
| 2 wk | 1,515 | 635 | 65 |
| 4 wk | 25 | 0 | 0 |
| 6 wk | 0 | 0 | 0 |
| 8 wk | 370 | 30 | 0 |

* The sera of the two rabbits showing the highest opsonic antibody responses (see Fig. 3) were pooled for these experiments.

FIG. 4. Agar gel diffusion test of antiserum (anti-pep M) from rabbit immunized with purified pep M24 against the homologous immunizing antigen (pep M) and a crude HCl extract (acid M) of type 24 streptococci.

TABLE V

Presence of TSM and Absence of NTSM Antibodies in Sera of Rabbits Immunized with Purified pep M24

<table>
<thead>
<tr>
<th>Rabbit serum</th>
<th>NTSM antibodies CF titers with M30</th>
<th>PHA titers with LTA-RBC</th>
<th>Precipitin reactions*</th>
<th>Opsonic titers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-pep M24</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rabbit no. 1</td>
<td>&lt;1:2</td>
<td>&lt;1:2</td>
<td>++++</td>
<td>1:256</td>
</tr>
<tr>
<td>no. 2</td>
<td>&lt;1:2</td>
<td>&lt;1:2</td>
<td>++++</td>
<td>1:256</td>
</tr>
<tr>
<td>no. 3</td>
<td>&lt;1:2</td>
<td>&lt;1:2</td>
<td>++</td>
<td>1:32</td>
</tr>
<tr>
<td>Anti-M24 (CDC)</td>
<td>1:512</td>
<td>1:256</td>
<td>++++</td>
<td>1:128</td>
</tr>
</tbody>
</table>

* Capillary precipitin reactions with standard hot acid extracts prepared from type 24 streptococci.

Polyacrylamide gels showed a single band of protein (Fig. 6) with a mobility consistent with an average mol wt of 33,500 daltons (Fig. 7).

Amino acid analysis demonstrated that the most abundant amino acid was alanine followed by glutamic acid, lysine, leucine and aspartic acid (Table VI). A small amount (2.7 residues per 100 amino acids) of phenylalanine was the only detectable aromatic amino acid. The absence of half-cysteine indicates that...
Disulfide cross-links are probably not involved in the structure of pep M24. The pep M contains approximately 2 methionine residues per 100 amino acids. Assuming a mol wt of 33,500, each M protein molecule should contain between six and seven residues of methionine and, therefore should be susceptible to degradation into smaller specific peptides by cyanogen bromide. Preliminary studies in our laboratory (unpublished observations) have indicated that cyanogen bromide cleaves the pep M into seven peptides.

**Amino Acid Sequence of the Amino Terminal Region of Pep M24.** Automated Edman degradation yielded the sequence of the first 29 amino acids of the amino terminal region of pep M24 (Fig. 8). The amino terminal amino acid was found to be valine. Only one new amino acid was detected at each step of Edman degradation, confirming the homogeneity of the purified pep M24.

**Discussion**

It has become apparent that the purified TSM protein antigen of group A streptococci is often closely associated with nontype-specific moieties that are shared among several different serotypes of M protein (2, 3, 33, 34). Widdowson
et al. (33) called the nontype-specific antigens "M-associated protein" or "MAP" because of the sensitivity of the antigens to trypsin digestion. In our studies we have referred to these antigens as simply NTSM because at least a portion of the antigens associated with M proteins appeared to be nonprotein in nature (21). Later we found that the nonprotein antigen is LTA (32).
### Table VI

**Amino Acid Analysis of Electrofocused pep M24**

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Residues/100 amino acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>20.8</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>17.8</td>
</tr>
<tr>
<td>Lysine</td>
<td>13.1</td>
</tr>
<tr>
<td>Leucine</td>
<td>12.4</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>11.3</td>
</tr>
<tr>
<td>Serine</td>
<td>7.0</td>
</tr>
<tr>
<td>Threonine</td>
<td>5.6</td>
</tr>
<tr>
<td>Arginine</td>
<td>3.2</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>2.7</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>2.3</td>
</tr>
<tr>
<td>Glycine</td>
<td>2.1</td>
</tr>
<tr>
<td>Methionine</td>
<td>1.7</td>
</tr>
<tr>
<td>Valine</td>
<td>0.2</td>
</tr>
</tbody>
</table>

**Vol - Ala - Thr - Arg - Ser - Glu - Thr - Asp - Thr - Ser -**

**Glu - Lys - Val - Gin - Glu - Arg - Ala - Asp - Ser - Phe -**

**Glu - Ile - Glu - Asn - Asn - Thr - Leu - Lys - Thr**

**FIG. 8.** Covalent structure of the NH2-terminal region of type 24 M protein extracted from streptococci with pepsin.

Heretofore, it has been difficult to separate the NTSM antigen from the TSM antigen in the molecular complex of M protein. Our previous studies had shown that digestion of purified conventional HCl extracts of M protein with pepsin at pH 5.0 partially abolished the NTSM antigens while preserving the antigenicity of TSM antigens (11). The optimal pH was then found to be 5.8 for extraction of TSM antigen from whole streptococci. The present study demonstrates that although the initial pepsin extract contains both NTSM and TSM antigens, the TSM antigen is readily separable in high yields by further purification including ion-exchange chromatography and isoelectric focusing.

There were several significant differences between the properties and composition of the purified pep M in the present study and the electrofocused fraction of M protein prepared from an alkaline extract during our previous studies (5). The purified alkaline extract of type 24 M protein has a mol wt of 36,000 daltons, and although it was a strong inhibitor of type-specific opsonization it failed to precipitate type-specific M antiserum. Moreover, it lacked immunogenicity in rabbits. In contrast, the purified pep M was shown in this study to have a mol wt of 33,500 daltons, to inhibit type-specific opsonization, and to precipitate homologous M antiserum, each of the latter two at relatively low concentrations of purified pep M. Perhaps most importantly the highly purified pep M24 was fully immunogenic in rabbits producing high titers of type-specific opsonic M anti-
body. Neither the purified alkaline-extracted M nor pep M possessed detectable NTSM antigens or nontype-specific skin reactivity in adult guinea pigs. Our previous studies have demonstrated that adult guinea pigs develop natural delayed hypersensitivity to NTSM antigens present in most streptococcal M protein preparations (15). Frederick et al. (35) recently demonstrated naturally acquired delayed hypersensitivity to LTA in guinea pigs which may account for at least a portion of the delayed skin reactivity of the crude M protein preparations.

In addition to the above differences, the two type 24 M protein preparations also differed considerably in their amino acid compositions. Whereas the most abundant amino acids in the alkaline M extract had been found to be glycine followed by aspartic acid, glutamic acid, proline, and lysine, the predominant amino acids in pep M were shown to be alanine followed by glutamic acid, lysine, leucine, and aspartic acid; the pep M had no detectable proline and only small amounts of glycine. These differences may account for the differences in the immunochemical properties of these two type 24 M protein preparations.

Our preliminary studies of the covalent structure of pep M protein indicate that valine rather than alanine (36) was the amino terminal amino acid. The appearance of only one new amino acid at each step of Edman degradation confirmed the homogeneity of the purified pep M24. The presence of an estimated six to seven methionine residues per molecule of M protein lend promise for attempts to cleave the molecule to its smallest active peptide with cyanogen bromide. The possibility that the relatively few phenylalanine residues, the only detectable aromatic amino acid, may be strategically placed in the molecule raise the prospects that chymotrypsin digestion may also result in smaller immunoreactive peptides.

These studies should provide an initial attack on the primary molecular structure of the type-specific moiety of M protein involved in protective immunity against group A streptococcal infections. Clarification of the structural features of the M protein molecule that render it serotype specific as compared to those structural features that account for serotype nonspecificity are important goals both in regard to the understanding of the pathogenesis of streptococcal infections and their sequelae and in regard to developing safe and effective vaccines against group A streptococcal infections.

**Summary**

M protein was extracted from type 24, group A streptococci with pepsin at pH 5.8 and was further purified by ammonium sulfate precipitation, ribonuclease digestion, ion-exchange chromatography, and isoelectric focusing. The purified pepsin extract of M (pep M) protein was shown to be free of nontype-specific immunoreactivity in (a) complement fixation tests with heterologous M antiserum, (b) skin tests in normal adult guinea pigs, and (c) passive hemagglutination tests for the presence of lipoteichoic acid sensitizing or antigenic activity. The pep M24 was highly immunogenic; two of three rabbits developed opsonic antibody titers of 1:256 and the third a titer of 1:32 6 wk after a single injection of 100-μg doses of pep M24 emulsified in complete Freund's adjuvant. The antisera lacked nontype-specific antibodies and produced single precipitin lines in agar
gel diffusion tests against crude HCl extracts of the homologous M protein. Thus, the type-specific antigenic determinant(s) of type 24 M protein appears to be separable from immunotoxic, cross-reactive antigens without loss of immunogenicity in rabbits.

The mobility of pep M24 upon electrophoresis in 10% sodium dodecyl sulfate polyacrylamide gel was consistent with an average mol wt of 33,500 daltons. Amino acid analysis demonstrated a predominance of alanine, followed by glutamic acid, lysine, leucine, and aspartic acid. Pep M24 contained an estimated six to seven methionine residues and approximately ten phenylalanine residues per molecule. No other aromatic amino acids were detected. Automatic Edman degradation of pep M24 yielded the sequence of the first 29 amino acids (the amino terminal amino acid being valine) of the amino terminal region of the molecule. The detection of only one new amino acid at each step of Edman degradation confirmed the homogeneity of the purified pep M24.

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References
PROPERTIES OF STREPTOCOCCAL M PROTEIN


