AMINO TERMINAL SEQUENCE OF TYPE 3 STREPTOCOCCAL M PROTEIN EXTRACTION PRODUCTS

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The amino terminal hypervariable part of the M protein molecule was chosen as a basis for the preparation of a synthetic peptide vaccine against group A streptococci. As part of the mapping of various serological types the main products of extraction of type M *Streptococcus pyogenes* with limited pepsin (Pep) hydrolysis at pH 5·5 and with phage-associated lysin (PAL) were sequenced. Two entirely different sequences were obtained. The sequence of PAL M 3 shows the absence of the α-helical potential in the shorter N-terminal region as is characteristic of the N-terminus of the M protein molecule. The main product of limited hydrolysis Pep M3 (pH 5·5), which shows the presence of the α-helical potential from the very amino terminal residue of its chain, does not involve most likely the proper N-terminus of the M protein. Extraction with pepsin under conditions of very limited proteolysis (pH 5·8) yielded a fragment with N-terminal sequence identical with that of PAL M3 (extracted nonproteolytically).

As part of our investigation of M proteins of various types of *Streptococcus pyogenes* aimed at the preparation of a synthetic peptide vaccine we examined in the present study type M3 M protein. From the viewpoint of type-specific immunity to group A streptococcal infections the most important part of the M protein molecule is that which is located close to its N-terminus. Evidence has been obtained that peptides corresponding to the N-terminal part of the M protein molecule can produce protective antibodies[1]. The knowledge of the N-terminal sequences of M proteins of several *Streptococcus pyogenes* types (refs[2,3]) provided a possibility of comparing these data with partial sequences of preparations of the M3 type.

EXPERIMENTAL

Streptococcus pyogenes type 3 No. 44/55NCCTC was cultivated in Todd–Hewitt broth (Difco) overnight at 37°C. The bacteria were separated from the culture medium by centrifugation.

The preparation of fragments of type 3 proteins was effected by peptic hydrolysis according to ref.[4]. The bacteria were suspended in 50 mM phosphate buffer at pH 5·5 or 5·8. Pepsin was added to the bacteria (16 mg per 100 g of bacteria dry weight) and the suspension was stirred 60 min at 37°C. Subsequently the pH of the enzymatic digest was adjusted to pH 8·0 and the digest was centrifuged. The supernatant was saturated to 0·7 saturation with ammonium sulfate. The precipitated proteins were centrifuged off, dissolved in 50 mM phosphate buffer at pH 8·0 and desalted on a column of Sephadex G-25 equilibrated with 20 mM-NH_4HCO_3 and lyophilized.

Preparation of type M3 *proteins by phage lysin* (N-acetylmuramyl-L-alanine amidase) was carried out as described in ref.[5]. The bacteria were suspended in 50 mM phosphate buffer at pH 7. The enzyme was added in the form of phage lysate and the pH of the reaction mixture was adjusted to pH 7·3. Incubation was then continued for 18 h at 37°C. The reaction mixture was centrifuged and the supernatant saturated with ammonium sulfate to 0·7 saturation. The precipitate which had formed was centrifuged off, dissolved in water and dialyzed against 20 mM acetate buffer at pH 4·5. The clear dialysate was applied to a CM-cellulose column equilibrated with 20 mM acetate buffer at pH 4·5. Proteins were displaced by stepwise elution using buffer 1, 20 mM sodium acetate buffer at pH 4·5, buffer 2, 10 mM sodium acetate, pH 5·5, and buffer 3, 1M sodium acetate at pH 8· The material eluted by buffer 2 and 3, showing the highest activity against homologous antibodies (antibodies to the same M protein type) when assayed by radial immunodiffusion, was subjected to further purification by affinity chromatography.

Affinity chromatography was effected on a column of adsorbent prepared by bonding of fibrinogen to AH-Sepharose[4,6]. In this chromatographic procedure[7] the material dissolved in 0·1M Tris–HCl, buffer, pH 8·0, containing 0·5M NaCl, 0·1% ethylene glycol and 0·02% sodium azide, was adsorbed to the column. The desorption of the material bound to immobilized fibrinogen was effected by elution with 50 mM citrate buffer at pH 3·0 containing 6M urea, 0·35M NaCl and 10% ethylene glycol. The material which had not been retained in the column at pH 8·0 as well as the material eluted from the column at pH 3·0 was desalted on the Sephadex G-25 column and lyophilized.

The molecular weight determination was carried out by electrophoresis in 10% polyacrylamide gel in the presence of dodecyl sulfate using the modification described in ref.[8]. Human serum albumin (68 000), ovalbumin (43 000), pepsin (35 000), chymotrypsinogen (25 000), hemoglobin (17 000) and lysozyme (14 000) were used as molecular weight standards.

Radioligand assays (RLA) were carried out according to the modified procedure described in ref.[9] by incubation of selected samples in microwells arranged in titration series. The free bonds in the microwell plates were blocked by the reaction with 0·2M PBS in 1% BSA. After overnight incubation with a constant concentration of 125I-labeled fibrinogen the microwells were washed and bound radioactivity was assayed in a Packard Crystal Gamma Counter.

Gel permeation chromatography was carried out with a Superose™ 12 column (Pharmacia) equilibrated in 50 mM NH_4HCO_3 or in 75% formic acid. The flow rate was 0·1 ml/min. The course of the chromatography was monitored by simultaneous measurement of the absorbance of the effluent at 220 and 230 nm. When chromatography was carried out in 50 mM NH_4HCO_3 some samples had to be applied as solutions in 8M urea because of their decreased solubility.

Sequence analysis was carried out with 0·1–0·3 mg of sample in Applied Biosystems Model 470A Protein Sequencer using program 03 RPTH. The amino acids degraded were determined as phenylthiohydantoins by HPLC on a Beckman Ultrasphere ODS column[10].

Amino acid analyses were carried out with 0·5 mg samples hydrolyzed 20 h in 5·6M-HCl at 110°C. Aliquots of the hydrolysates were analyzed in Durrum D-500 amino acid analyzer.

RESULTS AND DISCUSSION

Isolation and Purification of PAL M3

The action of phage lysin on the *Streptococcus pyogenes* type M3 cells resulted in the release of a highly complex material. A small part of the latter was capable of binding to fibrinogen immobilized on AH-Sepharose[4]. The main portion of the material bound to the column showed a mobility corresponding to 65 kD when analyzed by electrophoresis in polyacrylamide gel in the presence of sodium dodecyl sulfate. When this material was chromatographed on the Superose™ 12 column the presence of a major component was observed corresponding in its elution volume to serum albumin. This material showed after desalting all the characteristics of the M protein. When assayed by RIA it was able to bind a high titer of antibodies to streptococci of the homologous type and a considerably lower titer of antibodies of the heterologous type. In the RLA assay using fibrinogen the binding of labeled fibrinogen to this material was demonstrated. The material, however, was not completely homogeneous according to the results of N-terminal sequence analysis and was therefore purified by gel permeation chromatography on a column of Superose™ 12 in 75% formic acid. The purified product was homogeneous when assayed electrophoretically and by sequence analysis. It will be referred to in what follows as PAL M3. The 40-residue amino terminal sequence of PAL M3 was determined with the exception of residues 36 and 39 which we could not identify:

```
1                                   10
Asp-Ser-Arg-Ser-Val-Asn-Gly-Glu-Phe-Pro-Arg-His-Val-Lys-Leu-Lys-
          20                                                  30
Asn-Glu-Ile-Glu-Asn-Leu-Leu-Asp-Gln-Val-Thr-Gln-Leu-Tyr-Asn-Lys-
                    40
His-Asn-Asp-X-Tyr-Gln-X-Tyr
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When the sequence was determined by the phenylisothiocyanate method the degradation products of the first two cycles were contaminated by a compound which emerged from the HPLC column in a volume corresponding to that of PTH-alanine. We did not try to determine whether this compound is alanine liberated from a part of the cell wall together with the protein or whether its elution volume coincides with that of PTH-Ala. In spite of this drawback proteolytic hydrolysis by phage lysin is in our opinion a procedure of choice to obtain material with the intact N-terminal M protein sequence.

Isolation and Purification of Fragment Pep M3

The character of the material obtained by pepsin treatment of the type 3 *Streptococcus pyogenes* cells depends on the pH of the reaction mixture[4]. When the digestion is

carried out at pH 5·5, two major polypeptides of M_r = 15 and 25 kD are released into solution by pepsin. The smaller of these two peptides only can bind to immobilized fibrinogen. A polypeptide of M_r = 40 kD is the only product when the hydrolysis is performed at pH 5·8.

All three products which are liberated under the different conditions were isolated by gel permeation chromatography. The immunological assay of these products showed that all three possess characteristics typical of M proteins, both as regards the ability of specific binding to antibodies to homologous streptococci and also as regards decreased ability to bind to heterologous types. All three products were shown to bind to labeled fibrinogen in the RLA assay. This feature was also observed with the 25 kD fragment which did not bind to immobilized fibrinogen. The amino terminal sequences of various lengths of all three polypeptides were determined: fragment Pep M3 (15 kD), sequence of 42 residues, fragment Pep M3 (25 kD), 30-residues, fragment Pep M 3 (40 kD), which was obtained by pepsin hydrolysis at pH 5·8, sequence of 40 residues.

The N-terminal regions of polypeptides Pep M3 (15 kD) and Pep (25) kD show a completely identical amino acid sequence of the regions assayed. For this reason. the longer N-terminal sequence of peptide Pep M3 (15 kD) only is shown:

```
1                                   10
Glu-Lys-Asp-Asp-Leu-Glu-Lys-Glu-Val-Lys-Glu-Leu-Lys-Glu-Lys-Ile-
            20                                              30
Asp-Lys-Lys-Glu-Lys-Glu-Tyr-Gln-Asp-Leu-Asp-Lys-Asp-Phe-Lys-Leu-
                                40
Ala-Lys-Gln-Gly-Tyr-Val-Leu-Lys-Asp-Lys
```

α-Helical potential shows the region Glu(1)-Ala(33) beginning with N-terminal residue. The heptad periodicity of this region is illustrated in Fig. 1. The N-terminal region of polypeptide Pep M3 (40 kD), obtained by pepsin hydrolysis at pH 5·8, was also determined (32 residues). This part of the amino acid sequence is entirely identical to the sequence of polypeptide PAL M3 released by phage lysin. The relation between this part of the structure and the amino termini of other M proteins determined is apparent from the alignment in Fig. 2.

CONCLUSIONS

Limited proteolysis of streptococci by pepsin is the method usually used for the preparation of M protein fragments for sequence studies on the N-terminal parts of their molecules. In some M proteins types, however, the N-terminal regions contain bonds readily cleavable by pepsin. Hence, fragments comprising a larger N--terminal region of the original M protein molecule cannot be obtained by this procedure. We have made an effort in this study to obtain the N-terminal part of

the M protein molecule by extraction of streptococci with an enzyme which cannot hydrolyze peptide bonds. Phage lysin (N-acetyl-muramyl-L-alanine amidase) cleaves the peptidoglycan of the bacterial cell walls and a release of intact M protein molecules may thus be expected. When we compared the amino acid sequences of polypeptides prepared by the usual peptic hydrolysis under different conditions with the sequence of the protein liberated by phage lysin we observed that one polypeptide only of M_r = 40 kD, which was liberated at a pH least favorable for peptic digestion (pH 5·8), showed the N-terminal sequence identical with that of the product liberated by phage lysin. It follows from a comparison of the secondary structures of the N-terminal regions of various M proteins made by the method of Chou and Fasman

	a	b	c	d	e	f	g	
+								Glu -
2	Lys	Asp	- Asp	Leu	Glu	- Lys	- Glu	-
9	Val	Lys	- Glu	Leu	Lys	- Glu	- Lys	-
16	Ile	Asp	- Lys	Lys	Glu	- Lys	- Glu	-
23	Tyr	Gln	- Asp	Leu	Asp	- Lys	- Asp	-
30	Phe	Lys	- Leu	Ala	Lys	- Gln	- Gly	-
37	Tyr	Val	- Leu	Lys	Asp	- Lys	-	

FIG. 1

Heptad periodicity in N-terminal sequence of Pep M 3 (pH 5·5). Inner positions e and g lie next to the core whereas the amino acid side chains in outer positions b, c, and f are distant from the core and available for interaction with surrounding molecules[12]

M 5 (ref.[2])			T	V	T	R	G	T	I	S	D	P	Q	R	A	K	E	A	L	D	K	Y	E	L	E	N	H	D	L	K
M 6 (ref.[11])	R	V	F	P	R	G	T	V	E	N	P	D	K	A	R	E	L	L	N	K	Y	D	V	E	N	S	M	L	Q			
M 24 (ref.[3])		V	A	T	R	S	Q	T	D	T	L	E	K	V	Q	E	R	A	D	K	F	E	I	E	N	N	T	L	K			
M 3					D	S	R	S	V	N	G	E	F	P	R	H	V	K	L	K	N	E	I	E	N	L	L	D	Q			

M 5	T	K	M	E	G	L	K	T	E	N	R	G	L	K																			
M 6	A	N	N	G	K	L	T	T	E	N	N	N	L	T																			
M 24	L	K	N	S	D	L	S	F	N	N	K	A	L	K																			
M 3	V	T	Q	L	Y	N	K	H	N	D	X	Y	Q	Y																			

FIG. 2

Comparison of amino acid sequences of various M protein types with N-terminal sequence of M protein released by treatment of type 3 *Streptococcus pyogenes* with phage associated lysin

that the absence of the α-potential is a characteristic feature of this part of the molecule. This finding is in agreement with the sequence of regions PAL M3 and Pep M3 (pH 5·8). On the other hand the fragments which were obtained by pepsin hydrolysis at pH 5·5 correspond in their coiled-coil arrangement to the middle parts of the M protein molecules[11]. The identity of the N-terminal sequence of Pep M3 (pH 5·8) with the sequence of PAL M3 indicates that there was no additional cleavage of peptide bonds in the N-terminal region of the M protein molecule. Khandke and coworkers[12] concluded in their study on the amino acid sequence of fragment Pep M 49 that, judging by the N-terminal sequences, the M protein of streptococci causing nephritis clearly differs in its N-terminal regions from identical parts of the molecule of rheumatogenic type streptococci M proteins. This conclusion has received additional evidence from a comparison with our partial sequences of fragment Pep M 1 (ref.[13]) which, however, do not represent the proper N-terminus of the M protein molecule[14]. The analysis of the amino acid sequences of fragments PAL M3 and Pep M3 (pH 5·8) presented here does not support such a conclusion, even though the M 3 type belongs to the nephritogenic types. The partial amino acid sequence of the M3 type M protein will serve as a basis for the preparation of synthetic antigens.

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