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Complete localization of disulfide bonds in GM2 activator protein

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Abstract

Lysosomal degradation of ganglioside GM2 by hexosaminidase A requires the presence of a small, non-enzymatic cofactor, the GM2-activator protein (GM2AP). Lack of functional protein leads to the AB variant of GM2-gangliosidosis, a fatal lysosomal storage disease.

Although its possible mode of action and functional domains have been discussed frequently in the past, no structural information about GM2AP is available so far. Here, we determine the complete disulfide bond pattern of the protein. Two of the four disulfide bonds present in the protein were open to classical determination by enzymatic cleavage and mass spectrometry. The direct localization of the remaining two bonds was impeded by the close vicinity of cysteines 136 and 138. We determined the arrangement of these disulfide bonds by MALDI-PSD analysis of disulfide linked peptides and by partial reduction, cyanylation and fragmentation in basic solution, as described recently (Wu F, Watson JT, 1997, *Protein Sci 6*:391–398).

Keywords: disulfide bonds; GM2 activator protein; GM2 gangliosidosis AB variant, MADLI-MS; PSD

In vertebrate cells, the catabolism of sphingolipids with short carbohydrate chains is accomplished by lysosomal exoglycosidases, but requires the presence of small non-enzymatic cofactors, the sphingolipid activator proteins, SAPs or saposins (Sandhoff et al., 1992). Four of these activators, SAPs A-D, are closely related in structure and derive from a common precursor protein by proteolytic processing, the gene of which maps to human chromosome 10 (Fürst et al., 1988; O'Brien et al., 1988, Nakano et al., 1989). The fifth activator, GM2 activator protein (GM2AP), a lysosomal glycoprotein of 162 amino acids, is encoded by a gene on human chromosome 5 (Burg et al., 1985). It is essential for the degradation of GM2 (GalNAcβ1-4-(NeuAcα2-3)Galβ1-4Galβ1-1Cer) by β-hexosaminidase A (Hex A; β-N-acetyl-D-hexosaminidase; EC 3.2.1.52), and loss of its function leads to the rare AB-variant of Tay-Sachs disease. Clinically, this variant closely resembles the classical Tay-Sachs disease, where GM2, GA2, and related glycosphingolipids accumulate in neuronal tissues, leading to cell decay, severe neurological dysfunction, and finally to death of the affected individual (Sandhoff et al., 1992).

The genomic structure of GM2AP has been characterized to about 95%, identifying four exons (Klima et al., 1991). Full length cDNA clones have been isolated and characterized (Klima et al., 1991; Xie et al., 1991, Nagarajan et al., 1992). Its amino acid sequence has been deduced from the cDNA structure and has been confirmed by Edman degradation (Fürst et al., 1990).

The protein has been made available in large amounts by recombinant expression and refolding from *Escherichia coli* inclusion bodies (Klima et al., 1993; Wu et al., 1996a), and recently from expression in the baculovirus system without the necessity of refolding (T. Lemm, O. Bartelsen, & K. Sandhoff, unpubl. obs.).

In the last years, several hypotheses about the mode of action of the GM2 activator protein and the localization of its functional domains, such as the NeuAc binding site, its hydrophobic pocket, and the site of interaction with Hex A have been discussed (Wu et al., 1996b; Smiljanic-Georgijev et al., 1997). In the absence of any structural information, none of these hypotheses could be definitely supported.

For final understanding of the complex function of GM2AP, complete structural information will be necessary. In this work we elucidate the disulfide structure of GM2AP, thereby reducing the number of folding possibilities severely and presenting the first conformational information about this protein.

Results

The GM2 activator protein was expressed in insect cells using the baculovirus expression system (T. Lemm & K. Sandhoff, unpubl. obs.). SF21 cells were infected with a recombinant virus contain-

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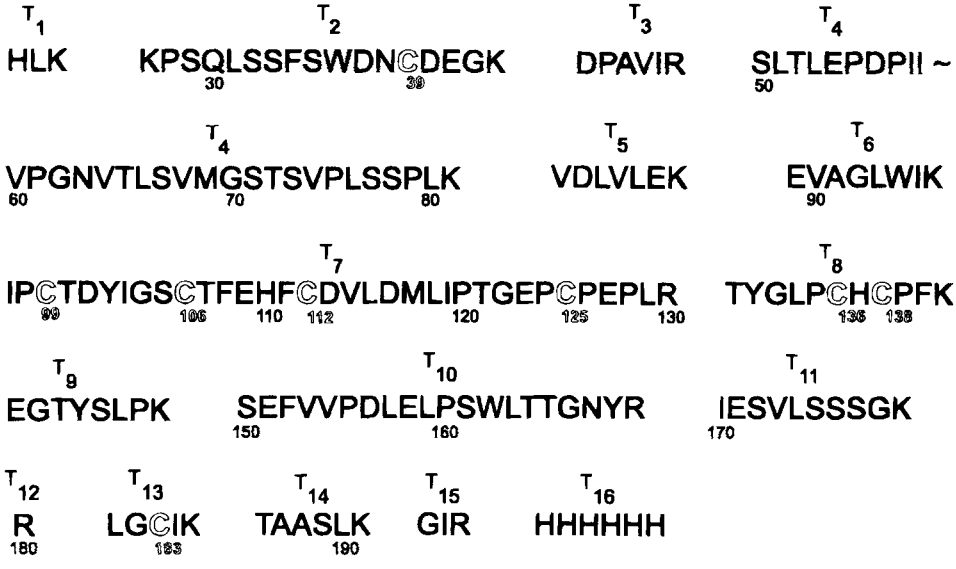

Fig. 1. Amino acid sequence of GM2AP from baculovirus expression. Possible tryptic cleavage sites are marked by blanks.

ing the GM2AP cDNA encoding for the GM2A-precursor protein and a C-terminal His_6-tag. The protein was purified from the medium of infected cells by immobilized metal chelate affinity chromatography as described elsewhere (Hochuli et al., 1988).

The activity of the recombinant GM2AP was found to be similar to GM2AP purified from human Gaucher spleen (70 AU/mg protein; 1 AU = 1 nmol ^{3}HGM2/U β-Hexosaminidase A * min) (Conzelmann & Sandhoff, 1978). The protein was shown to be homogeneous by MALDI-TOF MS and the molecular mass was determined to be 20,532 ±3 Da, consistent with a glycosylation of $(Hex)_3(HexNAc)_2(Fuc)_1$ and four disulfide bonds (theoretical mass 20,531 Da). This result also confirms the N-terminus after proteolytic removal of the signalling peptide to be His 24, as predicted previously (Glombitza et al., 1997).

Tryptic cleavage

The protein was subjected to trypsin digestion. Figure 1 shows the complete amino acid sequence of baculo-GM2AP with the predicted tryptic cleavage pattern. Figure 2 shows the MALDI-TOF mass spectrum of the entire tryptic digest, with peptide assignments given on individual peaks. Calculated and experimentally determined masses of the peptides are listed in Table 1. Note that the tryptic cleavage site at Lys 43 was selectively missed, resulting in a peptide T2/3 of mass 2,579.8. This peptide is linked to peptide T13 of mass 532.7, resulting in a peptide of total mass of 3,110.2 (average mass, Table 1), thus proving the existence of a disulfide bond between Cys39 and Cys183. Similarly, peptides T7 (4 Cys) and T8 (2 Cys) appear as a linked peptide of mass 5,070.8. This excludes the possibility of an internal disulfide bond between Cys136 and Cys138. Tryptic cleavage at Arg130 is proved by the addition of 18 Da in comparison with the uncleaved peptide.

The peptides were isolated by reverse-phase HPLC (Fig. 3). The MALDI spectrum of peptide T2/3 + T13 showed the total mass of 3,109.5 (monoisotopic mass) as well as the masses of the composing peptides (Table 2). The observation of the combined mass of both peptides proves the existence of a disulfide bond between Cys39 and Cys183. The synchronous appearance of their individual masses results from on-target- or gas phase cleavage of the S-S-bond, a well known phenomenon in MALDI-MS (Patterson & Katta, 1994).

V8 cleavage

Peptide T7 + T8 was isolated by RP-HPLC and digested by protease V8, thus splitting the peptide backbone at Glu109, separating T7 into two peptides containing two Cys each. The MALDI-MS spectrum showed two peptides, S1 (amino acid 97–109) with mass 1,446.3, corresponding to a peptide with an oxidized disulfide linkage between Cys99 and Cys106 (the reduced form would appear two daltons higher), and S2 (aa 110–130) + T8 (amino acid 131–141) of mass 3,643.7, containing two disulfide bonds (Table 3, Fig. 4).

The determination of the arrangement of the last two disulfide bonds was not possible by classical proteolytic cleavage and mass

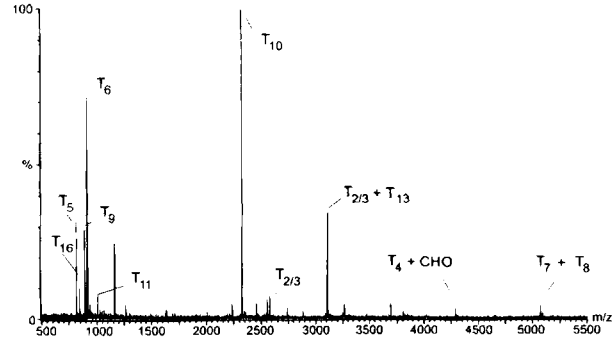

Fig. 2. MALDI-mass spectrum of the tryptic map of GM2AP expressed in the baculovirus system. DHB was used as matrix. Tryptic fragments are assigned according to Figure 1. (CHO: carbohydrate side chain:$(Hex)_3(HexNAc)_2(Fuc)_1$.)

Table 1. *Theoretical and experimental masses of tryptic peptides of baculo GM2AP*

Peptide	Theoretical mass[c]	Experimental mass[a,c]
T5	816.0	815.7
T16	841.9	842.2
T9	895.0	894.6
T6	916.1	916.4
T11	1,007.1	1,007.7
T10	2,324.6	2,324.7
T2/3	2,580.8	2,580.2
T2/3 + T13	3,111.5	3,111.2
T4 + CHO[b]	4,289.8	4,288.7
T7 + T8	5,073.9	5,071.8

[a]Masses in Da, average mass deviation < 500 ppm.
[b]CHO: carbohydrate side chain $(Hex)_3(HexNAc)_2(Fuc)_1$.
[c]Average mass.

analysis, since no protease exists which would cleave T8 between Cys136 and Cys138. We, therefore, used two different and independent methods for analyzing the arrangement.

PSD (Postsource decay) analysis

Peptide S2 + T8 was isolated by reversed-phase HPLC and cleaved further by Asp N to obtain three individual peptides linked by two disulfide bridges. Under the conditions used, cleavage also occurred on the N-terminal side of Glu, thus resulting in separation of S2 into four peptides, D1 (aa 110–112), D2 (aa 113–115), D3 (aa 116–122), and D4 (aa 123–130). After reversed-phase HPLC purification, a peptide of mass 2,606.9 was isolated, corresponding to D1 + D4 + T8 (Fig. 5). The MALDI-MS spectrum of this peptide shows the mass of the intact disulfide linked peptide, as well as signals at m/z 2,203.6 (D4 + T8), 1,670.0 (D1 + T8), and 940.2 (D4) (Table 4). The ions at 2,606.9, 2,203.6, and 1,670.0 were consecutively selected by ion gating and subjected to PSD

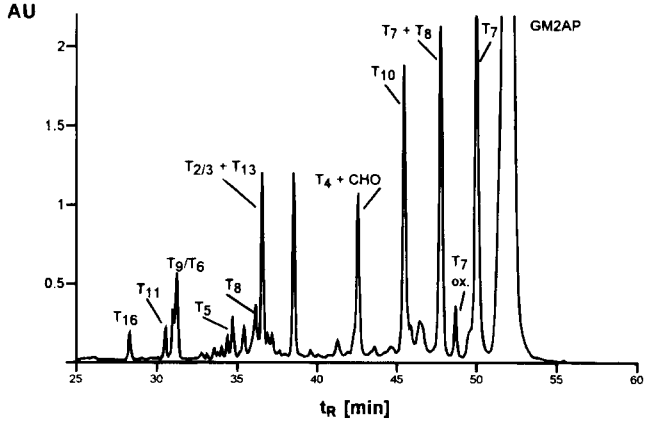

Fig. 3. C18-RP-HPLC separation of GM2AP subjected to tryptic digestion. Separation was achieved on a Vertex Nucleosil C18 column using a mixture of 70% acetonitrile, 30% isopropanol, and 0.06% TFA as eluent and running a linear gradient from 5 to 70% in 45 min. (CHO: carbohydrate side chain.)

Table 2. *MALDI-MS of isolated peptide T2/3 + T13*

Fragment	Theoretical mass	Experimental mass[a]
T2/3 + T13	3,109.5	3,109.4
T2/3	2,579.2	2,579.0
T13	5,33.3	—
T13 + Na	5,55.3	554.6

[a]Masses in Da, average mass deviation < 500 ppm.

analysis. The results are summarized in Table 5. Signals that are diagnostic for the disulfide linkage are those consisting of D1 or D4 and a fragment of T8 that resulted from cleavage of the backbone between Cys136 and Cys138. Those would be b6 and b7 as N-terminal fragments containing Cys136 and y″4 and y″5 as C-terminal fragments containing Cys138. The PSD spectrum of the ion at m/z 2,606.9 (Fig. 6) shows four such signals. Fragments y″4 and y″5 of T8 appear linked to D1, indicating a disulfide bond between Cys112 and Cys138. No signals deriving from a b6 fragment of T8 are detected, but fragment b7 of T8 appears to be linked to D4 and to the internal amino acid fragment PC, which can only derive from D4, containing a PCP sequence. As fragmentation N-terminal to Pro is usually very prominent (Kaufmann et al., 1994), PC should be a major fragment of D4. These results point toward the existence of a disulfide bond between Cys125 and Cys136. The PSD data of the partially opened peptides at 2,203.6 and 1,670.0 confirm this assignment (Table 5).

Partial reduction, cyanylation, and fragmentation

A method recently published by Wu and Watson (1997) provides a new possibility for analyzing disulfide linkages and is capable of discriminating cysteines in close vicinity. This method employs partial reduction of disulfide linked proteins or peptides by tris(2-carboxyethyl)phosphine hydrochloride (TCEP) at pH 3.0 and cyanylation of the free cysteines by 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP). After separation of the partially reduced and cyanylated isomers by reversed-phase HPLC, cleavage on the N-terminal side of cyanylated cysteines is achieved in alkaline solution, followed by reduction of the remaining disulfide bonds. One obtains specific fragments depending on the disulfide bridge opened in each isomer.

As the separation of the partially reduced species of the entire activator protein proved difficult, the protein was cleaved by protease V8, resulting in a S5 peptide (aa 110–142), which contained the two cystines in question (Fig. 7). The peptide was isolated by reversed-phase HPLC, partially reduced and cyanylated. In con-

Table 3. *Theoretical and experimental masses of the peptides obtained by S. aureus V 8 digestion of T7 + T8*

Peptide	Theoretical mass	Experimental mass[a]
S1	1,446.6	1,446.3
S2 + T8	3,642.7	3,643.7

[a]Masses in Da, average mass deviation < 500 ppm.

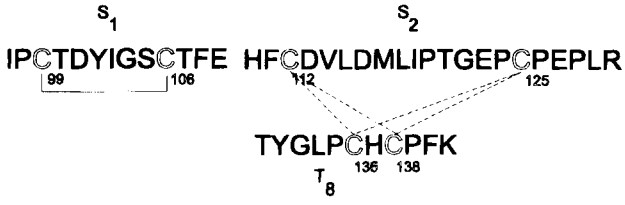

Fig. 4. Amino acid sequence of the peptides obtained by *S. aureus* V8 digestion of T7 + T8. Putative disulfide bonds are marked by broken lines, the bond between Cys 99 and Cys 106 is marked by a solid line.

Table 4. *Theoretical and experimental masses of the peptides obtained by Asp N digestion of S2 + T8*

Fragment	Theoretical mass	Experimental mass[a]
D1 + T8	1,668.7	1,669.2
D1 + D4 + T8	2,606.2	2,606.3
D4 + T8	2,203.0	2,203.3
D4	940.5	940.2

[a]Masses in Da, average mass deviation < 500 ppm.

trast to Wu and Watson, we found a 20–40 fold molar excess of TCEP over cystines to be necessary for obtaining good yields of partially reduced peptide. Interestingly, the two partially reduced isomers seemed to be more polar than the non-reduced peptide, since they eluted earlier in the HPLC separation (Fig. 8).

After isolation, the two isomeric partially reduced and cyanylated peptides were analyzed by MALDI-TOF MS. This showed cyanylation to be incomplete, since singly and doubly cyanylated species were present in each peak. We, therefore, expected to find partially cleaved fragments as well as doubly cleaved fragments and products of the side-reaction (β-elimination of thiocyanate) after cleavage in 1 M NH_4OH. This was confirmed in the MALDI-TOF analysis (Table 6, Fig. 9).

Several of the peptides possessing a C-terminus produced in the cleavage reaction showed an unusual isotopic pattern, suggesting a partially amidated C-terminus. This is consistent with the conditions of the cleavage (1 M NH_4OH) and will result in the monoisotopic mass appearing one dalton below the theoretical mass (Table 6).

The fragments confirmed the assignment of the disulfide bonds to be located between Cys125–Cys136 (isomer 1) and Cys112–Cys138 (isomer 2) as previously determined by PSD.

Discussion

The knowledge of disulfide bridge structure is an important prerequisite for structural predictions by molecular modeling and for analysis of functional domains. In this study, we have investigated the structure of disulfide linkages in GM2 activator protein expressed in the baculovirus system. Since the structural unit Cys-His-Cys was not amenable to the classical method of disulfide analysis by digestion and product analysis, we analyzed this arrangement by two different and independent methods involving mass spectrometry and protein chemistry. Post Source Decay (PSD) analysis of disulfide linked peptides for the analysis of disulfide bonds has been reported for the first time very recently in an independent report (Gorman et al., 1997). Although the method may not yield totally conclusive results, indication for the arrangement of disulfide bonds can be obtained in a fast and inexpensive way. The method of partial reduction presented by Wu and Watson proved to be a viable alternative for disulfide analysis, capable of discriminating cysteines in close vicinity.

The total determination of the disulfide linkages yields the results presented in Table 7.

Analyzing GM2 activator protein refolded from *E. coli* inclusion bodies yielded identical results (data not shown), thus proving the disulfide linkages to be formed correctly under refolding conditions.

In the absence of any detailed information on the tertiary structure of GM2AP, it proves difficult to link the disulfide bonding pattern found here to discrete structural elements or putative domains of the protein. With regard to its high stability against protease, heat and acid treatment, one can assume that the main task of the disulfide bridges is to make the tertiary structure of the molecule compact, stabilizing it against the aggressive conditions of its natural environment, the lysosome.

The linkage between the first and last cysteine along the sequence, the Cys39–Cys183-bridge, is a feature GM2AP shares with the functionally related sphingolipid activator proteins SAP B and C (Vacaro et al., 1995). The remaining three bridges fall within

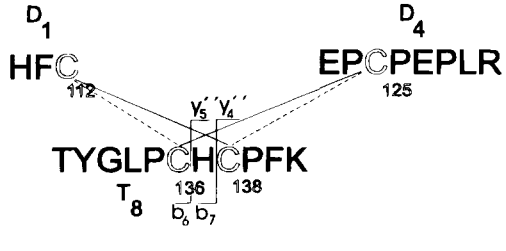

Fig. 5. Peptides obtained by Asp N digestion of S2 + T8. Putative disulfide bonds are marked by broken lines; the bonds which are experimentally found are marked by solid lines. Backbone cleavages as observed in PSD are marked, y'' meaning the C-terminal and b meaning the N-terminal fragment of the peptide after breaking the backbone in the peptide bond.

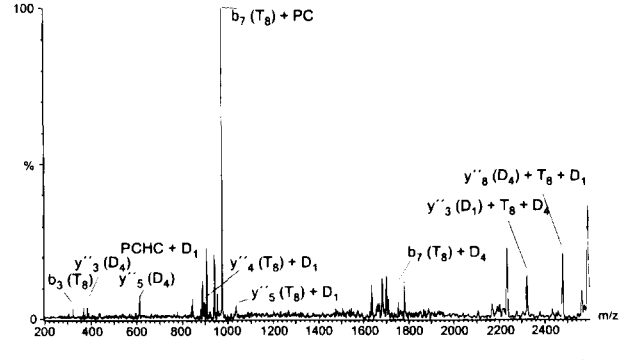

Fig. 6. PSD spectrum of T8 + D1 + D4. The peptide (mass 2,606.2) was selected by ion gating and PSD fragments were recorded by stepping the reflectron voltage in 12 steps and stitching the individual scans. The stitched spectrum is displayed.

Table 5. *Masses of PSD fragments, which are diagnostic for the disulfide arrangement*

Fragment	Theoretical mass	PSD of D1 + D4 + T8[a]	PSD of D4 + T8[a]	PSD of D1 + T8[a]
b7 (T8)	772.9	—	—	771.8
y″4 (T8) + D1	897.1	897.4	—	897.2
b7 (T8) + PC	971.2	972.5	971.4	—
y″5 (T8) + D1	1,034.3	1,034.2	—	1,034.2
b7 (T8) + D4	1,711.0	1,711.2	1,711.8	—

[a]Masses in Da, mass accuracy ±1 Da.

a stretch of 39 residues in the central third of the molecule, two of them (Cys99–Cys106, Cys125–Cys136) connecting cysteines arranged in a linear fashion along the sequence, while the third (Cys112–Cys138) forms a clamp around the Cys125–Cys136 bridge. This region also contains seven prolines out of seventeen in total, and the particular way in which they are arranged around Cys125, Cys136, and Cys138 may serve to keep the central part of the activator in a highly restricted conformation. That this structural element must play a critical role with regard to the stability and functionality of the molecule, becomes immediately apparent from the nonfunctional Cys138-Arg mutant described earlier (Schröder et al., 1991; Xie et al., 1992), in which the Cys112–Cys138 bridge cannot be formed.

A prediction of the secondary structure of GM2AP using the type-1 Discrete Space Model of Stultz, White and Smith (Stultz et al., 1993; White et al., 1994) for soluble, monomeric, globular proteins with no known sequence homologues, classifies GM2AP as belonging to the beta superclass ($p = 0.94$), typical members of which are, e.g., concanavalin A, macromycin, and rhizopuspepsin. According to this prediction, 81 of the 170 residues of the activator precursor (His24–Ile193) have high probabilities ($p > 0.5$) to be included in β-strands, while the rest may form β-turns and irregular regions, with very low probabilities for α-helix formation all over the sequence. Its structural macroclass might be beta sandwich (typical representative: macromycin), in which an array of more than five amphiphilic beta strands form a sandwich, the outer face of which is exposed to the solvent. In this model, the three central disulfide bridges fall within a region of less defined secondary structure. Thus, they may serve to structurally restrain a part of the molecule which would adopt an undesirably diffuse conformation in their absence, and it would be intriguing to speculate on the function of this particular "subdomain" of GM2AP. Depending on whether it protrudes into the interior of the activator or the solvent, it might form part of the lipid binding site or a region of contact between GM2AP and hexosaminidase A. Of special interest would be the function of His137, which, being flanked by two half-cystines and two prolines, probably takes a highly fixed position in space. Definite conclusions, however, on the three-dimensional shape of this protein and the significance of individual residues or sequence stretches must await the elucidation of its crystal structure.

$$\underset{110}{H}F\underset{112}{C}DVLDMLIPTGEP\underset{125}{C}PEPLRTYGLP\underset{138}{C}H\underset{138}{C}PFK\underset{142}{E}$$

Fig. 7. Amino acid sequence of the peptide S5 obtained by *S. aureus* V8 digestion of GM2AP. This peptide was isolated by RP-HPLC and used for the partial reduction and backbone cleavage.

Materials and methods

Modified trypsin was obtained from Promega as sequencing grade protease. α-Cyano-4-hydroxycinnamic acid, 2,5-dihydroxybenzoic acid (DHB), sinapinic acid, peptides for MALDI calibration, and CDAP were purchased from Sigma-Aldrich. Endoproteinase Asp-N and *S. aureus* V8 protease were obtained from Boehringer Mannheim as sequencing grade proteases. TCEP was purchased from Pierce.

Tryptic digestion of GM2 activator protein

Purified GM2AP (1 mg/mL in 0.2 M ammoniumbicarbonate buffer, pH 8.2) was treated with modified trypsin at an enzyme/protein ratio of 1/10 (w/w) at 37 °C for 18 h. Digestion was stopped by freezing.

S. aureus V8 digestion of peptide T7 + T8

The HPLC-purified and lyophilized peptide was redissolved in 25 mM ammoniumbicarbonate buffer, pH 8.2, at a concentration of 1 mg/mL. Digestion was accomplished by treatment with *S. au-*

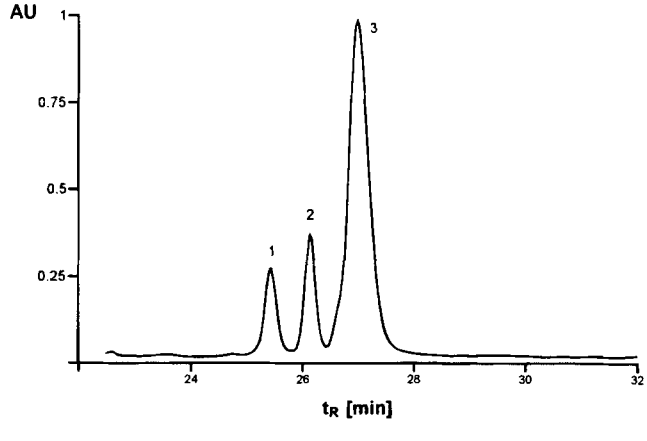

Fig. 8. C18-RP-HPLC separation of partially reduced and cyanylated isomers of peptide S5. 1: isomer 1, 2: isomer 2, 3: unreduced peptide. Separation was achieved on a Vertex Nucleosil C18 column, running a linear gradient from 30 to 60% acetonitrile, containing 0.1% TFA in 40 min.

Table 6. *Results of cleavage of S5 by the method of partial reduction and cyanylation*

Reduced Cys125–Cys136 (isomer 1)			Reduced Cys112–Cys138 (isomer 2)		
Fragment	Theoretical	Experimental[a]	Fragment	Theoretical	Experimental[a]
110–124	1,686.79	1,686.5	110–111	303.15	—[b]
125–135	1,270.63	1,269.6	112–137	2,894.33	2,893.40
136–142	888.35	—[b]	138–142	648.28	—[b]
125–142	2,114.96	2,114.77	112–142	3,498.60	3,498.04
110–135	2,913.40	2,913.23	110–137	3,153.47	3,152.18
β(125–142)	2,080.97	2,080.10	β(110–137)	3,119.48	3,118.48
β(110–135)	2,879.41	2,878.30	β(112–142)	3,464.62	3,464.06

[a]Masses in Da, average mass deviation < 500 ppm.
[b]Matrix suppression: 1,000 Da.

reus V8 protease at an enzyme/protein ratio of 1/10 (w/w) at 25 °C for 24 h.

Endoproteinase Asp-N digestion of peptide S2 + T8

The HPLC-purified and lyophilized peptide was redissolved in 50 mM phosphate buffer, pH 8.0, at a concentration of 0.2 mg/mL and treated with Asp N at an enzyme/protein ratio of 1/20 (w/w) at 37 °C for 3 h.

S. aureus V8 digestion of GM2 activator protein

Purified GM2AP (2 mg/mL in 25 mM ammoniumbicarbonate, pH 7.8, 0.5 M guanidine hydrochloride) was treated with *S. aureus* V8 protease at an enzyme/protein ratio of 1/8 at 25 °C for 15 h. Digestion was stopped by freezing.

HPLC separation

Narrow bore HPLC was performed on a Smart System (Pharmacia, Uppsala, Sweden) using a Vertex column (2 × 250 mm, Knauer, Berlin, Germany) containing Nucleosil C18–120 (3 μm, Macherey & Nagel, Düren, Germany) at a flow rate of 150 μL/min. Separation of tryptic fragments was performed with 0.1% trifluoracetic acid (TFA) in water (A) using a linear gradient of 5 to 70% B (0.06% TFA in 70% acetonitrile, 30% isopropanol) in 45 min. Other separations employed 0.84% TFA in acetonitrile as eluent B, running linear gradients from 12 to 60% B in 60 min (V8 and Asp N digestions of tryptic fragments) or 30 to 60% B in 40 min (V8 digestion of GM2AP or separation of partially reduced isomers).

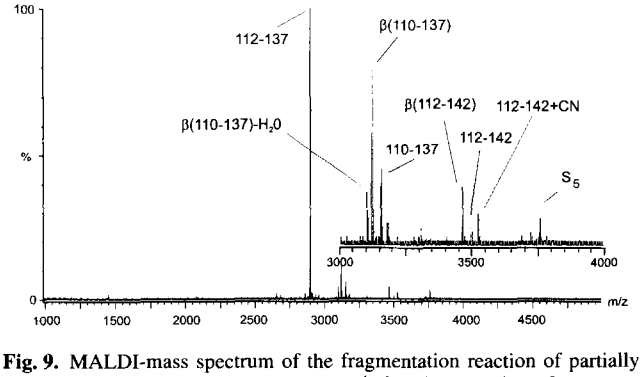

Fig. 9. MALDI-mass spectrum of the fragmentation reaction of partially reduced isomer 2. The strong signal at *m/z* 2.894 results from fragmentation at Cys 112 and Cys 138. Signals are labeled by amino acid sequence as in Table 6.

MALDI mass spectrometry

Mass spectrometric analysis was performed on a TofSpec E (MicroMass, Manchester, UK) mass spectrometer, operating at an acceleration voltage of 20 kV with a 337 nm nitrogen laser. α-Cyano-4-hydroxycinnamic acid (10 mg/mL in 50% acetonitrile, 0.1% TFA) was used as a matrix for HPLC fractions, DHB (10 mg/mL in 70% acetonitrile, 0.1% TFA) or sinapinic acid (10 mg/mL in 40% acetonitile, 0.1% TFA) were used for samples containing high salt concentrations and for intact proteins. 1 μL of analyte solution was mixed with 1 μL of matrix solution directly on the target and allowed to dry at room temperature. External calibration was performed as two point calibration, using commercially available peptides spanning the mass range of interest.

PSD was performed by stepping the reflectron voltage in 12 steps as implemented in the manufacturer's software.

Partial reduction of peptide S5 by TCEP

Five nanomoles of lyophilized peptide purified by HPLC were solubilized in 10 μL of 0.1 M citrate buffer (pH 3.0) containing 6 M guanidine hydrochloride. Partial reduction of the disulfide bonds was carried out by adding 2 μL of 0.1 M TCEP in 0.1 M citrate buffer (pH 3.0) and incubating at room temperature for 15 min.

Table 7. *Methods and results of disulfide bond localization*

Disulfide bond	Method of determination
Cys39–Cys183	Tryptic digestion
Cys99–Cys106	Tryptic digestion and V8 digestion
Cys112–Cys138	PSD analysis of digestion fragments and partial reduction
Cys125–Cys136	PSD analysis of digestion fragments and partial reduction

Cyanylation of nascent sulfhydryls

Eight microliters of 0.1 M CDAP in 0.1 M citrate buffer (pH 3.0) were added to the partially reduced peptide mixture and cyanylation performed at room temperature for 15 min.

Cleavage of singly reduced and cyanylated isomers

To the dried HPLC fractions of the isomers, 2 μL of 6 M guanidine hydrochloride in 1 M NH_4OH were added to redissolve the peptide. Then, 5 μL of 1 M NH_4OH were added and the cleavage was performed at room temperature for 1 h. Excess ammonia was removed in a vacuum system.

Complete reduction of remaining disulfide bonds

The mixture of cleaved peptides still linked by a residual disulfide bond was reacted with 5 μL of 0.1 M TCEP in 0.1 M citrate buffer (pH 3.0) at 37 °C for 30 min. Samples were diluted 1:10 prior to MALDI analysis.

Secondary structure predictions

A prediction of secondary structure was run on the PSA server (http://bmerc-www.bu.edu/psa) at the Biomolecular Research Engineering Center, Boston, using the type-1 Discrete Space Model (Stultz et al., 1993; White et al., 1994) for monomeric, single-domain, globular, and water-soluble proteins.

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