Disulfide Bonds of GM2 Synthase Homodimers

ANTIPARALLEL ORIENTATION OF THE CATALYTIC DOMAINS

Received for publication, August 16, 2000, and in revised form, September 22, 2000
Published, JBC Papers in Press, October 3, 2000, DOI 10.1074/jbc.M007480200

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GM2 synthase is a homodimer in which the subunits are joined by lumenal domain disulfide bond(s). To define the disulfide bond pattern of this enzyme, we analyzed a soluble form by chemical fragmentation, enzymatic digestion, and mass spectrometry and a full-length form by site-directed mutagenesis. All Cys residues of the lumenal domain of GM2 synthase are disulfide bonded with Cys520 and Cys476 forming a disulfide-bonded pair while Cys80 and Cys82 are disulfide bonded in combination with Cys412 and Cys529. Partial reduction to produce monomers converted Cys80 and Cys82 to free thiols while the Cys429 to Cys476 disulfide remained intact. CNBr cleavage at amino acid 330 produced a monomer-sized band under nonreducing conditions which was converted upon reduction to a 40-kDa fragment and a 24-kDa myc-positive fragment. Double mutation of Cys80 and Cys82 to Ser produced monomers but not dimers. In summary these results demonstrate that Cys429 and Cys476 form an intrasubunit disulfide while the intersubunit disulfides formed by both Cys80 and Cys82 with Cys412 and Cys529 are responsible for formation of the homodimer. This disulfide bond arrangement results in an antiparallel orientation of the catalytic domains of the GM2 synthase homodimer.

Ganglioside synthesis is regulated during differentiation, development, and malignant transformation (1–3) and occurs in the Golgi apparatus by the stepwise addition of monosaccharides to glycolipid acceptors by membrane bound glycosyltransferases. The simple gangliosides GM3,1 GD3, and GT3 are the precursors of the a-, b-, and c-series of gangliosides, respectively, and are synthesized by the addition of 1, 2, or 3 molecules of sialic acid to lactosylceramide (LacCer). Complex gangliosides are formed by the addition of GalNAc to simple gangliosides by the action of UDP-GalNAc:lactosylceramide/GM3/GD3 β,1,4-N-acetylgalactosaminyltransferase (GM2 synthase) followed by the attachment of Gal and additional sialic acid residues (1). Thus, GM2 synthase is a key enzyme in ganglioside biosynthesis, controlling the balance between the expression of simple and complex gangliosides (4). Genetic ablation of GM2 synthase in mice resulted in male sterility (5) as well as decreased myelination and axonal degeneration of the central and peripheral nervous system (6).

Previously we showed that GM2 synthase is a homodimer formed by disulfide bond(s) in the lumenal domain (7). The importance of Cys residues of glycosyltransferases has been shown in several functional and structural studies (8, 9) and also by the fact that the Cys residues of each glycosyltransferase family are conserved in spacing (10, 11). In addition a few glycosyltransferases have been shown to be disulfide bonded dimers although the majority are monomeric (see “Discussion”).

In this report we have utilized protein chemistry experiments, coupled with mass spectrometric analyses, to determine: 1) that all Cys of the soluble form of GM2 synthase are involved in disulfide bonds; and 2) which disulfides are responsible for dimer formation. These results demonstrate that in the dimer the NH2 terminus of one subunit is close to the COOH terminus of the other subunit in space.

EXPERIMENTAL PROCEDURES

GM2 Synthase—CHO cell clone GTm1 which stably expresses a soluble form of myc-tagged GM2 synthase was described previously (12). Large scale cell culture was achieved in roller bottles using the serum-free medium CHO-S-SFM II (Life Technologies) according to Kolhekar et al. (13) and in bioreactors at the National Cell Culture Center, Minneapolis, MN. The soluble form of GM2 synthase was partially purified from culture supernatants by SP-Sepharose chromatography using a 0–0.25 M NaCl gradient in 50 mM Hepes, pH 7.6, 5 mM MgCl2.

Liquid Chromatography/Electrospray Ionization-Tandem Mass Spectrometry

1 The abbreviations used are: GM3, NeuAc2,3Galβ1,4Glc-ceramide; GD3, NeuAc2,8NeuAc2,3Galβ1,4Glc-ceramide; GT3, (NeuAc2,8NeuAc2,8NeuAc2,3Galβ1,4Glc-ceramide; GM2 synthase, UDP-GalNAc:lactosylceramide/GM3/GD3 β,1,4-N-acetylgalactosaminyltransferase; LC, liquid chromatography; ESI, electrospray ionization; MS/MS, tandem mass spectrometry; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; CHO, Chinese hamster ovary.

This paper is available on line at http://www.jbc.org
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Spectrometric (LC/ESI-MS/MS) Analyses—A sample of the concentrated soluble form of GM2 synthase (0.05 nmol) was treated with a 20-fold molar excess of PEO-maleimide-activated biotin and immediately denatured with 8 M urea. The sample was incubated for 60 min in the dark at room temperature. The concentration of urea in the sample was decreased by adding water in an equimolar ratio (1/10 ratio, w/w, of trypsin/protein), and the mixture (50 μl) was incubated overnight at 37 °C.

Oligosaccharides were released from GM2 synthase by PNGase F treatment. PNGase F (Roche Molecular Biochemicals) was dissolved in 100 mM sodium phosphate, 25 mM EDTA at pH 7.2 at a concentration of 200 units/ml. PNGase F digestion was performed on data described below. GM2 synthase was added to PNGase F to a final concentration of 20 units/ml and incubating overnight at 37 °C.

GM2 synthase digests were separated using a capillary C18 column (150 × 0.18 mm; Nucleosil, 5-μm particle size) and analyzed on a Finnigan LCQ ion trap mass spectrometer (San Jose, CA) with a modified electrospray ionization (ESI) source. A detailed scheme of the experimental setup for the LC/ESI-MS/MS analyses is described elsewhere (14). Briefly, a positive voltage of 3 kV was applied to the electrospray needle, and a N2 sheath flow (65 scale) was applied to stabilize the ESI signal. The LC/MS analysis was conducted using a Hewlett-Packard 1050 HPLC system (Palo Alto, CA) coupled to the Finnigan LCQ. The mobile phase was split before the injector by a Tee-connector, and a flow rate of 2 ml/min was established for the separation. The HPLC eluate was split by a 10/1 ratio, which a flow rate of 0.1 ml/min using a ThermoQuest LCQ ion trap mass spectrometer (San Jose, CA) with a modification ESI source. The LC/MS/MS analysis was conducted using an automated data acquisition procedure, in which a cyclic series of three different scan modes were performed. Data acquisition was conducted using the full scan mode (m/z 300–2000) to obtain the most intense peak (signal > 1.5 × 10^6 counts) as the precursor ion, followed by a high resolution zoom scan mode to determine the charge state of the precursor ion and an MS/MS scan mode (with a relative collision energy of 38%) to determine the structural fragment ions of the precursor ion. The resulting MS/MS spectra were then searched against a protein database (Owl) by Sequest to confirm the sequence of tryptic peptides.

After analyzing for free Cys-containing peptides, a fraction of the digested protein (40 pmol) was digested with DTT (dithiothreitol, 200-fold molar excess over protein) at 65 °C for 20 min and alkylated with iodoacetamide (500-fold molar excess) in the dark for 30 min to detect cysteine residues. The sample was hydrolyzed using modified trypsin (Promega). Differences from the method of Jensen et al. (15) were the use of higher (20 mM) DTT and incubating overnight at 37 °C.

Partial Reduction—The soluble form of GM2 synthase (0.25 nmol) was incubated with 100 mM DTT in 100 mM Tris, pH 8.0, 0.5 mM EDTA under nitrogen for 2 h at room temperature after which a 10-fold excess of iodoacetamide (in 0.4 mM Tris, pH 8.0) over the DTT content was added, and incubation was continued under nitrogen for an additional 2 h at room temperature in the dark. Samples were separated on SDS-PAGE gels, Western blotted with anti-GM2 and cholera toxin for in vivo GM2 synthase activity, and cell extracts were analyzed by Western blotting with anti-MyC and in vitro assay for GM2 synthase activity as described elsewhere (7, 17).

RESULTS

Determination of the Disulfide-thiol Status of All Cys Residues—The strategy for determining the oxidation state of Cys residues was to alkylate all free Cys with a biotinylated form of maleimide under denaturing conditions, reduce and alkylate with iodoacetamide all other Cys, digest with trypsin and PNGase F, and analyze by LC/MS (14). When the soluble form of GM2 synthase was analyzed in this way, no biotinylated peptides were found, suggesting that no Cys residues with free thiol groups were present. Instead, five peptides were identified which contained alkylated Cys, and the sequence of each tryptic peptide was validated by MS/MS analysis. Thus, the following peptides were identified with reduced and alkylated Cys residues (all residue numbers refer to the position in full-length GM2 synthase): amino acids 69–95 containing Cys residues 67 and 70, 248–261 containing Cys residues 250 and 253, 367–379 containing Cys residues 370 and 373, 476, and 527–533 (plus the residues P and EQK of the myc tag) containing Cys residue 529. Fig. 1 shows the MS/MS spectrum for the reduced and alkylated peptide 398–414 as an example. As shown in this figure, fragment ions were produced at various sites along the peptide via peptide bond cleavage. Both COOH-terminal y_n (n = 8–15) and NH2-terminal b_n (n = 3, 5, 6, and 12) ions can clearly be identified in the spectrum. Fragment ion assignments are shown in the peptide structure of Fig. 1.

Identification of the Disulfide Bond Pairs—LC/MS analyses of a tryptic digest of unreduced GM2 synthase produced a triply charged ion at m/z 1373.5 and a quadruply charged ion at m/z 1030.8 (Fig. 2A). These ions correspond to the N-terminal cysteine residue of the mature protein and the reduced, alkylated sample (m/z 1373.5). The observed ion (4118.8) is equal to the mass of these two peptides minus the mass of two protons which are lost as the result of the disulfide bond formed, divided by the charge state (i.e., 3 or 4) of the ion. Thus, the molecular weight of peptide 418–442 is equal to 2718.1, and that of peptide 473–486 is 1402.6. Linking these peptides via a disulfide bond generates a dipeptide with an average mass of 4118.7, in good agreement

CNBr Cleavage—The soluble form of GM2 synthase (0.1 nmol) was organic-solvent precipitated (16). The dried protein pellet was resuspended in 0.04 ml of cleavage solution (20 mM sodium citrate, pH 4.5, 0.2% SDS) with or without CNBr (0.5 mM stock, Aldrich; final 1.2 mM which was at least a 50-fold molar excess over the Met content) followed by flushing of the reaction tubes with nitrogen, and incubation over-night in the dark at room temperature. Cleavage reactions were diluted 10-fold with water, frozen, and solvent removed by sublimation in a centrifugal vacuum concentrator. The remaining residue was solubilized in nonreducing SDS-PAGE sample buffer by heating at 100 °C for 5 min, separated on SDS-PAGE gels under reducing and nonreducing conditions, and stained with Coomassie Blue or Western blotted with anti-myc. Bands of interest were cut out and subjected to in-gel digestion and analysis by MALDI-TOF. Bands from nonreducing gels were also cut out of the gel, minced, boiled in reducing SDS-PAGE sample buffer, and the suspension of gel pieces loaded in the wells of new SDS-PAGE gels.

Site-directed Mutagenesis—Site-directed mutagenesis of full-length GM2 synthase was performed on a pcDNA3 plasmid containing GM2 synthase/myc cDNA using the Transformer™ Site-directed Mutagenesis Kit (CLONTECH), according to the manufacturer’s instructions. The sequence of the mutated construct at the mutation sites was confirmed by DNA sequencing (EPSciO Sequencing Center, University of Louisville and Biomedical Research Facility, University of Virginia). A sense and an antisense construct was tested for expression in the full-length GM2 synthase in vitro using the Taq coupled transcription/translation system (Promega, Madison, WI). Wild-type CHO cells were transfected, and cells stably expressing mutated GM2 synthase/myc were analyzed by anti-myc immunofluorescence screening as described previously (7, 17). Transfected cells were analyzed by flow cytometry with anti-GM2 and cholera toxin for in vivo GM2 synthase activity, and cell extracts were analyzed by Western blotting with anti-MyC and in vitro assay for GM2 synthase activity as described elsewhere (7, 17).

Induction of the Disulfide Bond Pairs—LC/MS analyses of a tryptic digest of unreduced GM2 synthase produced a triply charged ion at m/z 1373.5 and a quadruply charged ion at m/z 1030.8 (Fig. 2A). These ions correspond to the N-terminal cysteine residue of the mature protein and the reduced, alkylated sample (m/z 1373.5). The observed ion (4118.8) is equal to the mass of these two peptides minus the mass of two protons which are lost as the result of the disulfide bond formed, divided by the charge state (i.e., 3 or 4) of the ion. Thus, the molecular weight of peptide 418–442 is equal to 2718.1, and that of peptide 473–486 is 1402.6. Linking these peptides via a disulfide bond generates a dipeptide with an average mass of 4118.7, in good agreement
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![Fig. 1. MS/MS spectrum for the reduced and alkylated tryptic peptide of GM2 synthase containing Cys\(^{412}\). Both b- and y-dominant fragment ions as defined according to the nomenclature of Biemann (56) are shown, confirming the sequence (amino acids 398–414) and Cys\(^{412}\) alkylation.](image)

with the measured mass of 4118.4. MS/MS analysis of the triply charged ion at \(m/z\) 1373.5 (Fig. 2B) produced a spectrum with dominant fragments of \((Y_{13}X_{16})^3^+\) at \(m/z = 1548.7\), \((Y_{13}Y_{22})^3^+\) at \(m/z = 1838.9\), \((Y_{13}Y_{19})^{5^+}\) at \(m/z = 1649.9\), and \(y_9\) at \(m/z = 1022.6\). These ions were either generated from the COOH terminus of the peptide containing amino acids 418–442 (denoted as \(y_9\)), or from the COOH terminus of the peptide 418–442 (denoted as \(Y_{13}\) and \(Y_{19}\)) in combination with amino acids 473–486 at the COOH terminus of the peptide (denoted as \(Y_{13}\) and \(Y_{14}\)). The observed fragment peaks at \(m/z\) 1367.9 and 1305.7 in Fig. 2B also matched well with the calculated masses for the fragments \((M + ^2\text{H} - 18)^{3^+}\) and \((Y_{13}Y_{22})^{3^+}\), respectively. These data provided a complete verification of the proposed disulfide-bonded tryptic peptide pair.

To confirm this disulfide assignment, the GM2 synthase dimer separated on a nonreducing SDS-PAGE gel was cut from the gel, in-gel digested with trypsin followed by PNGase F, and analyzed by MALDI-TOF. The dipeptide containing the Cys\(^{229}\)-Cys\(^{476}\) disulfide was detected at 4118 (Fig. 3A). In addition a small peak consistent with a free thiol for the peptide containing Cys\(^{81}\) was also detectable (data not shown). However, that ion could be explained by the report of Patterson and Katta (18) that disulfide-linked peptides could be fragmented to reduced forms of peptides during MALDI-TOF. To prove that point, the sample was alkylated with iodoacetamide and reanalyzed. The same small peak for free thiol at Cys\(^{412}\) was still present indicating that it was not present prior to MALDI-TOF but instead was generated during MALDI-TOF. Thus, Cys\(^{229}\) and Cys\(^{476}\) form one disulfide bond.

Evidence for a second disulfide bonded set of tryptic peptides came from the presence of ions with 3 and 4 positive charges at \(m/z\) 1950.2 and 1462.8 (average \((M + H)^{+}\) ion at 5848.6) (Fig. 4A). These ions are consistent with the presence of a tripeptide composed of peptides linked via disulfide bonds, containing amino acids 69–95 (\(M_5 = 2835.2\), 398–414 (\(M_2 = 1724.0\)), and 527–533 plus the Pro and EQK of the C terminus (\(M_1 = 1291.5\) for LQCMTSQPEQK) with a calculated \((M + H) = 5847.7\). MS/MS analysis of the quadruply charged ion at \(m/z = 1462.8\) (Fig. 4B) showed that the dominant triply charged fragments, \((B_x_{11}B_{16}B_{22})^{3^+}\), \((Y_x_{11}B_{16}X_{16})^{3^+}\), and \((B_x_{11}B_{16}B_{22})^{3^+}\) were generated from the COOH or NH\(_2\) terminus of amino acids 527–533 plus PEQK (denoted as \(X_{11}\) and \(B_{11}\)), the NH\(_2\) terminus of amino acids 398–414 (denoted as \(B_{16}\) and \(B_{17}\)), and the COOH or NH\(_2\) terminus of amino acids 69–95 (denoted as \(Y_{22}\), \(B_{22}\), and \(B_{23}\) consistent with the proposed disulfide-bonded tripeptide structure. The results presented in these figures support the proposed tripeptide structure in which Cys\(^{80}\) and Cys\(^{82}\) are disulfide bonded to Cys\(^{412}\) and Cys\(^{529}\).

**Occupancy of Asn-linked Glycosylation Sites**—Haraguchi et al. (19) found that all three potential N-glycosylation sites of GM2 synthase were utilized during in vitro transcription/translation. Similarly, our data indicate that all three sites are occupied in the soluble form of GM2 synthase. Specifically, the measured mass \((M + H)\) for the doubly charged ion at \(m/z\) 1475.1 and triply charged ion at \(m/z\) 983.9 of the peptide (containing alkylated Cys residues 80 and 82 amino acids 69–95) was 2950 (Fig. 5A), rather than 2949 demonstrating that Asn\(^{79}\) was converted to Asp\(^{79}\) by PNGase F. Moreover, the MS/MS spectrum of the doubly charged ion at \(m/z\) 1475.1 (Fig. 5B) for this peptide confirmed that Asn\(^{79}\) had been converted to Asp since the difference between the mass of the NH\(_2\)-terminal fragments \(b_{10}\) at \(m/z\) 1110.7 (containing amino acids 69–78) and \(b_{11}\) at \(m/z\) 1225.6 (containing amino acids 69–79) is 114.9 (mass for Asp) rather than 114 (mass for Asn). In addition, MALDI-TOF analyses after PNGase F treatment also demonstrated the appearance of peptides containing Asn\(^{79}\) and Asn\(^{274}\), indicating that those residues were glycosylated (Fig. 6). Moreover, in the reflectron mode the detected mass for the latter peptide was 2503.28 rather than the calculated mass of 2502.36 demonstrating that Asn\(^{274}\) was converted to Asp\(^{274}\) by PNGase F (data not shown).

**Identification of the Intersubunit and Intrasubunit Disulfide Bonds**—Based on the disulfide pairs identified above, there are seven possible models for the intersubunit disulfide bonds responsible for dimer formation. These models include the following combinations of intersubunit bonds: 1) Cys\(^{429}\) to Cys\(^{476}\); 2) one or both of the disulfides connecting Cys\(^{80}\) and Cys\(^{82}\) with Cys\(^{412}\) and Cys\(^{529}\); or 3) both 1 and 2. To distinguish inter- from intrasubunit disulfides, initially we tested specific proteases to generate diagnostic fragments such as caspase 3 cleavage at residue 315 and furin cleavage at 414, but neither protease cleaved GM2 synthase (data not shown). Therefore, we resorted to two alternative strategies, partial reduction and CNBr cleavage.

Our rationale for partial reduction was to reduce GM2 syn-
thase without denaturation so that the more accessible inter-
subunit disulfide(s) would be reduced whereas the less ac-
cesible intrasubunit disulfide(s) would remain intact. Treatment
with 100 mM DTT followed by alkylation converted all of the
GM2 synthase dimer to the monomer (Fig. 7E). LC/MS of the
tryptic digest of this partially reduced sample (data not shown)
revealed the triply charged ion at m/z 1373.5 (monoisotopic (M
+ H)⁺ ion at 4117.3) described above (Fig. 2A) which is the
result of a disulfide-bonded tryptic dipeptide containing amino
acids 418–442 and 473–486. MS/MS analysis of this species
(data not shown) verified the structure of this tryptic peptide
pair as described above (Fig. 2B). Furthermore, we cut out the
monomer band produced by partial reduction and performed
in-gel digestion followed by MALDI-TOF (Fig. 3B). The Cys
429–476 dipeptide was detected at 4118.8 in agreement with
the LC/MS data. Following complete reduction, this dipeptide
band disappeared (Fig. 3C). In the partially reduced sample
there were also ions at 4175.7 and 4232.8. Since these differed
from the dipeptide peak and from each other by an average of
57 atomic mass unit, they are due to addition of acetamide.
Since these two peptides contain three His, it is likely that
these bands are due to alkylation of these His as described
previously (20, 21). Thus, at least a portion of the disulfide bond
between Cys429 and Cys476 remained intact under conditions in
which dimer was converted to monomer, meaning that the
Cys429–476 disulfide must be an intrasubunit bond.
LC/MS of the partially reduced and alkylated sample also
detected a doubly charged ion at m/z 1475.4 (monoisotopic (M
+ H)⁺ ion at 2948.4) which is the tryptic peptide containing
amino acids 69–95 as verified by MS/MS analysis (data not
shown; the results were identical to those shown in Fig. 5,
A and B). Thus, Cys69 and Cys82 were both reduced and alkylated
by the partial reduction conditions, indicating the likelihood
that both participate in intersubunit disulfide bonds. Ions for
peptides containing alkylated Cys412 and Cys529 were not de-
tected following partial reduction and alkylation. In summary

Fig. 2. Disulfide bond between Cys⁴²⁹ and Cys⁴⁷⁶. A, the full scan mass
spectrum of the dipeptide containing Cys⁴²⁹ and Cys⁴⁷⁶ at m/z 1373.5 for the
triply charged ion and m/z 1030.8 for the quadruply charged ion. B, the correspond-
ing MS/MS spectrum for m/z 1373.5 contains both b- and y-dominant fragment
ions, confirming the sequences.
Based on the partial reduction experiments, we eliminated any models for GM2 synthase intersubunit disulfide bonds that included Cys\textsuperscript{429} to Cys\textsuperscript{476} disulfides as intersubunit bonds.

We next used cleavage with CNBr to further identify the intersubunit disulfide bonds. The soluble form of GM2 synthase contains three Met residues at positions 330, 515, and 591.

**Fig. 3.** Partial reduction studies of the disulfide-coupled peptides 418–442 and 473–486. A, the MALDI-TOF (reflectron) mass spectrum from m/z 3400 to 5000 for the disulfide-linked dipeptide obtained from a GM2 synthase dimer band that was in-gel digested with trypsin and analyzed without reduction. B, the MALDI-TOF (reflectron) mass spectrum for the same dipeptide following partial reduction and alkylation. m/z = 4118 represents the dipeptide + H\textsuperscript{+}, while masses at 4175.7 and 4232.8 represent mono- and dialkylated species, respectively. C, the MALDI-TOF (reflectron) mass spectrum for the same peptide following complete reduction using DTT and alkylation with iodoacetamide.

**Fig. 4.** Cys\textsuperscript{80} and Cys\textsuperscript{82} are disulfide bonded in combination with Cys\textsuperscript{412} and Cys\textsuperscript{529}. A, the full scan mass spectrum of the tripeptide containing Cys\textsuperscript{80}, Cys\textsuperscript{82}, Cys\textsuperscript{412}, and Cys\textsuperscript{529} at m/z 1950.2 for the triply charged ion and m/z 1462.8 for the quadruply charged ion. B, the corresponding MS/MS spectrum for m/z 1462.8 contains both b- and y-dominant fragment ions, confirming the sequences.
530 (numbers refer to the position in the full-length enzyme with the amino terminus of the soluble form corresponding to residue 21 of the full-length enzyme). Treatment with CNBr when analyzed under nonreducing conditions produced a monomer-sized band while considerable dimer remained (Fig. 7B, lane 3). When the sample was run under reducing conditions (Fig. 7B, lane 6), considerable monomer remained indicating that cleavage with CNBr had not been efficient. The low efficiency of cleavage may have been due to the unusual conditions used for fragmentation which were originally chosen to ensure retention of a photoaffinity probe used for other studies.2

CNBr cleavage when analyzed under reducing conditions should produce fragments of GM2 synthase of about 40 kDa (amino acids 21–330, with the precise molecular mass depending on the actual Asn-linked oligosaccharide structures attached at residues 79, 179, and 274), 20.5 kDa (amino acids 331–515), 1.9 kDa (amino acids 516–530), and 1.6 kDa (amino acids 531–544 which includes the myc epitope). However, if CNBr only cut at residue 330, then a myc-positive, 24-kDa fragment consisting of residues 331–544 plus a 40-kDa fragment consisting of residues 21–330 would be present under reducing conditions. In fact CNBr did produce a set of bands ranging from about 20 to 24 kDa plus a 40-kDa band when analyzed under reducing conditions (Fig. 7B, lane 6). However, the most abundant band among the set of 20–24-kDa fragments was the 24-kDa fragment which was myc-positive (Fig. 7C, lane 3) by Western blotting indicating that in fact it contained an intact COOH terminus. It was this 24-kDa myc-positive fragment that resulted from single cleavage at 330 that enabled us to define the intersubunit disulfide pattern as described below. We should also note that we attribute the preferential CNBr cleavage at residue 330 to the generally inefficient cleavage conditions that we used as well as to the documented sluggish cleavage that occurs at Met-Thr (23) such as at position 530.

The acidic conditions used for CNBr cleavage produced a small amount of monomer when analyzed under nonreducing conditions (Fig. 7B, lane 2) which was less than that produced

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2 J. Li, manuscript in preparation.
in the presence of CNBr (Fig. 7B, lane 3). When analyzed under reducing conditions the acidic buffer produced several fragments (Fig. 7B, lane 5), some of which were detected by anti-myc Western blotting and which migrated at 5, 10, 27, and 50 kDa (Fig. 7C, lane 2). The nature of this fragmentation in citrate buffer, pH 4.5, is unknown. CNBr cleavage in the presence of formic acid has been cautioned against because the reducing power of that acid can convert disulfides to free thiols (22). However, the fragments seen in our control sample do not appear to be simply the result of reduction of dimers to monomers. Rather they appear to result from proteolysis, perhaps by a contaminating acid protease. Nevertheless, the fragments produced by CNBr could be clearly distinguished under reducing conditions from the fragments produced by the acidic buffer alone.

The 24- and 40-kDa CNBr fragments were cut from a reducing gel (Fig. 7B, lane 6) and subjected to in-gel digestion with trypsin followed by MALDI-TOF. Nearly all of the peptides were identified for the 24-kDa piece (Fig. 8A, Table I, and Fig. 9A). Although the myc epitope was not deleted, a peptide containing Met515 was detected (YPGSLDESMQMAK) indicating that in fact CNBr had not cut at Met515. The 40-kDa piece contained major ions for the predicted region of 21–330 (Fig. 8B, Table I, and Fig. 9B). These MALDI patterns combined with the myc-positive staining of the 24-kDa fragment confirmed that the 40-kDa fragment consists of residues 21–330 and the 24-kDa fragment consists of residues 331–544.

Based on the disulfide pairs identified above, there are seven possible combinations by which disulfides could link two monomers to form a dimer. However, only one of these combinations would allow CNBr cleavage at 330 to produce the pattern of fragments described above; namely, a monomer-sized fragment under nonreducing conditions that is converted to 24- and 40-kDa fragments upon reduction. That combination consists of intersubunit disulfides linking both Cys429 and Cys476 to Cys412 and Cys529 for a total of four intersubunit disulfides (22). However, the fragments seen in our control sample do not appear to be simply the result of reduction of dimers to monomers. Rather they appear to result from proteolysis, perhaps by a contaminating acid protease. Nevertheless, the fragments produced by CNBr could be clearly distinguished under reducing conditions from the fragments produced by the acidic buffer alone.

FIG. 6. Glycosylation sites are occupied. Peptides generated from in-gel tryptic digests of the GM2 synthase dimer band were further treated with PNGase F to demonstrate sites of glycosylation. MALDI-TOF (linear) spectra show peptides at 6109.5 Da (containing Asn179) and 2504.4 Da (containing Asn274) after hydrolysis (panel B), but not before (panel A). In addition the mass at 4118 Da consists of the Cys429 to Cys476 dipeptide shown also in Figs. 2 and 3.
40- and 24-kDa bands (Fig. 7D, lane 5), and the latter band was 
myc positive (data not shown). Neither the 40-kDa nor the 
24-kDa bands were produced from the dimer band of an un-
treated sample (Fig. 7D, lane 1), the dimer or monomer band 
from a sample treated under control conditions (Fig. 7D, lanes 
2 and 4, respectively), or the dimer band from a CNBr-treated 
sample (Fig. 7D, lane 3). Therefore, these results confirm that 
in fact the monomer band produced by CNBr cleavage was 
the source of these fragments. In summary our CNBr results 
demonstrate that intersubunit disulfides join Cys80 and

![MALDI-TOF spectra of tryptic peptides generated from the CNBr fragments.](image)

**Fig. 8. MALDI-TOF spectra of tryptic peptides generated from the CNBr fragments.** CNBr cleavage produced major fragments at 24 and 40 kDa as a result of preferential cleavage at Met330. Following electrophoretic separation, bands were taken for in-gel tryptic hydrolysis, and the resulting peptides were analyzed using MALDI-TOF (reflectron) analysis.

**TABLE I**

| Fragment | Peptidea | Residues | Sequence | MWb | Error (Da)c |
|----------|----------|----------|----------|-----|-------------|
| 24 kDa   | T54      | 520–524  | LLFFK    | 666.4105 | 0.03        |
|          | T30      | 335–340  | GWFAGR   | 692.3394 | 0.05        |
|          | T49      | 489–494  | LPWTSR   | 758.4075 | 0.02        |
|          | T44      | 448–454  | EVGFDPR  | 818.3922 | 0.00        |
|          | T35      | 370–376  | LVDVLER  | 842.4861 | −0.01       |
|          | T50      | 495–503  | DAGAETYAR| 952.4250 | −0.02       |
|          | T48–49   | 487–494  | LKLPTSR  | 999.5865 | −0.02       |
|          | T31      | 341–350  | NLAVSQVTK| 1059.5824| −0.01       |
|          | T36      | 377–387  | TPLDLVGGAVR| 1096.6240| −0.02       |
|          | T37      | 388–397  | EISGFAATTYR| 1143.5560| −0.01       |
|          | T52      | 506–517  | YPGSILDESMAK| 1324.5969| −0.03       |
|          | T47      | 473–486  | VGCSDDVVDHASK| 1458.6773| −0.02       |
|          | T46      | 458–472  | VAHLEFFLDGLOSLR| 1672.8936| −0.03       |
|          | T32      | 351–364  | YVLWYDDDFYPTAR| 1744.8460| −0.04       |
|          | T38      | 398–414  | QLLSVEPGAPGLNCLR| 1779.9302| −0.02       |
|          | T41      | 418–442  | GPHHELVGFGCVVTDGVVNFRLAR| 2773.3798| −0.07       |
| 40 kDa   | T21      | 255–260  | HPPNPR   | 716.3718 | 0.03        |
|          | T26      | 294–300  | ALITSIDR | 772.4907 | 0.02        |
|          | T7       | 60–66    | YAHIPVR  | 854.4763 | 0.01        |
|          | T12      | 105–112  | ADFPAELR | 917.4607 | −0.02       |
|          | T17      | 219–228  | QQLQTVSSSR| 1193.6404| −0.01       |
|          | T14      | 119–128  | EQEQFQASLR| 1253.6040| −0.03       |
|          | T6       | 48–59    | RPELPIDLAPEPR| 1388.7412| −0.01       |
|          | T5       | 35–47    | LPLAPWAPPQSPR| 1428.7877| −0.03       |
|          | T19      | 240–252  | FSTEHEAATIFR| 1464.6997| −0.03       |
|          | T28      | 302–319  | FYPTTVVIAADSDKPER| 2051.0211| −0.00       |
|          | T15      | 129–158  | SQSPADQGLLIAPANSPLQPLQGVEQPLR| 3228.7142| 0.18        |

a Tryptic digestion fragments from GM2 synthase.
b MW are the calculated monoisotopic masses of the sequences in the table. All cysteine residues were alkylated by iodoacetamide.
c Errors are the difference between the calculated monoisotopic masses and the masses detected from the samples.
The peptides shown in **bold** type are peptides bearing Asn-linked sugar chains which therefore could not be observed under these conditions.

The 24-kDa fragment yielded tryptic peptides corresponding to the masses shown in **bold underlined** type. In addition there were peptides corresponding to the masses shown in **bold** type only in B are peptides bearing Asn-linked sugar chains which therefore could not be observed under these conditions.

**Site-directed Mutagenesis—**

**FIG. 9. MALDI-TOF** coverage of tryptic peptides generated from the CNBr fragments. A, The peptides shown in the spectra of Fig. 8 were identified in Table I. Met**536** is shown in **bold** type and **italicized**; numbers refer to the position in full-length GM2 synthase. A, the 24-kDa fragment yielded tryptic peptides corresponding to the masses shown in **bold underlined** type. B, the 40-kDa fragment yielded tryptic peptides corresponding to the masses shown in **bold underlined** type. The peptides shown in **bold** type only in B are peptides bearing Asn-linked sugar chains which therefore could not be observed under these conditions.

**FIG. 10. Disulfide bond pattern of soluble GM2 synthase.** The soluble form is shown which extends from the NH₂ terminus (equivalent to amino acid 21 of the full-length enzyme and which would be attached to the transmembrane domain in the full-length enzyme) to the Cys-rich COOH terminus. Each monomer contains six Cys at positions 80, 82, 412, 429, 476, and 529 (**numbers** refer to the position in the full-length enzyme). Disulfide bonds are shown as **solid lines** linking the Cys residues which indicate that Cys**429–476** are intra-subunit bonds while Cys**80** and Cys**82** form inter-subunit bonds in combination with Cys**412** and Cys**529** of the opposite monomer.

Cys**82** of one monomer in combination with Cys**412** and Cys**529** of the other subunit (Fig. 10).

**Site-directed Mutagenesis—** The results obtained from chemical analyses demonstrate that GM2 synthase exists as a dimer in which disulfide bonds connect Cys**80** and Cys**82** with Cys**412** and Cys**529**. Site-directed mutagenesis was used to further support these findings. Thus, a double mutant (C80S/C82S) of full-length GM2 synthase was created to eliminate the inter-chain disulfide bonding pattern observed in the wild-type enzyme. These mutations should result in GM2 synthase existing only as a monomer. As shown in Fig. 11, the anti-**myc** immunofluorescence staining pattern of cells expressing this mutated enzyme was similar to that of clone C5 cells expressing full-length, wild-type GM2 synthase/myc (17) and right, clone D9 expressing the C80S/C82S double mutant. The width of the panel represents 175 microns. **Bottom**, cell extracts were Western blotted with anti-**myc** under nonreducing conditions. Lanes: 1, untransfected CHO cells; 2, wild-type GM2 synthase/myc, clone C5; and 3, C80S/C82S double mutant.

**DISCUSSION**

Our results demonstrate that the monomers of GM2 synthase homodimers are joined by intersubunit disulfide bonds that pair Cys**80** and Cys**82** of one subunit with Cys**412** and Cys**529** of the other subunit. We have not attempted to determine whether it is Cys**80** or Cys**82** that is joined to Cys**412** or which is paired with Cys**529**. Because a Ser is at position 81, there is no protease that can cleave between Cys**80** and Cys**82**. Future efforts using MALDI post-source decay and partial reduction, cyanylation, and fragmentation in basic solution (26) will be required to answer that question.

Most glycosyltransferases described to date are monomeric. These monomeric forms include the enzyme responsible for N-deacetylation and N-sulfation of heparan sulfate (27), a polysialyltransferase of Escherichia coli (28), UDP-GalNAc-polyepitope N-acetylgalactosaminyltransferase (11), SpA...
from *Bacillus subtilis* (29), heparan sulfate 6-sulfotransferase (34), and α,1,3-galactosyltransferase (30, 31). Two Golgi enzymes which have monomeric luminal domains but which dimerize through intersubunit disulfides in their transmembrane or cytoplasmic domains are β1,4-galactosyltransferase and α,1,3-fucosyltransferase VI (8, 32, 33, 35). In the present study we analyzed a soluble form of GM2 synthase. The full-length form of this enzyme has two additional Cys residues in the transmembrane domain (36). However, it is unlikely that these two Cys residues are involved in dimerization because: 1) when the luminal domain is proteolytically cleaved intracellularly and then secreted from the cell, it is a homodimer (7); and 2) mutation of Cys80 and Cys82 to Ser of full-length GM2 synthase resulted in only monomers being produced (Fig. 11).

Golgi proteins that are dimeric include the nucleotide sulfate transporter (37), ERGIC-53 intermediate compartment marker protein (38), GDPase (39), α-mannosidase II (40), and the GDP-mannose transporter (41). Two other glycosyltransferases in addition to GM2 synthase that have been shown to be dimers are the α,1,2-fucosyltransferase (H enzyme) (42) and GlcAT-I (43). Interestingly, the intersubunit disulfide bond of this latter enzyme occurs in the stem region at Cys52; thus, the enzyme forms a Y shape with two separate catalytic domains at each arm of the Y similar to the structure of IgG.

The structure of the GM2 synthase dimer that we report here is entirely different from that of GlcAT-I in that the disulfide bond pattern results in an antiparallel orientation of the luminal domains of the two monomers (Fig. 10). There are precedents for the antiparallel orientation of the monomeric chains of a dimer although there are none among the glycosyltransferases. Cys42 and Cys84 of one subunit of interleukin 5 are joined in an antiparallel manner to Cys44 and Cys52 of the other subunit (44, 45). Similarly, the monomeric subunits of platelet-derived growth factor are joined by two intermolecular disulfides between Cys43 and Cys52 to form an antiparallel arrangement (46, 47). Finally, homodimers of the small glycoprotein sGP of Ebola virus (48) are formed by intermolecular disulfides between Cys56 and Cys306 which results in an antiparallel orientation of the monomers and brings the amino terminus of one chain close to the carboxyl terminus of the other monomer, similar to the arrangement for GM2 synthase we describe here (Fig. 10).

To date we have not been able to generate a monomeric form of GM2 synthase that retains catalytic activity. Stepwise reduction and alkylation of GM2 synthase resulted in a parallel decrease in enzyme activity and conversion of dimers to monomers.3 Also, the double C80S/C82S mutation described here decreased in enzyme activity and conversion of dimers to monomers (34), and 3 M. L. Allende, unpublished observation.

With the goal of being able to use sequence similarities to predict folding similarities, a total of 553 glycosyltransferases were classified into 26 families based on sequence similarities (54). GM2 synthase was placed in family 12 which has only two members, GM2 synthase and the closely related GALGT2 enzyme (55). Therefore, family 12 may have a novel fold. Furthermore, it is possible that the antiparallel orientation of the monomeric luminal domains may be unique to family 12. Alternatively, we may speculate that in the full-length, membrane bound form of GM2 synthase this antiparallel arrangement forces the catalytic domain closer to the ganglioside GM3 substrate embedded in the Golgi membrane than if the polypeptide chains were extended away from the membrane. Therefore, it is possible that this antiparallel arrangement may be found in other membrane bound enzymes that act on lipid substrates.

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