

Elucidating the Three-Dimensional Structure of α -dystroglycan using Negative Staining

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ABSTRACT

Background: Abnormal glycosylation of α -dystroglycan causes many diseases, such as congenital muscular dystrophies. Determining the α -dystroglycan structure is very important for elucidating the pathogenesis of these disorders and treating them.

Methods: In this study, α -dystroglycan was purified using DEAE (diethylaminoethyl) Sephacel anion-exchange chromatography, WGA (Wheat Germ Agglutinin) affinity chromatography, and size-exclusion chromatography.

Results: The three-dimensional structure of α -dystroglycan was studied using negative staining and single-particle analysis, which revealed two globular domains connected by a flexible linker. The three-dimensional structure of α -dystroglycan is consistent with previously reported 2D images.

Conclusion: Clarifying the three-dimensional structure presented here of α -dystroglycan will help to reveal the molecular mechanisms underlying α -dystroglycanopathy and facilitate its treatment.

Keywords

α -dystroglycan

Three-dimensional structure

Negative staining

Single-particle analysis

INTRODUCTION

The dystrophin–glycoprotein complex (DGC) was originally isolated from skeletal muscle membranes [1, 2]. One of the chief component proteins of the DGC is dystroglycan. Molecular cloning of the dystroglycan gene revealed that the protein is generated from a single gene (DAG1), which is cleaved into α -dystroglycan and β -dystroglycan proteins [3]. α -Dystroglycan comprises three distinct domains: an N-terminal globular domain, a mucin-like central region, and a C-terminal globular domain [4]. The mucin-like central region is the predominant site of O-mannosylation (O-glycosylation). In humans, the O-glycosylation amino acids are 63, 317, 319, 367, 369, 372, 379, 381, 388, and 455, and the N-glycosylation amino acids are 141, 641, 649, and 661. β -Dystroglycan is an integral membrane protein and binds to the intracellular protein

dystrophin, which is a major component of the muscle fiber cytoskeleton. α -Dystroglycan binds to the extracellular part of β -dystroglycan, as well as to extracellular matrix components such as laminin, perlecan, agrin, neurexin, pikachurin, and slit. It is the interaction with laminin that is compromised by defects in the glycosylation of α -dystroglycan. Dissociation from the basal lamina or dissociation from laminin due to the abnormal glycosylation of α -dystroglycan seems to be the predominant cause of the main symptoms of the disorders discussed below.

Dystroglycanopathies represent the most common forms of congenital muscular dystrophy. Abnormal glycosylation of α -dystroglycan causes congenital muscular dystrophy with brain and eye abnormalities—such as Walker–Warburg syndrome, muscle–eye–brain disease, and Fukuyama-type congenital muscular dystrophy—and limb–girdle muscular

Short Communications

dystrophy, as well as cardiomyopathies [5-12]. α -Dystroglycan is also known to be an entry receptor for arenaviruses (Old World arenaviruses such as Lassa Fever, lymphocytic choriomeningitis, and Mobala viruses, and clade C New World arenaviruses such as Oliveros and Latino viruses) and a coreceptor for *Mycobacterium leprae* [13-17]. Determining the structure of α -dystroglycan is very important in elucidating the pathogenesis of these disorders and treating them.

The purpose of this study is to reveal the three-dimensional structure of α -dystroglycan. Brancaccio et al. reported the electron microscopic features of α -dystroglycan, which they determined using the rotary shadowing method [4]. Moreover, partial structures of the N-terminal region of α -dystroglycan without glycosylation were determined using X-ray crystallography [18-21]. The three-dimensional structure of the whole glycosylated protein, however, has not yet been determined.

Here, we report the whole three-dimensional structure of glycosylated α -dystroglycan, as elucidated using negative staining.

MATERIALS AND METHODS

Protein purification

α -Dystroglycan was purified from skeletal muscle using the method reported by Brancaccio, et al. [4], with some modifications according to Campbell, et al. [22], Ohlendieck, et al. [23], Ervasti, et al. [24, 25], Pall, et al. [26], Combs, et al. [27], Michele, et al. [28], Saito, et al. [29], Smalheiser, et al. [30], Wizemann, et al. [31], Nilsson, et al. [32], Mio, et al. [33], and Ogura, et al. [34]. All subsequent steps were carried out at 0-4 °C. Chicken muscles within 2 months of age were obtained from a local slaughterhouse and kept on ice for ~2 h before homogenization or freezing. Approximately 50 g of freshly frozen skeletal muscle was homogenized in a blender (Panasonic MX-X701) six times, 30 seconds each time, in five volumes of lysis buffer A (50 mM Tris/HCl, 200 mM NaCl, 0.02% sodium azide, and 0.2 mM phenylmethylsulfonyl fluoride (PMSF, Combi-Blocks); 0.1mM EDTA; and 125 μ l of protease inhibitor cocktail (Wako: ABESF 100 mmol/l, Aprotinin 80 μ mol/l, Bestatin 5 mmol/l, E-64 1.5 mmol/l, Leupeptin 2 mmol/l, Pepstatin A 1 mmol/l), pH 7.4). After centrifugation (for 10 minutes at 1,000 x g, TOMY MX-301), the supernatant was centrifuged again (for 20 minutes at 30,000 x g, Beckman 45Ti, Beckman OPTIMA L-80). The supernatant was filtered through six layers of cheesecloth, Advantec filter paper, and a 0.45 μ m filter (Millipore Sterivex-HV 0.45 μ m Filter Unit). The solution was loaded on a DEAE-Sephacel (GE Healthcare) column (3 x 14 cm) equilibrated with lysis buffer A. After extensive washing, the protein was step-eluted with 250 mM NaCl, 50 mM Tris/HCl, 0.02% sodium azide; 300 mM

NaCl, 50 mM Tris/HCl, 0.02% sodium azide; 350 mM NaCl, 50 mM Tris/HCl, 0.02% sodium azide; 400 mM NaCl, 50mM Tris/HCl, 0.02% sodium azide; 450mM NaCl, 50mM Tris/HCl, 0.02% sodium azide; and 500mM NaCl, 50mM Tris/HCl, 0.02% sodium azide. The eluted solution was applied directly to a 1 ml Wheat Germ Agglutinin agarose (WGA, Sigma, Lectin from *Triticum vulgaris*) affinity column (2x10 cm) equilibrated with buffer B (500mM NaCl, 50mM Tris/HCl, 0.02% sodium azide, 0.1mM EDTA, pH 7.4) and circulated overnight. After extensive washing, elution was carried out with two column volumes of the same buffer containing 300 mM N-acetyl-D-glucosamine. The eluate was concentrated with VIVASPIN6 Concentrator (VIVASCIENCE). The α -dystroglycan-rich fractions were purified using Superdex 200 size-exclusion chromatography (SEC) in a SMART System (Pharmacia) using buffer B (500mM NaCl, 50mM Tris/HCl, 0.02% sodium azide, 0.1mM EDTA, pH 7.4). The elution of the protein from the SEC column was monitored by measuring its UV absorbance at 280 nm and performing SDS-PAGE analysis. The peak corresponding to α -dystroglycan was collected for analysis by negative staining.

SDS-PAGE and immunoblotting

The standard Laemli method [35] was applied. Samples were mixed with a 1/4 volume of sample buffer containing 62.5 mM Tris-HCl pH 6.8, 2% SDS, 2 % glycerol, 40 mM dithiothreitol, and 0.01% bromophenol blue and then incubated at 100°C for 2 min. Proteins were separated in a polyacrylamide gel (SuperSep (TM) Ace 5-20%, Fujifilm) and visualized using silver staining. Electrophoresis started at 60 V for 15 min, followed by 180 V for 60 min at room temperature. The protein in the gel was analyzed using immunoblotting or visualized using silver staining after destaining the gel with a solution containing 50% methanol and 10% acetic acid. For immunoblotting, electrophoresed proteins in the gel were transferred to a polyvinylidene difluoride membrane (Immobilon-P, Millipore), as described by Towbin et al. [36], and analyzed with IHH6 (Millipore) and alkaline phosphatase-labeled anti-mouse antibodies (Promega).

Transmission electron microscopy

A volume of 2 μ l of purified α -dystroglycan was applied to glow-discharged (6mA) EM Cu grids (STEM) covered by a thin layer of continuous carbon film. Samples were washed with 10 drops of double-distilled water, negatively stained twice with 2% uranyl acetate solution for 30 seconds, blotted, and air-dried. Negatively stained EM grids were observed on a JEOL JEM-1230 transmission electron microscope operated at 100 kV. Images were recorded at 60-80k x using a 2K x 2K Gatan Orius camera.

Data processing

Fifty-one micrographs were imported (pixel size 0.93 Å, acceleration voltage 100 kV, spherical aberration 2.9 mm). The three-dimensional structure was analyzed using RELION [37] and cryoSPARC [38]. The contrast transfer function (CTF) parameters for each micrograph were determined using CTFFIND4 [39]. Exposures were curated: 50 exposures were accepted and 1 was rejected. Then, 1,185 particles with a certain shape were manually picked. After 2D classification, 8 of 25 classes and 584 particles were selected. Based on these selected classes, the template picker picked 7,703 particles, from which 5,043 particles were extracted. After 2D classification, 6 of 50 classes and 596 particles were selected. Junk 2D classes and particles belonging these 2D classes were excluded. The ab initio reconstruction, which was the initial 3D structure, was generated from these classes and particles. In addition, homogeneous refinement was performed using the ab initio reconstruction result as the initial 3D structure, and the 3D structure was generated. The homogeneous refinement result was visualized using UCSF chimeraX [40]. The resolution of the reconstruction was assessed using the Fourier shell correlation (FSC) criterion and a threshold of 0.143.

RESULTS

Purification of α -dystroglycan

SDS (sodium dodecyl sulfate) gel electrophoresis and immunoblotting of α -dystroglycan eluted from the affinity chromatography column showed smears at 150 kDa or more when using IIH6, a monoclonal antibody against α -dystroglycan. α -dystroglycan was eluted from the third size-exclusion chromatography step as a peak at 1.10 mL, corresponding to smears with molecular weights of approximately 150 kDa or more in SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) and immunoblotting (Figure 1) using IIH6 and silver staining.

Three-dimensional structure of α -dystroglycan using electron microscopy

DEAE (diethylaminoethyl) anion-exchange chromatography, WGA (Wheat Germ Agglutinin) affinity chromatography, and SEC (size-exclusion chromatography) were performed to purify skeletal muscle α -dystroglycan, which was obtained from the peak fraction. The purified product was adsorbed to electron microscopy grids immediately after elution, negatively stained, and examined using a transmission

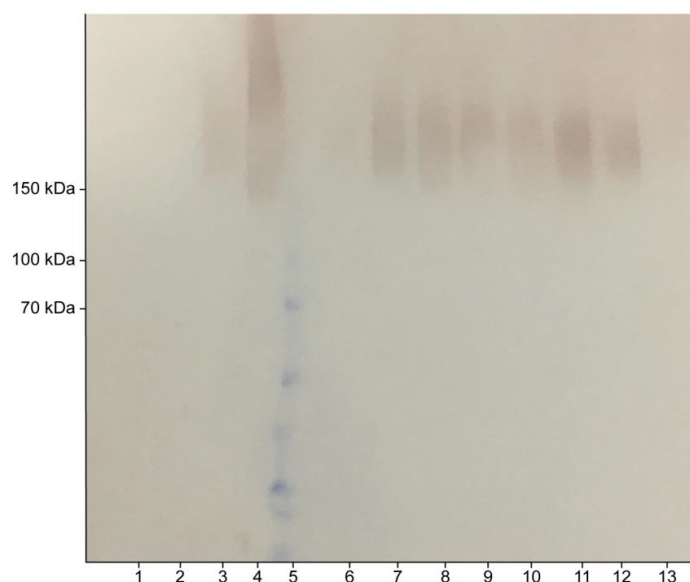


Figure 1: SDS-PAGE and immunoblotting results.

SDS-PAGE (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis: SuperSep (TM) Ace 5-20%, Fujifilm) and immunoblotting results of DEAE (diethylaminoethyl) Sephacel anion-exchange chromatography, WGA (Wheat Germ Agglutinin) affinity chromatography, and size-exclusion chromatography (SEC) products using the anti- α -dystroglycan antibody IIH6. Smears at 150 kDa or more are observed in SEC products: lanes 7-12. Lane 1-4 WGA affinity chromatography products. Lane 1: flowthrough; lane 2: washing; lane 3: elution; lane 4: concentration of lane 3 using VIVASPAN6 (x10); lane 5: size marker; lanes 6-13: size-exclusion chromatography products obtained using SMART system. Lanes 6-13: 2nd, 5th, 6th, 7th, 8th, 9th, 10th, and 14th products, respectively.

Short Communications

electron microscope. The results reveal that α -dystroglycan comprises two 15-30 nm globular domains connected by a flexible linker, which is string-shaped and frequently curved or sigmoid (Figure 2).

Two-dimensional classification results: The 2D classification

results show that the manually picked particles also have a stick- or string-like appearance.

Three-dimensional structure: The three-dimensional structure reveals two globular domains connected by a flexible linker (Figure 3, Movie 1). The resolution determined by the FSC

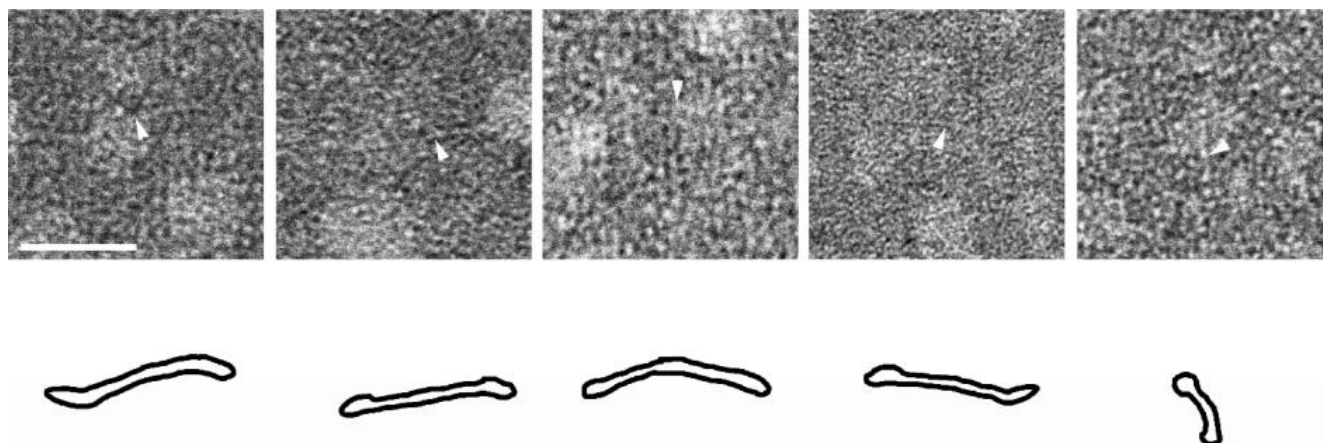


Figure 2: negative staining findings.

Electron microscopy of negatively stained α -dystroglycan revealed two balls and a stick (or a string) like appearance with two globular domains connected by a flexible linker, which is frequently curved or sigmoid (x80K). Arrowheads indicates α -dystroglycan molecules. Contours are shown below each row to delineate the α -dystroglycan. The scale bar represents 20 nm.

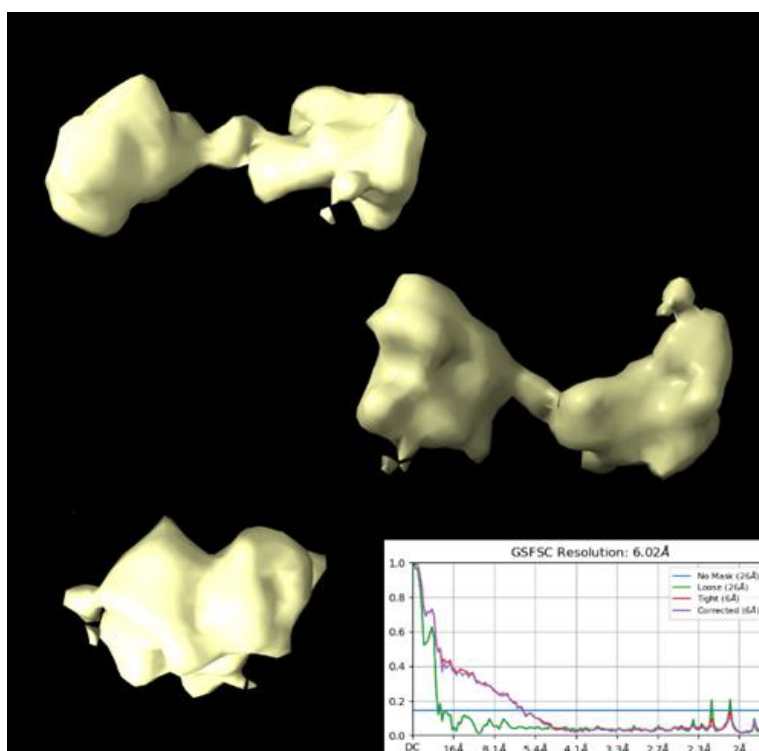
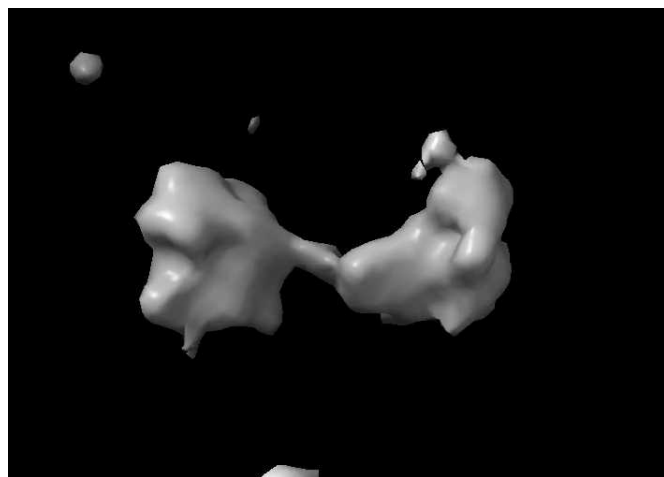


Figure 3: The 3D (dimensional) structure of α -dystroglycan and the Fourier shell correlation (FSC) curve.

The three-dimensional structure of α -dystroglycan reveals two globular domains connected by a flexible linker. These structures underwent homogeneous refinement using cryoSPARC and were visualized using UCSF chimeraX.

The resolution determined using the FSC (Fourier shell correlation) curve was 6.02 Å (lower right).

curve was 6.02 Å (Figure 3). We attempted to fit the N-terminal X-ray structure (PDB ID 5LLK) to the 3D blob and identify the N-terminal globular domain, mucin-like central region, and C-terminal globular domain (Figure 4). The N-terminal X-ray structure fits the right side of the blob. Therefore, the right blob seems to be the N-terminal globular domain.



Movie 1: Movie of 3D (dimensional) structure of α -dystroglycan visualized using UCSF chimeraX (Windows media player or other mp4 player).

DISCUSSION

Here, we report the three-dimensional structure of α -dystroglycan. The α -dystroglycan structure reveals two globular domains connected by a flexible linker. These features are consistent with previously reported 2D images [4]. The resolution determined using the FSC curve was 6.02 Å. However, this study is based on negative staining, and the true

resolution is likely to be lower. Negative staining has limited resolution and can introduce artefacts.

The structure of the mucin-like domain of α -dystroglycan, which was previously unknown, is reported in this study. The N-terminal domain structure was determined using X-ray crystallography [18], and the C-terminal domain structure was determined as a part of the dystrophin–glycoprotein complex using cryo-EM [41,42]. However, the mucin-like domain structure has not yet been determined.

A molecule consisting of two 15–30 nm balls and a stick (or a string)-like region, consistent with α -dystroglycan, was observed using negative staining after DEAE anion-exchange chromatography, WGA affinity chromatography, and size-exclusion chromatography. It appears as two globular portions and a string-like portion. These morphological features are consistent with the 2D image in a previous report [4]. The 3D structure provides three-dimensional information on the protein, adding to the 2D image. Thus, the 3D analysis provides more information on protein structure and will improve understanding of the function associated with its structure.

One method of purification of α -dystroglycan is to isolate the muscle membrane [22–26] using differential and sucrose gradient centrifugation, and then use WGA affinity chromatography. Another method involves DEAE anion-exchange chromatography, WGA affinity chromatography, laminin affinity chromatography, and/or cesium chloride centrifugation [4, 27]. With this method, the glycoprotein is visualized as smears. In this study, DEAE anion-exchange chromatography, WGA affinity chromatography, and size-exclusion chromatography—used as a method of differentiation

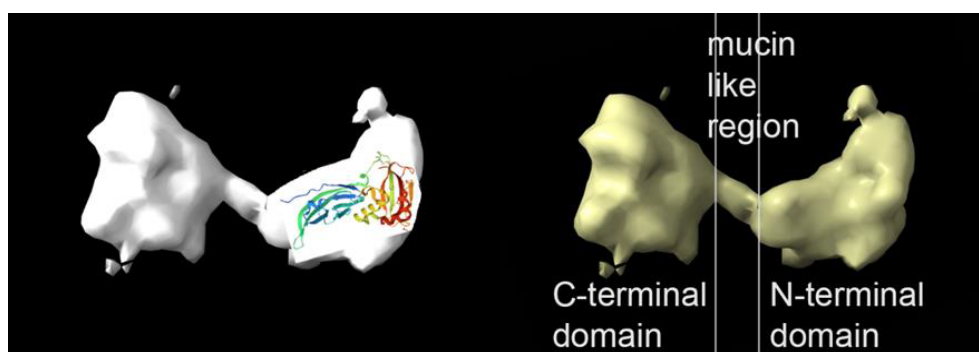


Figure 4: The fitting of the X-ray structure with annotations to highlight key structural features of α -dystroglycan.

Left: The N-terminal X-ray structure of α -dystroglycan (5LLK: Protein Data Bank accession number, previously reported 18)) fitted to the 3D blob. The N-terminal X-ray structure fits the right side of the blob. Therefore, the right blob seems to be the N-terminal globular domain.

Right: A 3D reconstruction of α -dystroglycan, annotated to highlight key structural features: the N-terminal globular domain, the mucin-like central region, and the C-terminal globular domain.

based on molecular weight instead of cesium chloride centrifugation—were used. Size-exclusion chromatography has not been previously used for the purification of α -dystroglycan. For single-particle analysis, size-exclusion chromatography is usually the last step of purification. Therefore, size-exclusion chromatography was used for the last step of purification in this study.

The N-terminal and C-terminal domains were identified by fitting the N-terminal X-ray structure (PDB-ID: 5LLK) to the 3D blob (Figure 4). The N-terminal X-ray structure fits the right blob in Figure 3. Therefore, the right blob seems to be the N-terminal globular domain. The C-terminal globular domain is the site that binds to β -dystroglycan [43], whereas the mucin-like central region is the main site of O-glycosylation and the binding site for laminin and other extracellular proteins.

Mutations in enzymes involved in O-mannosylglycan biosynthesis (O-glycosylation) cause α -dystroglycanopathies such as congenital muscular dystrophy, and this is attributed to dissociation from laminin and other proteins because of the abnormal glycosylation of α -dystroglycan. POMT1 (protein O-mannosyl transferase 1) mutations give rise to Walker-

Warburg syndrome [44, 45]. POMT2 (protein O-mannosyl transferase 2) mutations cause a ‘MEB (muscle-eye-brain disease)-like’ phenotype, Walker-Warburg syndrome, and limb-girdle muscular dystrophy [46-48]. Mutations in B3GALNT2 (β -1,3-N-acetylgalactosaminyltransferase) cause congenital muscular dystrophy [49], and POMGNT1 (protein O-linked mannose β -1,2-N-acetylglucosaminyltransferase 1) mutations cause muscle-eye-brain disease [50]. A retrotransposal insertion in fukutin causes Fukuyama-type congenital muscular dystrophy [51], and FKRP (fukutin-related protein) mutations cause muscle-eye-brain disease, Walker-Warburg syndrome, and limb-girdle muscular dystrophy type 2I [52-54]. Missense mutations in TMEM5 (transmembrane protein 5) have been found in α -dystroglycanopathy patients [55]. Mutations in LARGE (acetylglucosaminyltransferase-like) are associated with congenital muscular dystrophy type 1D (MDC1D) [56]. Walker-Warburg syndrome, muscle-eye-brain disease, and Fukuyama-type congenital muscular dystrophy are characteristic congenital muscular dystrophies related to α -dystroglycanopathy.

The biosynthetic pathway of O-mannosylglycan (O-glycosylation) in mammals was previously reviewed [57] (Table 1). The steps in this pathway are as follows: 1: The POMT1-POMT2 complex adds mannose to a Ser (serine)/Thr (threonine) residue. 2: POMGNT2 (protein O-linked mannose β -1,2-N-acetylglucosaminyltransferase 2) adds GlcNAc (N-acetylglucosamine) to mannose. 3: B3GANLT2 (β -1,3-N-acetylglactosaminyltransferase2) adds GalNAc (N-acetylglactosamine) to GlcNAc. 4: POMK (protein O-mannose kinase) adds phosphate to mannose. 5: FKTN (fukutin)-POMGNT1 (complex) adds ribitol to GalNAc. 6: FKRP adds ribitol to ribitol. 7: TMEM5 adds Xyl (xylose) to ribitol. 8: B4GAT1 (β -1,4-glucuronosyltransferase) adds GlcA (glucuronic acid) to xylose. 9: LARGE (acetylglucosaminyltransferase-like) adds Xyl and GlcA to GlcA.

The blob-like structures correspond to the N-terminal globular domain and C-terminal globular domain identified previously. Information at a higher, near-atomic resolution can be correctly mapped to specific domains of α -dystroglycan. The flexible linker corresponds to the mucin-like central region, which is the predominant site of O-glycosylation. Therefore, the site interacting with molecules such as laminin seems to be the flexible linker or mucin-like central region. It is the flexible linker or mucin-like central region that is likely impacted by glycosylation defects. If the structures of normal α -dystroglycan and pathological α -dystroglycan are available at higher, near-atomic resolution, their comparison can advance the understanding of α -dystroglycanopathies and

Table 1: Biosynthetic pathway of O-mannosylglycan in mammals [57].

Enzyme	Substrate or added sugar Ser/Thr
1. POMT1-POMT2 (complex)	mannose
2. POMGNT2	GlcNAc
3. B3GALNT2	GalNAc
4. POMK	P
5. FKTN-POMGNT1 (complex)	ribitol
6. FKRP	ribitol
7. TMEM5	Xyl
8. B4GAT1	GlcA
9. LARGE	Xyl, GlcA

Abbreviations: B3GALNT: β -1,3-N-acetylglactosaminyltransferase; B4GAT1: β -1,4-glucuronosyltransferase; FKTN: fukutin; FKRP: fukutin related protein; GalNAc: N-acetylglactosamine; GlcA: glucuronic acid; GlcNAc: N-acetylglucosamine; LARGE: acetylglucosaminyltransferase-like; P: phosphate; POMGNT1: protein O-linked mannose β -1,2-N-acetylglucosaminyltransferase 1; POMGNT2: protein O-linked mannose β -1,2-N-acetylglucosaminyltransferase 2; POMK: protein O-mannose kinase; POMT: protein O-mannosyl transferase; Ser: serine; Thr: threonine; TMEM: transmembrane protein; Xyl: xylose.

Table 2: Previous reports of electron microscopic features or structures of α-dystroglycan.						
Author	Ref.no.	Year	Method	Part	Glycosylation	Mutation
Brancaccio et al.	4	1995	rotary shadowing	Whole	+	
Bozic et al.	18	2004	X-ray crystallography	N-terminal	-	
Bozzi et al.	20	2015	X-ray crystallography	N-terminal	-	T190M
Covaceuszach	19	2017	X-ray crystallography	N-terminal	-	
Covaceuszach	21	2017	X-ray crystallography	N-terminal	-	V72I, D109N, T190M
Liu et al	41	2025	SPA	C-terminal	+	
Wan et al	42	2025	SPA	C-terminal	+	
Present study		2025	SPA	Whole	+	

SPA: single-particle analysis.

assist in developing glycosylation-targeted strategies for their treatment.

Electron microscopic findings and X-ray crystallographic structures of α-dystroglycan have been previously reported (Table 2). Brancaccio et al. reported dumbbell-like molecules with two globular units connected by a 20-30 nm long rod-shaped and frequently curved segment using the rotary shadowing method with electron microscopy [4]. The results of the present study are consistent with Brancaccio’s 2D findings. Bozic et al. revealed the N-terminal structure using X-ray crystallography [18]. They confirmed the presence of two autonomous domains in the N-terminal region of α-dystroglycan: the first was an Ig-like domain, and the second resembled ribosomal RNA-binding proteins. Bozzi et al. reported the X-ray crystal structure of the missense variant *T190M* of the N-terminal domain of murine α-dystroglycan [20]. They reported that α-dystroglycan showed an overall topology (Ig-like domains followed by a basket-shaped domain reminiscent of the small subunit ribosomal protein S6) very similar to that of the wild-type structure. Covaceuszach, et al. reported the crystal structure of the wild-type human α-dystroglycan N-terminus and compared it to the murine structure [19]. Moreover, they analyzed both structures in solution. Small-angle X-ray scattering (SAXS) revealed the existence of two main protein conformation ensembles. Covaceuszach, et al. also investigated the crystallographic and solution structures of pathological point mutations; namely, *V72I*, *D109N*, and *T190M* [21]. Scattering analysis revealed that these mutations affect the structures in solution, altering the distribution between compact and more elongated conformations. Liu, et al. studied the dystrophin–glycoprotein complex (DGC) structure [41] and determined the structure of the C-terminal domain of α-dystroglycan (DG). However, the mucin-like domain of α-DG was not visible in their DGC structure, indicating that it may have a flexible disposition. Wan

et al. also investigated the dystrophin–glycoprotein complex structure [42]. In their study, the structure of the C-terminal domain of α-dystroglycan was determined, but the mucin-like domain was disordered. Therefore, an advantage of the present study is its revelation of the structure of the mucin-like domain of α-dystroglycan.

Fortunately, we were able to purify and reconstruct a certain 3D structure of α-dystroglycan. The purification of the α-dystroglycan protein is challenging. Moreover, since the mucin-like central region or the flexible linker is very soft and flexible, the determination of its structure using single-particle analysis is very difficult. Even AlphaFold [58] was unable to predict the whole structure. Since the mucin-like central region or flexible linker is very soft and flexible, α-dystroglycan shows many heterogeneous formations and seems to be able to adopt many structures. Therefore, the structure revealed in the present study might be the only one certain folded structure of α-dystroglycan. Individual-particle electron tomography or a new method of single-particle analysis using cryo-electron microscopy may enable the acquisition of a higher-resolution structure of α-dystroglycan in the future.

CONCLUSIONS

In this study, we elucidated the three-dimensional structure of α-dystroglycan using negative staining. The α-dystroglycan structure revealed two globular domains connected by a flexible linker. Clarifying the three-dimensional structure of α-dystroglycan will help to reveal the molecular mechanism underlying α-dystroglycanopathy and facilitate its treatment.

DATA AVAILABILITY STATEMENT

The dataset generated and analyzed during the current study is available in the protein data bank repository, wwPDB Deposition: D_1300054657.

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