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Backbone and side chain chemical shift assignments of apolipophorin III from *Galleria mellonella*

Karin A. Crowhurst,

Department of Chemistry and Biochemistry, California State University Northridge, 18111 Nordhoff St., Northridge CA 91330-8262, karin.crowhurst@csun.edu, phone: 818-677-4288, fax: 818-677-4288

James V.C. Horn, and

Department of Chemistry and Biochemistry, California State University Long Beach, 1250 Bellflower Blvd., Long Beach CA 90840-9507

Paul M.M. Weers

Department of Chemistry and Biochemistry, California State University Long Beach, 1250 Bellflower Blvd., Long Beach CA 90840-9507

Abstract

Apolipophorin III, a 163 residue monomeric protein from the greater wax moth *Galleria mellonella* (abbreviated as apoLp-III_{GM}), has roles in upregulating expression of antimicrobial proteins as well as binding and deforming bacterial membranes. Due to its similarity to vertebrate apolipoproteins there is interest in performing atomic resolution analysis of apoLp-III_{GM} as part of an effort to better understand its mechanism of action in innate immunity. In the first step towards structural characterization of apoLp-III_{GM}, 99% of backbone and 88% of side chain ¹H, ¹³C and ¹⁵N chemical shifts were assigned. TALOS+ analysis of the backbone resonances has predicted that the protein is composed of five long helices, which is consistent with the reported structures of apolipophorins from other insect species. The next stage in the characterization of apoLp-III from *G. mellonella* will be to utilize these resonance assignments in solving the solution structure of this protein.

Keywords

apolipophorin III; exchangeable apolipoprotein; apoLp-III; *Galleria mellonella*; NMR; chemical shift assignment

Apolipophorin III (apoLp-III) is an exchangeable apolipoprotein that reversibly associates with diacylglycerol-enriched lipoproteins in insect hemolymph. The protein is relatively small (~18 kDa) and monomeric in the lipid-free form, although up to 16 apoLp-III can cluster on the lipoprotein surface during insect flight when diacylglycerol transport is greatly

Correspondence to: Karin A. Crowhurst.

Conflict of Interest

The authors declare that they have no conflict of interest in the publication of this manuscript.

stimulated. Due to structural similarities with vertebrate apolipoproteins apoLp-III is used as a prototype to investigate the structure and function of exchangeable apolipoproteins (Narayanaswami et al. 2010). Like many apolipoproteins, apoLp-III is able to solubilize phospholipid vesicles into discoidal complexes, and this is mediated by the presence of amphipathic α -helices, a common structural feature of apolipoproteins.

X-ray and NMR solution structures have been solved for apoLp-III from the African migratory locust *Locusta migratoria* and the tobacco hornworm *Manduca sexta* (Breiter et al. 1991; Wang et al. 2002; Fan et al. 2003). These species are unrelated and their apoLp-III proteins share less than 12% amino acid sequence identity. However, they display a similar globular fold of a bundle of five amphipathic α -helices organized in an up-and-down topology. Both proteins have been extensively studied for their role in lipid transport processes in vivo and to improve our understanding of apolipoprotein structural changes upon interaction with lipid surfaces (Weers et al. 2006; Van der Horst et al. 2010). A third apoLp-III, of interest here, is primarily studied for its function in innate immunity (Zdybicka-Barabas et al. 2013a). This apoLp-III, from the greater wax moth *Galleria mellonella* (and abbreviated as apoLp-III_{GM}), upregulates expression of antimicrobial proteins, associates with lipopolysaccharides, and binds and deforms bacterial membranes (Gotz et al. 1997; Weise et al. 1998; Oztug et al. 2012; Zdybicka-Barabas et al. 2013b). *G. mellonella* and *M. sexta* are from the *Sphingidae* family, and apoLp-III from these species have a high degree of sequence similarity (Weise et al. 1998).

Due to the increasing interest in the function of exchangeable apolipoproteins in innate immunity, it is critical to have a detailed knowledge of the three-dimensional structure of apoLp-III_{GM}. A structure-guided approach would be extremely beneficial in order to improve our understanding of its novel function. As the first step in the structural characterization of this protein, backbone and side chain ^{15}N , ^1H and ^{13}C chemical shift assignments were carried out.

Methods and Experiments

Protein expression and purification

All isotopes were obtained from Cambridge Isotope Laboratories.

Recombinant apoLp-III_{GM} was expressed at 37 °C in an established bacterial expression system using BL21 cells in M9 minimal media (47.75 mM sodium phosphate, 22 mM potassium phosphate, 8.65 mM sodium chloride, 2 mM magnesium sulfate, 0.1 mM calcium chloride, 0.25% U- ^{13}C -glucose- C_6 (m/v), 18.7 mM $^{15}\text{NH}_4\text{Cl}$, pH 7.2) (Niere et al. 1999). When the OD₆₀₀ reached 0.6, over-expression was induced with the addition of IPTG to a final concentration of 1 mM and the cells were then grown at 37°C for 4 additional hours before harvesting. Cells were removed by centrifugation at 7,000×g for 15 min, and the supernatant was concentrated with a regenerated cellulose membrane (10 kDa) in a stirred ultrafiltration cell (Millipore). ApoLp-III_{GM} was purified by size-exclusion chromatography using Sephadex G-75 resin (GE Healthcare). Further purification was achieved by RP-HPLC (Ultimate 3000) HPLC system, Thermo Scientific) using a Zorbax 300SB-C8 5 μm 9.4×250 mm column (Agilent Technologies). A linear gradient of water and acetonitrile in

0.05% TFA (v/v) was used for protein elution. To remove solvent, the apoLp-III_{GM} fraction was freeze-dried; the protein was then dissolved in 6 M guanidine-HCl and dialyzed against 3 L of 200 mM potassium phosphate buffer, pH 6.1 at 4°C with three additional buffer changes over 48 hours.

The final NMR sample contained 1.2 mM ¹³C/¹⁵N apoLp-III_{GM} in 200 mM potassium phosphate, pH 6.1, 2 mM NaN₃, 0.2 mM DSS, 10% D₂O.

Assignment experiments

All experiments were recorded at 25 °C on a 600 MHz Agilent DD2 spectrometer with a room-temperature probe. Standard assignment experiments included 2D ¹⁵N HSQC, ¹³C aliphatic HSQC, ¹³C aromatic HSQC, Hb(CbCgCd)Hd, and Hb(CbCgCdCe)He spectra, as well as 3D HNCaCb, CbCa(CO)NH, HNHA, HNCO, HCC-TOCSY-NH, CCC-TOCSY-NH, HCCH-TOCSY, aromatic HCCH-TOCSY, ¹⁵N NOESY-HSQC, ¹³C NOESY-HSQC and aromatic NOESY experiments. The ¹³C and aromatic NOESY experiments utilized 100 ms mixing times and the ¹⁵N NOESY experiment utilized a 150 ms mixing time. There were challenges in assignment due to missing resonances and ambiguity due to multiple similar sequence segments. As a result, a number of spectra were recorded in addition to these standard experiments. These included HNHb (Archer et al. 1991), HbHa(CO)NH (which correlates Hβ and Hα at (i-1) with ¹⁵N and ¹H_N at (i))(Grzesiek et al. 1993), HNN (which correlates ¹⁵N and ¹H_N at (i) with ¹⁵N and ¹H_N at (i+1) and ¹⁵N and ¹H_N at (i-1))(Panchal et al. 2001), and TOCSY-HSQC (providing intraresidue correlation of aliphatic ¹H with ¹⁵N and ¹H_N)(Zhang et al. 1994).

Data were processed using NMRPipe v.8.2 (Delaglio et al. 1995) and analyzed with NMRViewJ v.9.0.0-b114 (Johnson 2004; Johnson 2013).

Assignments and Data Deposition

Backbone assignments for apoLp-III_{GM} are 99% complete (not counting the amide nitrogens from the four Pro residues or from the N-terminus). This average is comprised of 100% amide nitrogen and hydrogen, 98% Hα, 100% Cα and 99% carbonyl carbon atoms assigned. An annotated ¹H-¹⁵N HSQC is provided (Fig. 1).

Assignments of side chain resonances (including Cβ and Hβ), at 88%, are somewhat less complete than those of the backbone. The following were not included in the calculation of the total number of side chain atoms: labile protons and their associated heteroatoms (such as amine groups on lysines or hydroxyl groups on serines) with the exception of NH₂ groups on Asn and Gln, methyl groups at the end of Met side chains, carbons that have no attached hydrogens, and groups with degenerate assignments. Many of the unassigned resonances are a result of missing lysine side chain resonances: only 65% of the 17 lysine side chain atoms were successfully assigned. If lysines are excluded from the calculation, side chain assignment of apoLp-III_{GM} is 93% complete.

Although the RunAbout semi-automated chemical shift assignment tool in NMRViewJ (Johnson 2004) was employed initially, it quickly became apparent that the majority of the

assignment of apoLp-III_{GM} would have to be performed manually. This was due to significant resonance overlap in the center of the ¹H-¹⁵N HSQC spectrum (Fig. 1), weak or missing peaks in various 3D experiments, as well as similar or repeated segments of the sequence which required in-depth interpretation and additional experiments in order to determine the correct assignments (Fig. 2a). It is notable that apoLp-III_{GM} contains 17 lysine, 17 glutamine and 24 alanine residues; these three amino acids therefore represent 36% of the overall sequence (compared to 18% on average for all proteins in the July 2015 UniProt database (UniProt 2015)).

TALOS+ analysis (Shen et al. 2009) of the backbone chemical shifts (¹H_N, ¹⁵N, ¹H_α, ¹³C_α and ¹³C_β) predicts that apoLp-III from *Galleria mellonella* contains 5 helices (Fig. 2b), which is consistent with the solved structures of apolipoporphins from other species. These chemical shift assignments constitute the first step in the structural characterization of apoLp-III_{GM}. Studies as described in this report (and the subsequent solution structure which is forthcoming) will provide critical insight into the mechanism by which *G. mellonella* apoLp-III contributes to stimulating immune processes, and may shed light onto parallel activities of apolipoproteins in humans (Oztug et al. 2012).

Backbone and side chain assignments of apoLp-III_{GM} at pH 6.1 have been deposited online at the BMRB (accession number 26638).

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Fig. 1. ^1H - ^{15}N HSQC spectrum of 1.2 mM $^{13}\text{C}/^{15}\text{N}$ apoLp-III_{GM} in 200 mM potassium phosphate buffer, pH 6.1, recorded at 25 °C on a 600 MHz NMR spectrometer. Each peak assignment is labeled with a one-letter code and residue number. Side chain NH_2 groups from Asn and Gln residues are indicated with δ_1/δ_2 and ϵ_1/ϵ_2 , respectively. Insets at the upper and lower right side show enlargements of the side chain amide region and center of the spectrum, respectively, in order to clearly show assignment labels. Backbone (99% complete) and side

chain (88% complete) resonance assignments can be found online at the BMRB repository (BMRB accession number 26638).

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a)

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1 50
DASTPLQDLE KHAAEFQKTF SEQLNAFTNS KDTKEFNTAL KEGSDSVLQQ
51 100
LNALASSLQK ALNDANGKAK EALEQTRTNL ERTAEELRRA HPDVERQAGA
101 150
LRDRLQTAVQ ATVQETQKLA KTVGANLEET NKKLAPQIKS AYDDFVKQAQ
151 163
EVQKKLHEAA SKQ
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Fig. 2.

a) Sequence of apoLp-III_{GM}; b) TALOS+ prediction of protein secondary structure, utilizing $^1\text{H}_\text{N}$, ^{15}N , $^1\text{H}_\alpha$, $^{13}\text{C}_\alpha$ and $^{13}\text{C}_\beta$ chemical shifts (Shen et al. 2009).