NMR study of macro domains (MDs) from betacoronavirus: backbone resonance assignments of SARS–CoV and MERS–CoV MDs in the free and the ADPr-bound state

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Received: 5 July 2021 / Accepted: 11 October 2021 / Published online: 22 October 2021
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Abstract
SARS-CoV and MERS-CoV Macro Domains (MDs) exhibit topological and conformational features that resemble the nsP3b macro (or “X”) domain of SARS-CoV-2. Indeed, all the three domains (SARS-CoV-2, SARS-CoV and MERS-CoV MDs) fold in a three-layer α/β/α sandwich structure, as reported by crystallographic structural investigation of SARS-CoV MD and MERS-CoV MD. These viral MDs are able to bind ADP-ribose as many other MDs from different kingdoms. They have been characterized also as de-ADP-ribosylating enzymes. For this reason, these viral macrodomains recently emerged as important drug targets since they can counteract antiviral ADP-ribosylation mediated by poly-ADP-ribose polymerase (PARPs). Even in presence of the 3D structures of SARS-CoV MD and of MERS-CoV MD, we report herein the almost complete NMR backbone (1H, 13C, 15N) of SARS-CoV MD and MERS-CoV proteins in the free and ADPr bound forms, and the NMR chemical shift-based prediction of their secondary structure elements. These NMR data will help to further understanding of the atomic-level conformational dynamics of these proteins and will allow an extensive screening of small molecules as potential antiviral drugs.

Keywords SARS–CoV · MERS–CoV · Non-structural protein · Solution NMR-spectroscopy · Macro domains · NMR backbone assignment

Biological context

According to a number of reports, the very first case of a severe acute respiratory syndrome (SARS), was initially observed in Guangdong region, a province in China, back in November of 2002. This case was not immediately reported to the World Health Organization (WHO) and it was only one year later when a WHO officer, who examined a patient in Hanoi in Vietnam, reported a significant outbreak to the WHO main office in March of 2003. SARS is a viral respiratory disease, an atypical, highly contagious pneumonia, caused by an airborne virus of the coronavirus (CoV) family, namely SARS–CoV and affected 32 countries over the first half of 2003. CoVs affecting humans are referred to as human coronaviruses (HCoVs) and they usually cause multiple respiratory diseases, such as common cold, pneumonia, bronchitis. CoVs are responsible also for the Middle East respiratory syndrome (MERS) which was reported first in June 2012 in Jeddah, Saudi Arabia. MERS is caused by a coronavirus called Middle East Respiratory Syndrome Coronavirus (MERS–CoV) and till these days the World Health Organization (WHO) has reported approximately 2500 cases in 27 countries and an overall 35% fatality. In general, CoVs can be rapidly transmitted by means of aerosols and they exhibit high mortality rate; thus, making SARS and MERS potential global threats. Although SARS and MERS were put under control, SARS–CoV–2 caused the recent COVID-19 pandemic, and it has affected more than 170 million of people worldwide with more than 3.5 million of deaths.

CoVs are positive-sense, single-stranded RNA viruses featuring the largest viral RNA genomes known to date (Marra et al 2003; Rota et al 2003; Ruan et al. 2003). The SARS–CoV
genome is comprised of ~29,700 nucleotides and its replicate (bearing >27,000 nucleotides) encodes two overlapping polyproteins of 486 kDa (pp1a) and 790 kDa (pp1ab), which are processed by two viral proteases to generate 15 or 16 mature nonstructural proteins (nsPs). These polyproteins mediate or participate in all viral processes which are required for viral replication and transcription (Thiel et al. 2001). The largest nsP is nsP3, a multidomain protein with different functional properties, which contains one or more macro or macro-like domain (Neumann et al. 2008; Tan et al. 2009; Till and Ladurner 2009). MERS–CoV, like SARS–CoV, has a length of about 30 kb, one of the longest amongst RNA viruses and present a similar organization compared to SARS-CoV (Rohaim et al., 2021).

In general macro domains are ancient, highly evolutionary conserved domains, consisting of 150–180 amino acids and initially, they were called as “X–domain” since their function was unknown. Later, they were called as “macro” domains due to their structural homology with MacroH2A histone. They exhibit an α/β/α sandwich-like fold and recent reports revealed that they are characterized by their ability to bind ADP-ribose (ADPr), either as a free molecule or as a tag covalently bound to other proteins, playing thus a significant role in ADPr signaling pathway (Rack et al. 2016). Furthermore, mammalian and other macro domains are also found to be able to enzymatically remove the ADPr from other proteins. Due to this property, these domains have been characterized also as de-ADP-riboseylating enzymes. The viral macromdomains are emerging as important drug targets since they can counteract antiviral ADP-riboseylation mediated by poly-ADP-ribose polymerase (PARPs) (Abraham et al 2018; Fehr et al. 2018).

Additionally, several structural studies on ADPr-free and ADPr-bound macro domains indicate that despite some similarities of the binding mode, each macro domain may exhibit unique ADPr recognition and binding properties because of the adaptability of the macro polypeptide that might tune its binding mode and the affinity for ADPr molecules (Neuvonen et al. 2009; Zapata-Pérez et al. 2017; Ferreira-Ramos et al 2020; Lin et al. 2021). In summary Coronavirus macro domains have the ability to block the innate immune response of the host and separately to promote in vivo replication (Fehr et al. 2016).

The present study reports the almost complete backbone resonance assignment of the SARS–CoV MD and MERS–CoV MD in their free form and in their ADPr bound form.

**Methods and experiments**

**Construct design**

The coding sequences of the SARS–CoV macro domain (residues 183–354 of nsP3) and of the MERS–CoV macro domain (residues 254–425 of nsP3) were obtained as codon optimized for expression in E. coli synthetic genes from GenScript, (Piscataway, NJ), already cloned into pET20b(+) expression vector, containing an N-terminal His6-tag and a C-terminal His6c-tag respectively. The SARS–CoV macro domain contained one artificial N-terminal residue (M-1) and six histidines, preceding the native protein sequence while the one corresponding to MERS–CoV macro domain one artificial N-terminal residue (M-1), four artificial C-terminal residues (LELE) and six histidines.

**Protein expression and uniform ¹⁵N and ¹⁵N/¹³C labeling**

Both macro domains were expressed according to the following procedure. An LB preculture was inoculated with E. cloni® EXPRESS BL21(DE3) (Lucigen) E. coli cells transformed with the plasmid of interest, and was grown overnight at 37 °C, 180 rpm with 1 mg/mL ampicillin. A culture of 0.5 L M9 medium (40 mM Na2HPO4, 22 mM KH2PO4, 8 mM NaCl) containing 0.5 g ¹⁵N labeled NH4Cl and 2 g unlabeled or ¹³C D-glucose 1 mL from a stock solution containing 0.5 mg/mL biotin and 0.5 mg/mL thiamine, 0.5 mL 1 M MgSO4, 0.15 mL 1 M CaCl2, 1 mL solution Q (40 mM HCl, 50 mg/L FeCl2·4H2O, 184 mg/L CaCl2·2H2O, 64 mg/L H2BO3, 18 mg/L CuCl2·6H2O, 4 mg/L CuCl2·2H2O, 340 mg/L ZnCl2, 710 mg/L Na2MoO4·2H2O, 40 mg/L MnCl2·4H2O), and 0.1 mg/mL ampicillin was inoculated with the preculture. When the OD₆₀₀ reached 0.6–0.8, IPTG was added to final concentration of 1 mM and the culture was incubated at 18 °C. Sixteen hours (16 h) after induction the cells were harvested by centrifugation.

**Protein purification and sample preparation**

The cell pellet was re-suspended with 25 mL lysis buffer containing 10 mM imidazole, 50 mM Tris–HCl pH 8, 500 mM NaCl and 25 mL of protease inhibitor cocktail (Sigma Aldrich® P8849) and was sonicated (PMisonix®, Sonicator 4000). In case of SARS-CoV before the sonication step the suspension was frozen in liquid nitrogen followed by immersion in a water bath at 42 °C, 3 times. After the sonication took place, 25 mL DNase (1 mg/mL) were added to the suspension and it was incubated for 10 min on ice, then it was centrifuged at 4 °C and 20,000 rpm (Thermo Scientific®, Sorvall Lynx 6000) for 30 min. The soluble fraction containing the His₆c-tagged macro domain was loaded onto a HisTrap™ FF affinity column (GE Healthcare) that had been previously equilibrated with 0.1 M NiSO₄·6H₂O and binding buffer (10 mM imidazole, 50 mM Tris–HCl pH 8, 500 mM NaCl). The column
was washed with a step gradient of imidazole in binding buffer (10, 20, 40, 100, 200, 400 mM). Macro domains eluted mostly in 100 mM imidazole as it was verified by a 15% SDS-PAGE gel. Using an Amicon® Ultra 15 mL Centrifugal Filter membrane (nominal molecular weight cutoff 10 kDa) the protein was concentrated to final volume of 1 mL, and as well buffer exchange was performed, from the elution buffer to 10 mM HEPES, pH 7, 20 mM NaCl, 2 mM EDTA, 2 mM DTT. As a final step, size exclusion chromatography was performed using Superdex 75 Increase 10/300 GL GE Healthcare column. The fractions containing the pure protein were pooled together and were concentrated to final volume of 500 mL. The final NMR sample was prepared by adding 10% D2O and 0.25 mM DSS.

Data acquisition, processing and assignment

Protein NMR samples in the free and ADPr bound forms of SARS–CoV MD and MERS–CoV MD were prepared in 500 μL buffer at pH 7.0 containing 10 mM HEPES, 20 mM NaCl, 10% D2O, 2 mM DTT, 2 mM EDTA, 2 mM NaN3, protease inhibitor cocktail (Sigma Aldrich® P8849) and 0.25 mM DSS (4,4-dimethyl-4-silapentane-1-sulfonic acid) as internal 1H chemical shift standard. 13C and 15N chemical shifts were referenced indirectly to the 1H standard using a conversion factor derived from the ratio of NMR frequencies (Wishart et al. 1995). The NMR sample protein concentration was 0.54 mM for SARS–CoV MD and 0.8 mM for MERS–CoV MD in the free form and 0.65 mM for SARS–CoV MD and 0.6 mM for MERS–CoV MD in the ADPr bound form (protein to ADPr ratio 1:10). All NMR experiments were recorded at 298 K on a Bruker Avance III High-Definition four-channel 700 MHz NMR spectrometer equipped with a cryogenically cooled 5 mm 1H/13C/ 15N/D Z-gradient probe (TCI). The acquired NMR experiments used for sequence specific assignment are summarized in Table 1. Backbone assignments for SARS–CoV and MERS–CoV MDs in the free and in the ADPr bound form were obtained from the following series of heteronuclear experiments: 2D [1H,15N]–HSQC and 2D [1H,15N]–TROSY, 3D HN(CO)CA, 3D HN, 3D TROSY CBCA(CO)NH, 3D TROSY CBCANH, 3D HN(CA)CO, 3D HNCO, 3D HBHA(CO)NH (Table 1). All NMR data were processed with TOPSPIN 4.1.1 and analyzed with CARA 1.9.2a4 (Keller 2004).

Extent of assignments and data deposition

The two macro domains of SARS–CoV and MERS–CoV shared a highly sequence homology as shown in Fig. 1, indeed the percentage of identity is about 44%. The sequence homology is 73% between SARS–CoV MD and SARS–CoV–2 MD. The high percentage of identity suggest a similar fold for all these MDs.

The 2D 1H, 15N-HSQC spectrum shows well-dispersed amide signals as shown in Fig. 2a for free form of SARS–CoV MD and in Fig. 2b for ADPr-bound form of SARS–CoV MD, respectively.

For the free form of SARS–CoV MD we assigned 96.8% of the resonances of the backbone atoms (HN, N, CO, Cα and Cβ). The unassigned HN and N resonances of SARS–CoV belong to G228, G229, G230, V231 and I313 and the N of all the prolines (detectable only through 13C direct detection experiments). All the missing residues belong to loop regions or to unstructured regions or part of loops indicating some conformation variability and flexibility that hampers their detection. The ADPr bound we were not able to assign only a fraction of the backbone resonances (assigned 86.6%). The signals lost are belonging to same region that were hard to identify in SARS–CoV-2 MD, as well (Cantini et al 2020). Specifically, for the segments spanning the residues A221 to K237, I313 and F314, we could not detect and assign any signals. The disappearance of the above-mentioned set of resonances might suggest conformational variability and flexibility upon binding.

For the free form of MERS–CoV MD we assigned 98.5% of the resonances of the backbone atoms (HN, N, CO, Cα and Cβ). The unassigned backbone resonances of

| Table 1 | List of NMR experiments acquired, including the main parameters used, to perform the sequence specific assignment of the backbone SARS–CoV MD and MERS–CoV MD in the free and ADPr bound form |
|---------|---------------------------------------------------------------------------------|
| Time domain data size (points) | Spectral width (ppm) | ns | Delay time (s) |
| t1 | t2 | t3 | F1 | F2 | F3 |
| 1H–15N HSQC | 512 | 2048 | 44.0 (15N) | 16.0 (1H) | 8 | 1.0 |
| 1H–15N TROSY | 512 | 2048 | 40.0 (15N) | 14.0 (1H) | 2 | 1.0 |
| TROSY-HN(CO)CA | 96 | 40 | 1024 | 72.0 (15N) | 44.0 (15N) | 14.0 (1H) | 16 | 1.0 |
| TROSY-HNCACB | 96 | 40 | 1024 | 72.0 (15N) | 44.0 (15N) | 14.0 (1H) | 16 | 1.0 |
| HN(CA)CO | 64 | 40 | 1024 | 18.0 (13C) | 44.0 (15N) | 14.0 (1H) | 8 | 1.0 |
| HNCO | 64 | 40 | 1024 | 18.0 (13C) | 44.0 (15N) | 14.0 (1H) | 8 | 1.0 |
| HN(CA)CA | 80 | 40 | 1024 | 42.0 (13C) | 44.0 (15N) | 14.0 (1H) | 8 | 1.0 |
MERS–CoV are the N of all the prolines (detectable only through $^{13}$C direct detected experiments), H299, G300, G301, and G302 and last residue Q425 of the native aminoacidic sequence (Fig. 3a). For the ADPr-bound form, we were able to assign almost the entire backbone (95.7%) missing only the same set of resonances as in the free form (Fig. 3b). Even in the bound form the three glycines (G300, G301, and G302) and the two adjacent residues (H299, I303) are missing. All these missing residues belong to a very conserved loop that is broadened beyond detection in both forms and for this loop this suggests conformational variability and flexibility upon binding. This is comparable with previously assigned MERS–CoV macro domain proteins in the free (BMRB_ID 26657) and bound (BMRB_ID 50393) forms even though the previously deposited assignments are reported in different buffer and acquisition conditions (Huang et al. 2016; Lin et al. 2021).

Secondary structure prediction for both MDs in their free and bound form (SARS–CoV MD and MERS–CoV MD) has been obtained by using chemical shift assignments of five atoms (HN, Ha, Ca, Cβ, CO, N) for each residue in the sequence running the TALOS + software (Shen et al. 2009). The secondary structure elements for free SARS–CoV MD protein (172 a.a.) are organized α/β/α sandwich-like fold as follows from N- to C-terminal residues of the native sequence: β/β/α/β/α/β/α/β (Fig. 4). The order of the secondary structure segments corresponds almost exactly to that of the other viral MDs (Melektis et al. 2015; Makrynitsa et al. 2015; Lykouras et al. 2018). Similarly free MERS–CoV MD protein (172 a.a.) has α/β/α sandwich-like fold which compares almost exactly with other SARS-CoV MD (Fig. 5). We also report that upon interaction with ADPr no change in secondary structure elements has been noted in both MDs.

These proteins have a high degree of similarity of secondary structure identity in comparison with SARS–CoV 2 MD and other viral MDs (i.e., MAYV and VEEV) (Tsika et al. 2019; Makrynitsa et al. 2019). The dihedral angles predicted by TALOS + for free SARS–CoV MD and MERS–CoV and their respective ADP-ribose bound forms are in excellent agreement with the dihedral angles found in the free (2ACF for SARS–CoV MD and 5HIH for MERS–CoV MD) and ADPr bound (2FAV for SARS–CoV MD and 5HOL for MERS–CoV MD) crystal structures (Cho et al. 2016), implying that ligand binding does not alter the overall the secondary structure within the MDs.

Chemical shift values for the $^1$H, $^{13}$C and $^{15}$N resonances of SARS–CoV and MERS–CoV macro domains in the free state and in the ADPr bound state have been deposited at the BioMagResBank (https://www.bmrbr.wisc.edu) under accession numbers 50993, 50970, 50969 and 50971, respectively.
Fig. 2 700 MHz $^1$H, $^{15}$N–HSQC assigned spectrum of the 0.54 mM $^{13}$C, $^{15}$N–labelled SARS-CoV MD in the free state (a) and 0.65 mM $^{13}$C, $^{15}$N–labelled SARS-CoV MD with the ADPr bound (b) in 10 mM HEPES pH 7.0, 20 mM NaCl, 2 mM EDTA, 2 mM DTT, 0.25 mM DSS and 10% D$_2$O acquired at 298 K. Amino acid numbering is according to the sequence of the multi-domain non-structural protein 3 (nsP3).
Fig. 3  700 MHz $^1$H, $^{15}$N–HSQC assigned spectrum of the 0.8 mM $^{13}$C, $^{15}$N–labelled MERS-CoV MD in the free state (a) and 0.6 mM $^{13}$C, $^{15}$N–labelled MERS-CoV MD with the ADPr bound (b) in 10 mM HEPES pH 7.0, 20 mM NaCl, 2 mM EDTA, 2 mM DTT, 0.25 mM DSS and 10% D$_2$O acquired at 298 K. Amino acid numbering is according to the sequence of the multi-domain non-structural protein 3 (nsP3)
Acknowledgements This work was supported by the INSPIRED (MIS 5002550) which is implemented under the Action ‘Reinforcement of the Research and Innovation Infrastructure,’ funded by the Operational Program ‘Competitiveness, Entrepreneurship and Innovation’ (NSRF 2014–2020) and co-financed by Greece and the European Union (European Regional Development Fund). EU FP7 REGPOT CT-2011-285950—“SEE-DRUG” project is acknowledged for the purchase of UPAT’s 700 MHz NMR equipment.

Funding The research was funded by General Secretariat for Research and Technology & EU - NSRF 2014–2020, Grant No (INSPIRED MIS 5002550), FP7 Research Potential of Convergence Regions, Grant No (EU FP7 REGPOT CT-2011-285950 – “SEE-DRUG”).

Declarations

Conflict of interest The authors declare no conflict of interest.

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