Preferential domain orientation of HMGB2 determined by the weak intramolecular interactions mediated by the inter-domain linker

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Abbreviations used:

HSQC, heteronuclear single quantum coherence; TROSY, transverse relaxation optimized spectroscopy;
NOE, nuclear Overhauser effect; IPAP-HSQC, in-phase and anti-phase HSQC; RDC, residual dipolar
coupling; SAXS, small-angle X-ray scattering; DIORITE, determination of the induced orientation by
troty experiments

Abstract

High mobility group box protein 2 (HMGB2) contains homologous tandem HMG box DNA-binding domains, boxes A and B. These two boxes are linked by a short basic linker having a sequence characteristic of an intrinsically disordered element. The combined use of NMR and small angle X-ray scattering (SAXS) showed that the two boxes assume a preferred orientation to make their DNA binding surface in opposite directions, although the linker does not keep any specific conformation. A series of site directed mutations to the residues in the linker showed that a network of CH- π interactions connects the N-terminal part of the linker to box A. The mutants having impaired intramolecular CH- π interactions changed the interdomain dynamics and their dynamic averaged orientation relative to the wild-type. This work demonstrates that the apparently unstructured linker plays a role in defining the preferential domain orientation through the intramolecular CH- π interactions, even though the interactions are weak and transient.

Keywords: high-mobility group protein; weak alignment; DIORITE; NMR spectroscopy; CH- π interaction

1. Introduction

Accurate view on the conformational state of the modular protein having the folding units joined by apparently unstructured short linkers should be essential for understanding its function. The type of proteins including intrinsically unstructured parts plays pivotal roles in biological processes through its structural flexibility that allows adopting various forms in binding to partner molecules [1]. In elucidating how the proteins work by using its structural plasticity, we have to see how the modular proteins exist in solution. The high resolution techniques do not give the right insights in the particular regards. X-ray crystallography may determine the conformation of the modular protein whose domain arraignment is artificially defined by crystal packing, in addition to the lack of the electron density for the flexible linker parts. NMR can give solution structures of such protein, but NOE-based distance restrains are often scarce in the dynamic interdomain linker, thus the obtained domain arrangements do not represent the realistic conformational state.

Alternative approaches are available for the structural elucidation of the modular proteins. Another type of NMR experiment using residual dipolar couplings (RDCs) provides long-range structural information that defines the relative domain orientation and interdomain dynamics [2-8]. Small angle X-ray scattering (SAXS) is also used for the same purpose by its ability to give molecular shape information [9].

Independent use of each approach, however, cannot accurately determine the structure of modular protein. The RDCs define the domain orientation but cannot give the inter-domain distance. SAXS gives rather limited structural details due to its intrinsically low resolution, in particular for the cases of medium sized proteins, although it can accurately determine the molecular radii [9]. NMR with the RDCs and SAXS are, therefore, the complementary methods in determining the modular protein structure determination [2,3,10].

High mobility group box 2 (HMGB2) protein consists of two DNA-binding domains, HMG boxes A and B and a short inter-domain linker ('l-region'), which connects the HMG boxes (Fig. 1a). HMGB2 and its homologous protein HMGB1 are non-histone chromosomal proteins abundant in eukaryotic cells [11-13]. HMGB1 and 2 share high sequence homology with their orthologues (Fig. 1a). The preceding works have solved the structures for the two HMG boxes in HMGB1, both of which showed characteristic 'L-shaped' structures [14,15]. The roles for each HMG boxes were characterized; box A has preferential affinity to the deformed DNA structure, while the box B shows strong DNA binding and bending ability [16,17]. The tandem aligned HMG boxes with different DNA binding ability have cooperative functions in

DNA binding and inducing DNA bending [18-20]. The accumulated insights into the functional cooperation of the boxes in HMGB1 and 2 suggest that the spatial arrangement of the boxes has some functional meanings, which prompted us to see how the boxes in HMGB2 stay in solution with the combined use of NMR and SAXS.

In this work, we found the interdomain linker defines the relative domain orientation of the HMG boxes. A series of site-directed mutagenesis to the linker showed that a part of the linker has specific contact with box A; which is mediated by a network of CH- π interactions [21] as elucidated by the box A structure in HMGB1 in comparison with the counterpart in HMGB2 determined in this work [15]. HMG boxes in the wild-type HMGB2 showed preferential orientation to locate their DNA binding surfaces in the opposite side of the molecule. The interdomain linker mutants having impaired CH- π interactions changed the relative domain orientation with increased interdomain dynamics. This finding adds another functional role associated with the interdomain linker of HMGB2.

2. Materials and Methods

2.1. Plasmids

The cDNA encoding porcine HMGB2 was provided by Prof. Yoshida. The fragments of HMGB2 used in this work are schematically described in Fig. 1b; HMGB2-A (residues 1-77), HMGB2-B (88-165), HMGB2-AI (1-87) and HMGB2-AIB (1-165). **The box A used in this work has C23S mutation to improve the sample stability according to the previous work [15].** The gene encoding each fragment was cloned into pET28a (Merck Chemicals, Germany) using *NdeI* and *EcoRI* sites. Site-directed mutagenesis of HMGB2 was performed using QuickChange (Stratagene) and KOD-plus mutagenesis (Toyobo), to make the following mutants (P80G/P81G, K82G, D84G, K85G, K86G and Y78G).

2.2. Expression and purification of HMGB2 fragments

Each HMGB2 fragment was purified from *E. coli* grown in M9 minimal medium. For backbone assignment, medium also contained $^{15}\text{NH}_4\text{Cl}$ and ^{13}C glucose. The purification procedure is briefly described below. Cells were grown at 37°C to $\text{OD}_{600} = 0.6$ and IPTG (isopropyl β -D-thiogalactopyranoside) was then added to the medium to induce protein expression (final conc. 0.5 mM). After induction, cell growth was continued for 5 hours. Cells were collected by centrifugation and resuspended in buffer

solution (50 mM Tris-HCl, pH 8.0; solution A), and then subjected to sonication. Cell debris was removed by centrifugation (15,000 rpm for 30 min) and supernatant was applied to a HisTrap affinity column (GE Healthcare). The column was washed with solution A containing 20 mM imidazole followed by the elution of the His-tagged HMGB2 fragment by solution A containing 500 mM imidazole. The His-tag was cleaved by thrombin for 12 hours at 4°C in a dialysis bag in solution A. The resultant protein was applied to Hi-Trap SP cation-exchanger equilibrated by solution A. The desired protein was purified using a linear NaCl concentration gradient from 0 mM to 800 mM in solution A. The purified protein was dialyzed against buffer solution (50 mM sodium phosphate, pH 6.4) prior to use in NMR experiments. All HMGB2 fragments used in this work were obtained using the above protocol. A three-residue attachment from the construct, having the sequence, GSH, is attached to the N-terminal part of the proteins used.

2.3. NMR experiments

NMR experiments were performed using solutions typically containing 1 mM protein; the buffer solution used was 50 mM sodium phosphate, pH 6.4, containing 1 mM Pefabloc (Sinus Biochemistry and Electrophoresis GmbH). All experiments were performed at 293 K or 298 K on a Bruker DMX600 or DRX700 spectrometer. The backbone resonance assignments were determined for the fragments HMGB2-A, HMGB2-B, HMGB2-A1, and HMGB2-A1B using a standard set of triple resonance spectra [22] collected in a non-uniform sampling manner [23] on a DMX600 spectrometer. 3D ¹⁵N-edited NOESY (mixing time 100 msec) and ¹⁵N-edited TOCSY (mixing time 40 msec) were also used for confirming the backbone resonance assignments [22]. The triple-resonance data were processed with the Roland NMR toolkit[24]. The ¹⁵N-edited NOESY and TOCSY data were processed using the program, NMRPipe [25]. The backbone resonance assignments were determined on the NMRview software platform [26] using the KUIRA suites [27].

Structures of the boxes A and B of HMGB2 were determined in a conventional manner using CYANA [28] and XPLOR-NIH [29]. The structure statistics are shown in Tables S1 and S2: the overlaid 30 structures and their energy minimized averaged structure are displayed in Fig. S1. The chemical shift assignments for the atoms in the boxes A and B of HMGB2 were deposited to the Biological Magnetic Resonance Bank (BMRB): codes for box A and B are 11429 and 11430, respectively. The structures were registered in the Protein Data Bank (PDB): the accession codes are 1J3X (box A) and 1J3D (box B).

2.4. SAXS experiments

SAXS measurements were carried out at BL-10C, Photon Factory, Tsukuba, Japan [30]. The wavelength of the X-ray was adjusted to 1.488 Å using a Si monochromator. All samples were prepared in 50 mM sodium phosphate, pH 6.4, containing 5 mM DTT. The scattering profiles were collected at 20°C using an on-line imaging plate detector (R-Axis VII, Rigaku). The exposure time was 10 min. The obtained two-dimensional data were circularly averaged, and then the final one-dimensional data was obtained by subtracting the profile of the sample buffer solution. To eliminate inter-particle interference from the observed profiles, a series of the profiles at five different concentrations, ranging from 17.5 to 3.5 mg/ml, were extrapolated to obtain that at a concentration of zero.

2.5. Determining the alignment tensors for the HMG domains in HMGB2

The relative orientation of the HMG domains in the HMGB2-A1B fragment was estimated based on the alignment tensors obtained for each domain. The alignment induced TROSY shift changes were used to determine the alignment tensor for each HMG domain, which is referred to as DIORITE [5,31]. Because of the ‘L-shape’ of each HMG domain and the entirely extended structure of the HMGB2-A1B fragment, which has two HMG domains, the signals showed severe broadening under weak-alignment conditions; most of the anti-TROSY components on the IPAP-HSQC [32] spectra for the HMGB2-A1B fragment showed poor signals or disappeared. The alignment tensor determination was done according to the procedure described in our previous paper using optimized ^{15}N CSA tensor parameters [31]. The ^{15}N CSA tensor used for the residues in α -helix was $\Delta\sigma = -173.0$ ppm, $\eta = 0.23$, and $\beta = 19.8^\circ$. In the present analysis, the ^{15}N CSA tensor value that is specific for α -helix was used.

The weak-alignment of proteins was achieved using stretched acrylamide gels[33]. Acrylamide and *N,N'*-methylenebisacrylamide (bis-acrylamide) copolymer was prepared at a monomer concentration of 5% with a 75:1 molar ratio of acrylamide to bis-acrylamide. Polymerization was achieved using 0.05% (w/v) ammonium persulfate (APS) and 0.2% (v/v) *N,N,N',N'*-tetramethylethane-1,2-diamine (TEMED). One gel was cast in a Teflon block to make a rod of 4 mm diameter, which was used as the ‘reference gel’. The other gel was a rod with an ellipsoidal cross-section having 7 mm and 5 mm diameters, which was used as the ‘aligning gel’. The gels were extensively dialyzed against buffer (50 mM sodium phosphate,

pH 6.4), for over three days to completely remove residual reagents from polymerization. Gels were then soaked in 250 μ l protein solution containing 1 mM protein. The solutions were incubated at 4°C for three days to allow complete uptake of protein into the gel rod. Each gel was inserted into an NMR tube with open ends (New Era) using a Teflon device having a tapered head [34]. The NMR tube was sealed at one end with an Ultem plug (New Era). At the other end, 50 μ l sample solution was poured onto the gel and sealed with an inner glass rod from a Shigemi micro NMR tube (Shigemi). The NMR tube was then incubated for five days at 4°C to allow it to achieve a steady-state gel length. After adjusting the inner rod position to exclude the solution squeezed from the gel, the sample was subjected to NMR measurement.

Before TROSY measurements, the ^2H split was measured to correct the chemical shift drift caused by residual quadrupole splitting of the ^2H signal used for the frequency lock. In the HMGB2-AIB fragment experiments, the residual quadrupole ^2H split was kept around 2 Hz for the weakly aligned samples. The reference chemical shifts were measured for the sample in the reference gel, where protein is not aligned but is dissolved in the same acrylamide non-stretched gel. It was confirmed that no ^2H split was observed for the sample solution in the reference gel.

The NMR refined structure coordinate HMGB2-A and -B domains were used for the alignment tensor calculation; PDB codes for boxes A and B are 1J3X and 1J3D, respectively.

The error estimation for the obtained tensor parameters was done by the jack-knife method [35]; 128 times with 10% of the experimental data reduction at random in each calculation. In random number generation, ‘Mersenne twister’ algorithm [36] was used to improve the sampling.

2.6. Rigid-body minimization

The overall structure of the wild-type and Y78G mutant HMGB2-AIB were determined using the $\Delta\delta_{\text{TROSY}}$ and SAXS data using XPLOR-NIH 2.31 software platform [37]: $\Delta\delta_{\text{TROSY}}$ values were incorporated into the calculation as pseudo CSA (PCSA) restraints for amide nitrogen atoms [38], which assumes the ^{15}N CSA to the above values for the α -helix part. The used python routine was generated with modifications from the one for CSA restraints in XPLOR-NIH. In the calculation, the structures for boxes A and B were treated as ‘rigid bodies’ by keeping the coordinates fixed, while the residues in the N-terminal and inter domain linkers were allowed to have free dihedral angle rotations during the calculation [6,7]. In the

calculation, a low-temperature torsion angle dynamics simulated annealing, followed by a standard conjugate gradient minimization [6,7]. The calculation procedure used for determining the overall structure of HMGB2-AIB with the residual PCSA (RPCSA) and SAXS scattering data as structural constraints followed the preceding work [39].

The initial structures for the wild-type and Y78G mutant, which were subjected to the rigid-body refinement, were generated in the following way. The box A in the wild-type HMGB2-AIB was generated from the box A in HMGB1 (PDB: 1AAB) [15] by the homology modeling with the program MODELLER 9v8 [40]; the generated box A structure has altered orientation in the third helix and attached C-terminal segment comprising residues 78 — 84. In the case of the Y78G mutant, the box A NMR structure (residues 1—77) was used without any modifications. The box B structure (residues 88—165) was the NMR structure determined in this work. The HMGB2-AIB structure coordinate was generated by combining the boxes A and B with the computer generated interdomain linker (residues 85—87 for the wild-type, and residues 78—87 for the Y78G mutant) followed by energy minimization by XPLOR-NIH.

2.7. Linker peptide titration experiments

Synthetic peptide having the same sequence as the linker, Ac-YVPPKGDKKG-NH₂, was purchased in an HPLC purified grade (GL Biochem, Shanghai, China). The peptide was dissolved in NMR buffer (50 mM sodium phosphate, pH 6.4), and its pH was adjusted to pH 6.4 using 0.1 M NaOH solution. The peptide was titrated against ¹⁵N-labeled HMGB2-A or HMGB2-AIB fragment solutions at 0.7 mM protein. A series of HSQC spectra were collected for the samples with different molar ratios of the peptide (0.2, 0.5, 1.0, 1.5, 2.0 and 4.0) against the HMGB2 fragments.

3. Results and Discussion

3.1. Specific interaction of the interdomain linker with box A revealed by chemical shift changes

The primary sequence of boxes A and B in HMGB2 are shown with those of the homologous HMGB1 proteins and the orthologues (Fig. 1a): the boxes A and B domains consist of the residues 1—77 and 88—165, respectively (Fig. 1b) [16]. It is noted that the residue number adopted here is for the immature HMGB proteins having initial methionine.

The sequence position of the box A in this work is different from that defined in the homologous

HMGB1 box A (residues 1—84) [15]; the present box A position was defined according to the original report on HMGB2 by Yoshida and co-workers [16,17]. The box A in HMGB2 (residues 1—77) was determined based on the HMGB1 box A structure [14,41]: the HMGB1 box A has the structured part ended at T77 and the following segment, residues from Y78 to E84, is rich in amino acids of Pro, Gly, and Lys, which segment can be recognized as an unstructured linker due to its characteristic amino acid compositions (Fig. 1a) [42]. In also considering the box B structure [14], the part of the residues 78—87 in HMGB2 was defined as the linterdomain linker in this work (Fig. 1a, shown in green). The box B position in HMGB2 was defined as for the case of HMGB1 [14]. The fragments used in this work are schematically summarized with domain structures of the entire protein (Fig. 1b); the interdomain linker is denoted as ‘I’, and the ‘A1’ stands for the fragment having the domain A with the interdomain linker, for example.

The ^1H - ^{15}N heteronuclear single quantum coherence (HSQC) spectra for the fragments HMGB2-A, B and A1B are overlaid for comparison (Fig. 2a): each spectrum with resonance assignments is available as Supplementary material (Fig. S2). The spectral comparison showed the significant differences between the signals for HMGB2-A and the box A in HMGB2-A1B, while the signals for HMGB2-B mostly overlapped to those of the box B in HMGB2-A1B (Figs. 2a, 2b). The observed chemical shift differences are plotted against the residue number of HMGB2-A1B (Fig. 2c). The chemical shift changes were observed in the limited parts of box A, which may demonstrate that the inter-domain linker, Y⁷⁸VPPKGDKKG⁸⁷, specifically interacts with the N- and C- termini of the box A. The residues showing the changes over the average plus one standard deviation (σ), and those in the rage from an average + 0.5 σ to + 1.0 σ are marked in red and orange on the HMGB2-A structure, respectively (Fig. 3a): the HMGB2-A structure was determined in this work (Fig. S1a).

3.2. Estimation of the affinity of the interdomain linker to the box A using a synthetic peptide

We elucidated the affinity of the inter-domain linker to the box A by the NMR titration experiments using a synthetic peptide of the sequence, Ac-YVPPKGDKKG-NH₂. The four equimolar amount of the peptide input to ^{15}N -labeled HMGB2-A caused no apparent spectral change (Fig. S3). The linker has, thus, only limited affinity to the box A; the K_D should be greater than 2×10^{-4} M, in assuming that a 1% population of the bound form gives observable spectral changes.

The same peptide was titrated to the ^{15}N labeled HMGB2-A1B fragment, which also caused no

apparent spectral changes (data not shown).

The results showed that the interdomain linker has limited affinity to the box A in HMGB2; its tethering to the box A is essential to cause the specific contact of the linker with the parts in the box A, which showed the significant spectral changes.

3.3. The interdomain linker contact to box A is primarily mediated by Y78

The spectral comparison between the HMGB2-A1B and A1 fragments has shown that the signals for HMGB2-A1 mostly overlapped onto the corresponding part of the signals for HMGB2-A1B (Figs. S4a and S4b). Plot for the chemical shift changes along the residue number demonstrated no apparent spectral differences for the box A part between HMGB2-A1 and HMGB2-A1B fragments (Fig. S4c). In focusing on the residues in the interdomain linker, two amide-containing residues (Y⁷⁸V⁷⁹) in the N-terminal four-residue segment, Y⁷⁸VPP⁸¹, showed the signals resonating very close to those of the corresponding part in the A1B fragment (Fig. S4c). The two residues are in remarkable contrast to the following interdomain linker residues, 82—87, showing apparent spectral changes; the changes should be ascribed to the end-effect by the truncation of the following part originally existing in the A1B (Fig. S4c). Although the four-residue segment, residues 78—81, is apparently unstructured in the HMGB1 box-A structure (Fig. 3b) [15], the spectral comparison between the A1 and A1B fragments shows the N-terminal two residues, at least, in the interdomain linker should specifically contact to the box A, irrespective of the existence of the following part (Fig. S4c).

A series of the site-directed mutation to the residues in the interdomain linker was done to identify the residues primarily engaged in the contact to box A (Fig. S5). The results demonstrated that the Y78G mutation mostly reproduced the spectral changes observed for the spectral comparison between the HMGB2-A1B and HMGB2-A fragments (Figs. 4b and 2c); P80G/P81G mutation, also caused spectral changes, but the changes were rather limited to the C-terminal part of the box A (Fig. 4a). Y78 was, thus, identified as the primarily responsible residue for the interdomain linker contact to the box A.

3.4. Structural insight into the interdomain linker contact to box A

The structural details were explored in respect to the interaction between the interdomain linker and the box A (Fig. 3b). HMGB2-A lacks the segment (Y⁷⁸VPPK⁸²) that follows the structured part, while the box

A in HMGB1 has the C-terminal extension as a part of the interdomain linker (Y⁷⁸IPPKE⁸⁴) (PDB accession code: 1AAB) (Fig. 3c) [15]. HMGB1 and 2 have high sequence identity (Fig. 1a); the HMGB1 box A, residues 1—84, has only 11 different residues against the box A in HMGB2 (87% sequence identity) (Fig. 1a). The high sequence identity allows for exploring the binding mode of the interdomain segment, Y⁷⁸VPPK⁸², to the box A through the structural comparison of the boxes A in HMGB1 and 2 (Figs. 3a and 3b).

The five-residue segment, Y⁷⁸VPPK⁸², in the HMGB1 box A contacts the N-terminal part through Y78 (Fig. 3b). The side chain contacts in the NMR structure suggest that they are mediated through CH- π interactions; Y78 ring moiety may play as an acceptor π -system for the CH donors in P80, K8 and P9 (Fig. 3d). Similarly, Y71 may also be in the CH- π interactions with P9 and K12 (Fig. 3d); the Y71 mediating interactions were noted as hydrophobic contacts in the original report on the structure of box A in HMGB1 (Fig. 3b) [15]. Because Y78 is in the floppy part in the overlaid structure presentation, the intramolecular interactions mediated by Y78 were not focused in that original report [15].

The CH- π interaction is a type of non-bonded interaction giving a stabilization energy of about 0.5—1.0 kcal/mol per interaction, being comparable to single hydrogen bond; CH- π interaction is formed between aliphatic CH donors and aromatic π -acceptors as in tyrosine-rings [21]. The structural details in the atomic interactions associated with Y71 and Y78 are summarized in Table S3, which demonstrate that they are recognized as CH- π interactions according to the criteria defined in previous report [21].

The role of Y78 found in the HMGB1 box A structure, which mediates the interactions between the interdomain linker and box A, consistently explains the chemical shift changes by Y78G in HMGB2-A1 fragment (Fig. 4b): the spectral changes caused by the lack of the C-terminal part, after Y78, to the residues in the box A in HMGB2 were mostly reproduced by the Y78G mutation to the HMGB2-A1 (Figs. 2c and 4b). The residues showing the significant spectral changes by Y78G are consistent with the residues in the CH- π interaction network (Figs. 3a and 3b).

Y78 in the unstructured interdomain linker, therefore, may direct the linker and the C-terminal half of the third helix of the box A in HMGB2, as expected by the structure comparison (Fig. 3c). It should be noted that the interdomain segment of the residues 78—84 in HMGB1 box A is not fixed in that conformation, but it may be in a dynamic equilibrium between the bound and unbound states (Fig. 3c): the

distance restraints from NOEs bias the contact form of the segment. The structure fluctuation is evident for the interdomain segment by the low $^{15}\text{N}\{^1\text{H}\}$ NOE values, less than 0.5 (Fig. 4). The Y78 mediated interactions to direct the interdomain segment, therefore, should be weak and transient.

The role of Y78 in connecting the interdomain segment to box A was confirmed by the ^{15}N edited NOESY spectra (Fig. S6): the NOE signal between K8 amide proton and Y78 ring proton (H_ϵ) was observed in the HMGB2-A1 and the wild-type HMGB2-A1B (Figs. S6a and S6b), whilst the corresponding NOE signal was absent in the HMGB2-A1B (Y78G) mutant (Fig. S6c). It is noted that the NOE intensity observed between the residues K8 and Y78 in the wild-type HMGB2-A1B was extremely small relative to the sequential NOE between amide protons of K7 and K8, which may also suggest that the Y78 mediated interdomain segment contact to box A is transient (Fig. S6b).

3.4. The change in the dynamics of the interdomain linker by the mutation to Y78

The engagement of Y78 in the transient interactions of the interdomain linker to box A was found as described above. The changes in the dynamics, in psec-nsec time range, of the interdomain linker by Y78G and P80G/P81G mutation were elucidated by $^{15}\text{N}\{^1\text{H}\}$ NOEs (Fig. 5).

The P80G/P81G mutation slightly increased the order of the interdomain linker and also that of the C-terminal segment downstream of the third helix (residues 72 – 77) but significantly reduced the order of G4 (Fig. 5a). The unexpected increase in the order of the interdomain linker may be related to the intrinsically elongated shape of the polyglycine sequence as revealed by SAXS analysis [43]. The Y78G mutant reduced the order of the linker and the part of the residues 69—77 following the third helix (Fig. 5b).

The change in the $^{15}\text{N}\{^1\text{H}\}$ NOE profile for the Y78G mutant demonstrated that the impaired CH- π network mediated by Y78 increased the flexibility of the linker segment (Fig. 5b). The loss of the Y78 mediated interactions should have made the closed contact forms between the linker and the box A less populated. Although the Y78 mediated CH- π interactions have limited stabilization energies, the lack of them significantly increased the flexibility of the linker.

3.5. The altered reorientation dynamics of the tandem HMG boxes caused by linker mutation

The changes in the fluctuation of the interdomain linker by mutations, P80G/P81G and Y78G, should alter

the interdomain dynamics in HMGB2-A1B. The interdomain dynamics was elucidated through the alignment tensors determined by the residual pseudo- ^{15}N CSA (RPCSA), which are measured as the TROSY chemical shift differences between the isotropic and aligned states [4,5,31,38].

The observed TROSY shift changes, $\Delta\delta_{\text{TROSY}}$, induced by weak-alignment are shown on the overlaid spectra for the wild-type, P80G/P81G and Y78G mutants (Figs. 6a-c, left). The correlations between the observed and back calculated $\Delta\delta_{\text{TROSY}}$ values using the alignment tensors for the boxes A and B are also shown (Figs. 6a-c, right). In considering the possible structural difference between the isolated box A lacking the C-terminal segment Y⁷⁸VPPK⁸² and the box A in the HMGB2-A1B (Fig. 3c), we limited the residues used in the alignment tensor calculation for the box A; the residues having shown the chemical shift changes by mutations were omitted (see Materials and Methods and the legend to Table 1). The determined alignment tensors and the root mean square deviation (rmsd) values, with corresponding Q-factors, between the observed and back calculated $\Delta\delta_{\text{TROSY}}$ are listed in Table 1. The good correlations between the observed and back calculated values, as evidenced by the small values for the rmsds and Q-factors, credit the tensors are well determined.

The alignment tensor magnitude, D_a , shows the extent of the domain reorientation dynamics. The larger difference in D_a values between boxes A and B demonstrates the increased independency for their reorientation motions [3]. Significant difference in D_a values was observed between boxes A and B in both P80G/P81G and Y78G mutants, while the D_a values were coincident within the errors for the wild-type (Table 1). The greater alignment magnitude, as indicated by large D_a value, for box B than for box A show that box B has preferential contact with the aligning medium [3]. The changes in the aligning magnitudes for the proteins (Table 1) show the two boxes in the wild-type HMGB2-A1B reorient within the restricted space, whilst the boxes in the Y78G mutant reorient rather independently. The boxes in P80G/P81G mutant, showing the moderate differences in D_a values imply the less significant interdomain dynamics relative to Y78G mutant.

The $^{15}\text{N}\{^1\text{H}\}$ NOE profiles for the P80G/P81G and Y78G mutants shared the cooperative reduction in the values for G4 and G83 relative to the wild-type (Fig. 5). The increased flexibility to the parts including G4 and G83 residues, thus, may be responsible for enhancing the interdomain dynamics, irrespective of the flexure of the following segment to the residues; G83, in contact to G4, might play as a joint to link the reorienting domains (Fig. 5).

The wild-type HMGB2-AIB showed close alignment tensor magnitudes, D_a and D_r , between boxes A and B, which allows for determining the relative domain orientation by the alignment tensors of the individual domains. P80G/P81G and Y78G mutants, instead, showed significantly different alignment tensor magnitudes, therefore their domain orientations are not readily determined [2,3,44]. In the mutants, two domains are simply described as in a pronounced motion in a cone with approximate half angles of the bounding cone for P80G/P81G and Y78G, 47 degs. and 53 degs., respectively [44].

3.6. Relative domain orientation of the boxes A and B in HMGB2-AIB

The pair-distance distribution functions $P(r)$ from SAXS measurements for the wild-type and the Y78G mutant HMGB2-AIB fragments were compared (Fig. 7). The profiles were significantly different; the wild-type has greater values over the mutant in the longer pair-distance (r) region over 30 Å (Fig. 7). This observation may imply that the boxes in the wild-type HMGB2-AIB are populated to form more extended forms relative to the Y78G mutant (Fig. 6). The Kratky plots for the wild-type and Y78G mutant showed the increased unfolded structural parts in the mutant, as demonstrated by the greater values for the mutant in the q -range greater than 0.3 Å⁻¹ (Fig. S7).

The RPCSA values for boxes A and B and the scattering data from SAXS measurements for the wild-type HMGB2-AIB were directly used as structural restraints to determine the entire structure: it is noted that the RPCSA-based structure calculation was possible due to the close aligning magnitudes for the boxes A and B in the wild-type HMGB2-AIB (Table 1). The structures fulfilling the experimental restraints from RPCSA and SAXS were obtained by the rigid-body minimization using XPLOR-NIH (Fig. 8). The SAXS scattering profile was reproduced within experimental errors by the resultant structure (Fig. 8a), while the correlation between the observed and back calculated $\Delta\delta_{\text{TROSY}}$ values became worse relative to those for the isolated domains (Fig. 8b and Fig. 6a): the reduced correlation would come from the neglecting interdomain dynamics assumed in the present calculation, which is significant in reality. In spite of the reduced fitting quality, the correlation for the RPCSA was still good, which implies the interdomain dynamics in the wild-type HMGB2-AIB should be rather limited. The lowest energy structure, **the best consistent structure with the experimental data**, is depicted in the ribbon representation (Fig. 9a), which demonstrates the dynamically averaged structure for the HMGB2-AIB in solution. It is noted that the sole SAXS data without RPCSA merely ambiguously defines the domain orientation, although the ensemble

structures reproducing the SAXS scattering profile share the extended arrangement of the boxes (Fig. S8).

Because of the larger domain dynamics for the Y78G mutant, as evident by the mismatch in the alignment tensor magnitudes for the boxes, the RPCSA based orientation restraints cannot be used to determine the structure. The entire structure of the Y78G mutant was calculated by only SAXS data; the structure giving the closest scattering profile to the observed data is depicted (Fig. 9b). The structure comparison demonstrates that the wild-type has more elongated structure than that of the Y78G mutant (Fig. 9), which is consistent with the $P(r)$ profiles (Fig. 7). The relative domain angle differences between the wild-type and Y78G seems close to the estimated half angle of the bounding cone, 52 degs., which was calculated from the difference in the alignment magnitudes between the boxes A and B in Y78G mutant (Table 1) [44].

4. Conclusions

The present work has shown that the interdomain linker (residues 78 – 87) has a role to define the overall structure having preferential domain orientation. The N-terminal part of the interdomain linker, Y⁷⁸VPP⁸¹, is particularly connected to the N-terminus of box A through a CH- π interaction network primarily mediated by the conserved Y78 (Fig.3d). The weak and transient intramolecular interactions make the DNA binding surfaces of the boxes A and B preferentially stay in the opposite direction (Fig. 9a). The Y78G mutant having impaired intramolecular interactions altered preferential domain orientation and the interdomain dynamics, as revealed by the alignment tensors determined by RPCSA (Table 1), which suggests Y78 mediating weak and transient interactions are structurally significant. The usefulness of the hybrid use of SAXS and NMR with RPCSA was also emphasized to the structure analysis of the modular protein like HMGB2.

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Legends to Figures

Figure 1. Multiple alignment of HMGB2 and HMGB1 ortholog sequences from four representative species, which was prepared by ClustalW [45] (a). Gene ID and the species are indicated for each sequence. The HMGB2 sequence from *Sus scrofa* is shown on the top line, with the secondary structures defined in the NMR structures of boxes A and B (PDB: 1J3X and 1J3D). The domains in HMGB2 are defined as: box A (residues 1 – 77), linker (residues 78 – 87), box B (residues 88 – 165), joiner (residues 166 – 185) and the acidic tail (186 – 210). Identical amino acids are shown in red. **The residue C23 in the present HMGB2 was changed to serine to increase the sample stability [15]; the residue is marked with asterisk.** Schematic drawings of the HMGB2 fragments used in this work with full-length HMGB2 indicated at the top (b). The ‘l’ denotes the linker region (residues 78 – 87); A1 and A1B stand for the fragments of box A with the linker and boxes A and B linked by the linker, respectively.

Figure 2. ^1H - ^{15}N HSQC spectra for HMGB2-A (red) and HMGB2-B (cyan) overlaid onto that for HMGB2-A1B with the resonance assignments (black) (a). Expanded part (boxed in a) of the overlaid ^1H - ^{15}N HSQC spectra (b). The chemical shift differences observed between the spectra for HMGB2-A1B and those for HMGB2-A and HMGB2-B are plot against the residue number (c). Changes in backbone ^1H - ^{15}N shifts ($\Delta\delta$) are shown in Hz units. The lines represent the values of the average change plus one (red) and half (orange) standard deviations, respectively. **The colored boxes on the top of the graph represent the positions for the helices in the HMGB2-A1B, which were determined by the NMR analyses in this work.**

Figure 3. HMGB2-A residues that showed significant chemical shift differences in the spectral comparison with HMGB2-A1B (Fig. 2c) are colored on the NMR structure (PDB: 1J3X) (a); the residues in red and orange showed chemical shift changes over the lines in red and orange in Fig. 2c, respectively. The residues in possible CH- π interactions are drawn in ball-and-stick format on the NMR structure of HMGB1 box A (PDB: 1AAB) (b): two tyrosine residues Y71 and Y78 are colored in red and orange, respectively, while prolines and lysines are colored in cyan and dark blue, respectively. Overlay of the structures of box A in HMGB1 (gray) and of box A in HMGB2 (green) (c). The potentially interacting residues in box A of HMGB1 are shown in the ball-and-stick representation and the residues showing

significant chemical shift differences in box A in HMGB2 are colored as in Fig. 3a. The structure overlay was calculated to maximally fit the backbone atoms (N, C α , C') in the helical parts; H1 (residues 15 – 29), H2 (39 – 51) and H3 (54 – 71). The calculation was performed using the program PyMol (Schrödinger, LLC) and the rmsd for the atomic displacement was 1.1 Å. A close-up view of the potentially interacting residues in box A in the HMGB1 structure (d). The pseudo atom position was assumed to be the center of the aromatic ring in the distance calculations for the parts defined by the dotted lines.

Figure 4. Chemical shift changes caused by the mutations to the interdomain linker. Chemical shift differences observed on the ^1H - ^{15}N HSQC spectra between the wild-type HMGB2-AIB and P80G/P81G mutant (a), and Y78G mutant (b). The colored boxes on each graph show the positions of the helices.

Figure 5. Comparison of the $^{15}\text{N}\{^1\text{H}\}$ heteronuclear NOE profiles between the wild type and each mutant. The wild type (black bars) versus the P80G/P81G mutant (red bars) (a). The wild type (black bars) and the Y78G mutant (yellow bars) (b). The rectangular boxes above the graphs represent the positions of α -helices (red and blue boxes for boxes A and B, respectively) and the linker part (residues 78 – 87; green box).

Figure 6. Alignment tensor determination based on the ^{15}N TROSY shift changes induced by weak alignment. The expanded part of the overlaid ^1H - ^{15}N TROSY spectra collected for the protein in isotropic (black) and aligned (red) states; wild type (a), P80G/P81G mutant (b) and Y78G mutant (c). The chemical shift differences observed for the signals collected in the isotropic and aligned states are drawn on the spectra in Hz; the chemical shift correction for the aligned spectrum is not applied to those spectra displayed, which is required due to the residual quadrupole splitting of the deuterium lock signal. In the alignment tensor calculation, the correction to each shift difference was applied based on the directly observed deuterium signal in the aligned state. The correlation between the observed $\Delta\delta_{\text{TROSY}}$ and the back calculated $\Delta\delta_{\text{TROSY}}$ from the determined alignment tensor is plotted on the right side of each observed spectrum. In each correlation plot, blue and red dots represent data from the residues in box A and box B, respectively. In the calculations, only residues in the secondary structure parts were considered in calculation.

Figure 7 . Atom-pair distributions for HMGB2-A1B fragments. The $P(r)$ -versus- r profiles for the wild-type and Y78G mutant are plot in red and blue, respectively.

Figure 8. Quality of the wild-type HMGB2-A1B domain arrangement determined using the RPCSA ($\Delta\delta_{\text{TROSY}}$) and SAXS derived restraints. Experimental scattering profile for the wild-type HMGB2-A1B (●) and the calculated scattering curve in red (a). The correlation between the experimental RPCSA ($\Delta\delta_{\text{TROSY}}$) values for the wild-type HMGB2-A1B and those calculated from the refined structure by XPLOR-NIH calculation (Fig. 9a) (b). Q-factor, and rmsd for the fitting quality against the experimental RPCSAs were 0.30, and 4.1 ppb (0.29 Hz), respectively.

Figure 9. Structures for the wild-type and Y78G mutant HMGB2-A1B. The lowest energy structure of the wild-type HMGB2-A1B refined by the constraints from RPCSA and SAXS (a). The lowest energy structure of the Y78G mutant HMGB2-A1B determined by only the constraints from the SAXS scattering data (b).

References

- [1] A.K. Dunker, C.J. Oldfield, J. Meng, P. Romero, J.Y. Yang, J.W. Chen, V. Vacic, Z. Obradovic, V.N. Uversky, *BMC Genomics* 9 Suppl 2 (2008) S1.
- [2] H. Tossavainen, O. Koskela, P. Jiang, J. Ylanne, I.D. Campbell, I. Kilpelainen, P. Permi, *J Am Chem Soc* 134 (2012) 6660.
- [3] M.W. Fischer, J.A. Losonczi, J.L. Weaver, J.H. Prestegard, *Biochemistry* 38 (1999) 9013.
- [4] S. Tate, A. Imada, N. Hiroguchi, Complementary use of NMR to X-ray crystallography for the analysis of protein morphological change in solution, InTech, Rijeka, Croatia, 2011.
- [5] S. Tate, *Anal Sci* 24 (2008) 39.
- [6] N.K. Goto, N.R. Skrynnikov, F.W. Dahlquist, L.E. Kay, *Journal of Molecular Biology* 308 (2001) 745.
- [7] N.R. Skrynnikov, N.K. Goto, D. Yang, W.Y. Choy, J.R. Tolman, G.A. Mueller, L.E. Kay, *Journal of Molecular Biology* 295 (2000) 1265.
- [8] A. Bax, A. Grishaev, *Current opinion in structural biology* 15 (2005) 563.
- [9] S.E. Tsutakawa, G.L. Hura, K.A. Frankel, P.K. Cooper, J.A. Tainer, *Journal of Structural Biology* 158 (2007) 214.
- [10] X. Wang, H.-W. Lee, Y. Liu, J.H. Prestegard, *Journal of Structural Biology* 173 (2011) 515.
- [11] M. Bustin, R. Reeves, *Prog Nucleic Acid Res Mol Biol* 54 (1996) 35.
- [12] M. Bustin, M.P. Crippa, J.M. Pash, *Crit Rev Eukaryot Gene Expr* 2 (1992) 137.
- [13] M. Bustin, D.A. Lehn, D. Landsman, *Biochim Biophys Acta* 1049 (1990) 231.
- [14] H.M. Weir, P.J. Kraulis, C.S. Hill, A.R. Raine, E.D. Laue, J.O. Thomas, *EMBO J* 12 (1993) 1311.
- [15] C.H. Hardman, R.W. Broadhurst, A.R. Raine, K.D. Grassier, J.O. Thomas, E.D. Laue, *Biochemistry* 34 (1995) 16596.
- [16] A. Yamamoto, Y. Ando, K. Yoshioka, K. Saito, T. Tanabe, H. Shirakawa, M. Yoshida, *J Biochem* 122 (1997) 586.
- [17] Y. Nakamura, K. Yoshioka, H. Shirakawa, M. Yoshida, *J Biochem* 129 (2001) 643.
- [18] K. Yoshioka, K. Saito, T. Tanabe, A. Yamamoto, Y. Ando, Y. Nakamura, H. Shirakawa, M. Yoshida, *Biochemistry* 38 (1999) 589.
- [19] M. Webb, J.O. Thomas, *J Mol Biol* 294 (1999) 373.
- [20] Y. Nakamura, M. Shimizu, M. Yoshida, *J Biochem* 131 (2002) 153.
- [21] M. Brandl, M.S. Weiss, A. Jabs, J. Sühnel, R. Hilgenfeld, *Journal of Molecular Biology* 307 (2001) 357.
- [22] J. Cavanagh, W.J. Fairbrother, A.G. Palmer III, N.J. Skelton, *Heteronuclear NMR Experiments*, Academic Press, Inc., New York, 1996.
- [23] D. Rovnyak, D.P. Frueh, M. Sastry, Z.-Y.J. Sun, A.S. Stern, J.C. Hoch, G. Wagner, *Journal of Magnetic Resonance* 170 (2004) 15.

- [24] J. Hoch, C., A. Stern, S., NMR Data Processing, John Wiley & Sons, Inc., New York, 1996.
- [25] F. Delaglio, S. Grzesiek, G.W. Vuister, G. Zhu, J. Pfeifer, A. Bax, *J Biomol NMR* 6 (1995) 277.
- [26] B.A. Johnson, R.A. Blevins, *Journal of Biomolecular NMR* 4 (1994) 603.
- [27] N. Kobayashi, J. Iwahara, S. Koshiha, T. Tomizawa, N. Tochio, P. Güntert, T. Kigawa, S. Yokoyama, *Journal of Biomolecular NMR* 39 (2007) 31.
- [28] P. Güntert, *European Biophysics Journal* 38 (2009) 129.
- [29] C.D. Schwieters, J.J. Kuszewski, N. Tjandra, G. Marius Clore, *Journal of Magnetic Resonance* 160 (2003) 65.
- [30] T. Ueki, Y. Hiragi, M. Kataoka, Y. Inoko, Y. Amemiya, Y. Izumi, H. Tagawa, Y. Muroga, *Biophysical chemistry* 23 (1985) 115.
- [31] S. Tate, H. Shimahara, N. Utsunomiya-Tate, *J Magn Reson* 171 (2004) 284.
- [32] M. Ottiger, F. Delaglio, A. Bax, *J Magn Reson* 131 (1998) 373.
- [33] J. Chou, S. Gaemers, B. Howder, J. Louis, A. Bax, *Journal of Biomolecular NMR* 21 (2001) 377.
- [34] J.J. Chou, S. Gaemers, B. Howder, J.M. Louis, A. Bax, *J Biomol NMR* 21 (2001) 377.
- [35] F. Mosteller, J. Tukey, *Data Analysis and Regression: A Second Course in Statistics*, Addison-Wesley Publishing Co., Don Mills, Ontario, 1977.
- [36] M. Matsumoto, T. Nishimura, *ACM Trans. Model. Comput. Simul.* 8 (1998) 3.
- [37] C.D. Schwieters, J.J. Kuszewski, N. Tjandra, G.M. Clore, *J Magn Reson* 160 (2003) 65.
- [38] A. Grishaev, J. Ying, A. Bax, *J Am Chem Soc* 128 (2006) 10010.
- [39] A. Grishaev, J. Wu, J. Trehwella, A. Bax, *Journal of the American Chemical Society* 127 (2005) 16621.
- [40] R. Sanchez, A. Sali, *Methods Mol Biol* 143 (2000) 97.
- [41] S.H. Teo, K.D. Grasser, C.H. Hardman, R.W. Broadhurst, E.D. Laue, J.O. Thomas, *EMBO J* 14 (1995) 3844.
- [42] T. Tanaka, Y. Kuroda, S. Yokoyama, *J Struct Funct Genomics* 4 (2003) 79.
- [43] S. Ohnishi, H. Kamikubo, M. Onitsuka, M. Kataoka, D. Shortle, *Journal of the American Chemical Society* 128 (2006) 16338.
- [44] J.R. Tolman, J.M. Flanagan, M.A. Kennedy, J.H. Prestegard, *Nat Struct Biol* 4 (1997) 292.
- [45] M.A. Larkin, G. Blackshields, N.P. Brown, R. Chenna, P.A. McGettigan, H. McWilliam, F. Valentin, I.M. Wallace, A. Wilm, R. Lopez, J.D. Thompson, T.J. Gibson, D.G. Higgins, *Bioinformatics* 23 (2007) 2947.
- [46] M. Ottiger, A. Bax, *J Biomol NMR* 13 (1999) 187.

Table 1.Summary of the alignment tensors^a determined from $\Delta\delta_{\text{TROSY}}$.

Sample	Box ^b	D_a / Hz	D_r / Hz	Euler angles / deg.			Q-factor ^c
				α	β	γ	[rmsd Hz]
HMGB2-A1B	A	-3.3(0.4)	-1.4(0.7)	100 (7)	100 (4)	113 (9)	0.17 [0.17]
wild-type ^d	B	-3.9 (0.5)	-1.6(0.5)	111 (7)	66 (2)	104 (4)	0.20 [0.19]
HMGB2-A1B	A	-3.8 (0.5)	-1.5 (0.4)	120 (16)	104 (5)	124 (5)	0.24 [0.23]
P80G/P81G ^d	B	-5.3 (0.8)	-3.2 (1.2)	100 (7)	68 (4)	110 (7)	0.35 [0.24]
HMGB2-A1B	A	-3.5 (0.6)	-2.0 (0.6)	139 (8)	105 (8)	122 (5)	0.21 [0.21]
Y78G ^d	B	-7.2 (0.8)	-3.6 (1.0)	95 (5)	63 (2)	109 (4)	0.17 [0.31]

^a Values in parentheses are the standard errors estimated by the jack-knife method [35]; 128 times with 10% of the experimental $\Delta\delta_{\text{TROSY}}$ data reduction at random in each calculation. The calculation was done by home-written program using ‘Mersenne twister’ algorithm for random number generation [36].

^b The lowest energy structure among the NMR refined coordinates was used for each analysis. In each structural refinement, the RDC derived constraints were also used (Kurita *et al.* in preparation). The PDB codes for boxes A and B were 1J3X and 1J3D, respectively.

^c The quality factor Q is defined as $Q = \sqrt{\sum_{i=1,N} (\Delta\delta_{\text{TROSY}}^{\text{obs.}} - \Delta\delta_{\text{TROSY}}^{\text{calc.}})^2 / \sum_{i=1,N} (\Delta\delta_{\text{TROSY}}^{\text{obs.}})^2}$, which is in accordance with the Q-factor defined for the RDC analysis [46].

^d In the tensor determination, the residues were selected according to the following criteria; the residues 1) being in the secondary structure, 2) showing $^{15}\text{N}\{^1\text{H}\}$ NOE value greater than 0.6, 3) giving small chemical shift changes (less than 10 Hz) on the ^1H - ^{15}N HSQC spectra between the mutant, P80G/P81G or Y78G, and the wild-type HMGB2-A1B. As exceptions, the residues S15 and F18, which showed 23 Hz and 29 Hz shift changes caused by Y78G mutation, respectively, were incorporated in the tensor calculation for the wild-type, with intention to increase the number of input data: the addition of these two data did not significantly change the resultant tensor value. The residues used in the calculation were the followings: the wild-type (box A), residues 15 – 30 and 38 – 55, the P80/P81G mutant (box A), residues 15 – 30 and 38 – 64, and the Y78G mutant (box A), residues 15 – 30 and 38 – 71. For the box B, the residues 102 – 116 and 120 – 161 were used in all the fragments, the wild-type, P80G/P81G and Y78G mutants. Some residues showing severe signal overlaps and low signal intensities were omitted in the calculation.

(a)

(b)

Fig. 1

Fig. 2

(a)

(c)

(b)

(d)

Fig. 3

(a)

(b)

Fig. 4

Fig.5

(a)

(b)

(c)

Fig. 6

Fig. 7

(a)

(b)

Fig. 8

(a)

(b)

Fig. 9

G A

NMR

SAXS

+

=