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R3H DOMAIN COMPLEXES WITH MONONUCLEOTIDES

DOMĒNA R3H KOMPLEKSI AR MONONUKLEOTĪDIEM

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Introduction

The R3H domain has previously been identified as a conserved sequence motif in putative nucleic-acid-binding proteins from diverse range of organisms [1]. It is named after the presence of an invariant arginine residue and a highly conserved histidine residue that are separated by three residues. The 3D solution structure of the R3H domain from human Sμbp-2 has been determined by NMR spectroscopy and shown to represent the R3H domains in general [2]. The conserved arginine, histidine and another highly conserved arginine residues cluster on the same side of the R3H domain and could play role in nucleic acid recognition. The functions of other domains in proteins containing the R3H domain also indicate that the R3H domain might interact with nucleic acids [1].
Here, we report the results obtained from our binding studies of the R3H domain from human Sμbp-2 and mononucleotides.

Results and discussion

Protein production

The R3H domain from human Sμbp-2 with a C-terminal His-tag (**Fig. 1**) was produced as described previously [2]. An unlabeled sample was used for the determination of the dissociation constants of the R3H domain complexes with mononucleotides. ^{15}N labeling was introduced for further investigation of the binding of dGMP with the R3H domain using heteronuclear two-dimensional NMR spectroscopy.

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1    MGSLNGGSPE GVESQDGVDH FRAMIVEFMA SKKMQLEFPP SLNSHDRLRV 50
51   HQIAEEHGLR HDSSGEGKRR FITVSKRAGS HHHHHH               86
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Fig. 1. Amino acid sequence of the R3H domain from human Sμbp-2. The conserved arginines and histidine residues are typed in bold. The residues not present in the native protein domain are typed in italic

Determination of the dissociation constants of the R3H domain complexes with mononucleotides

Several NMR techniques can be used to detect and quantify the binding of small molecules to proteins (for a review see [3]). We chose to observe the ^{1}H NMR chemical shifts of the protein signals in the high-field-shifted methyl group region as a function of the small molecule concentration. Thereby we benefit from short experimental time and the possibility to map the shifts of the methyl-group signals to the previously determined structure of the R3H domain.
For a molecule in fast exchange between the bound and free forms any NMR parameter is the mole-fraction weighted average of that belonging to the two different states [3]. Thus, the chemical shift of a protein signal in solution with a ligand is

$$\delta_{(obs)} = X_{(free)}\delta_{(free)} + X_{(bound)}\delta_{(bound)}, \quad (1)$$

where $X_{(free)}$ and $X_{(bound)}$ are the mole-fractions of the free and bound species and $\delta_{(free)}$ and $\delta_{(bound)}$ are the respective chemical shift values of the protein signals in free and bound forms.
The complex dissociation constant (K_D) is

$$K_D = [P][L]/[PL] = ([P_0]-[PL])([L_0]-[PL])/[PL], \quad (2)$$

where [P], [L] and [PL] are the equilibrium concentrations of protein, ligand and complex; P_0 and L_0 are the total concentrations of protein and ligand, respectively. By substituting

$$X_{(free)} = ([P_0]-[PL])/[P_0], \quad (3)$$

$$X_{(bound)} = [PL]/[P_0] \text{ and} \quad (4)$$

$$[PL] = \frac{K_D + [L_0] + [P_0] - \sqrt{(K_D + [L_0] + [P_0])^2 - 4[L_0][P_0]}}{2}, \quad (5)$$

we can rewrite the equation for the observed chemical shift of a protein signal:

$$\delta_{(obs)} = (\delta_{(bound)} - \delta_{(free)}) \frac{(K_D + [L_0] + [P_0]) - \sqrt{(K_D + [L_0] + [P_0])^2 - 4[P_0][L_0]}}{2[P_0]} + \delta_{(free)}. \quad (3)$$

The two unknown parameters, K_D and $\delta_{(bound)}$ can be determined by acquiring a series of experiments and applying non-linear least-squares fitting of simulated data to experimental data.
Proton 1D spectra at different R3H to mononucleotide ratios were recorded, and the chemical shifts of the signals of R3H in the methyl group region were measured. The experimental data points were fitted to a simulated binding isotherm by changing the two parameters, K_D and $\delta_{(bound)}$.
Results from the titration of the R3H domain with guanosine mono-phosphate (GMP) are shown in **Fig. 2a**. Any of the shifting signals can be chosen for the calculation of the dissociation constant but it is convenient to choose a well resolved signal for a precise measurement of the chemical shift value at different protein-ligand ratios. In this example the singlets at 0.26 ppm and -0.43 ppm were chosen. The relationship between $\delta_{(obs)}$ and GMP concentration along with the fitted binding curve that determines the K_D is shown in **Fig. 2b**. The results of the NMR chemical shift titrations of the R3H domain with mononucleotides are summarized in **Table 1**.
The R3H domain has a substantial preference for binding with deoxyribonucleotides over ribonucleotides, especially for the purine-containing bases and dGMP is the best binder. Our results show that unphosphorylated nucleosides do not bind to R3H at all. The R3H domain does not bind mononucleotide di-phosphates and tri-phosphates not present in the DNA and the RNA. Furthermore the R3H domain has no affinity towards 3'-phosphorylated mononucleotides (data not shown). Therefore we conclude that the mono-phosphate moiety on the ligand is absolutely necessary for binding to take place, and that the binding of di- and tri-phosphates is abolished due to steric hindrance. The cause of not binding the 3'-phosphorylated mononucleotides is less clear, but it may also be related to steric obstacles, which may mean that either the R3H domain binds only the terminus of a 5'-phosphorylated DNA (RNA) or that the impact on the ligand binding affinity of the sugar moiety and the nucleic base is rather substantial. The reason for the high selectivity of the different mononucleotides must be addressed to specific interactions of the different bases with the R3H domain. These data are supported by previous reports on the Sμbp-2 proteins from human, mouse and hamster to bind single-stranded DNA with a 5'-phosphorylated guanine-rich sequence.

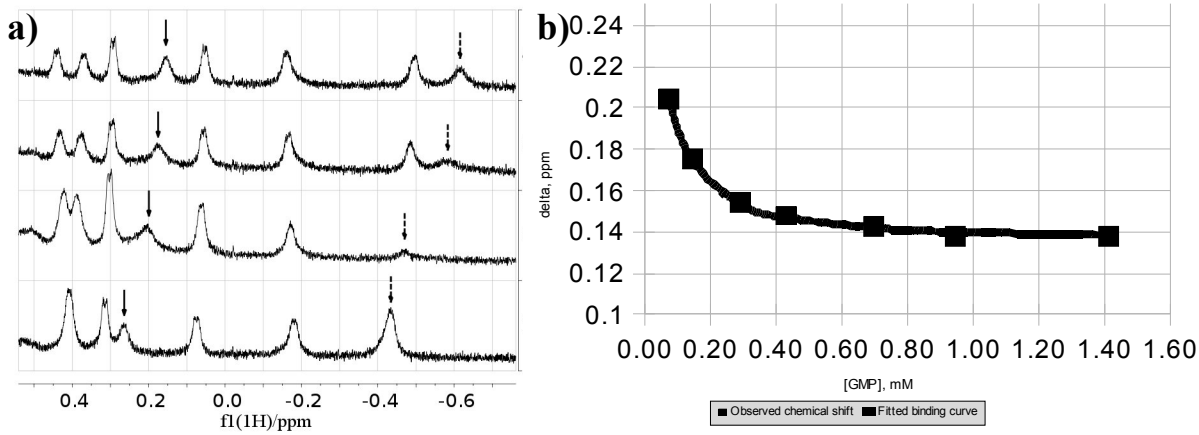

Figure 2. a) Methyl-group region of the proton 1D spectra of R3H at different R3H to GMP concentration ratios. The methyl-group signals at 0.26 ppm and -0.43 ppm shift with increasing concentration of the ligand until an equilibrium is reached and the receptor is saturated

b) The chemical shift of the methyl group signal of R3H at 0.26 ppm as a function of [GMP] (mM) at constant [R3H] = 0.1 mM. The squares are the experimental points, and the line is the fitted binding isotherm. The estimated K_D is 33.6 ± 1.0 ($\delta_{(free)}$=0.264 ppm, $\delta_{(bound)}$ =0.135 ppm)

Table 1

Dissociation constants of the R3H domain complexes with mononucleotides

Nº	**Nucleotide (abbreviation)**	**K_D, µM**	**Nº**	**Nucleotide (abbreviation)**	**K_D, µM**
1	GMP	33.6 ± 1.0	5	dGMP	8.0 ± 0.1
2	AMP	109.0 ± 4.0	6	dAMP	58.7 ± 4.7
3	UMP	106.4 ± 10.6	7	dTMP	268.5 ± 54.5
4	CMP	290.5 ± 30.5	8	dCMP	276.0 ± 43.0

Characterization of the binding of dGMP on the R3H domain

Our results from NMR chemical shift titration experiments verify that the R3H domain binds nucleic acids and that it has the highest affinity towards dGMP. To identify the putative DNA binding site on the structure of the R3H domain, we monitored the chemical shift changes in the [^{1}H, ^{15}N] heteronuclear single quantum coherence (HSQC) spectrum upon addition of dGMP (**Fig. 3**). Most of the shifting signals were found to be located in the helix α2 and the strands β2 and β3 as well as the loop between strands β2 and β3 (**Fig. 4**). The strongest shifting signals were those of residues 45-52, 55-56, 63, 72-73 and 75. The residues 45-52, 55-56 and 63 are located on the same side of the R3H domain as the residues R47, H51 and R70 that are thought to be involved in nucleic acid recognition. Indeed, our results suggest that the negatively charged phosphate group of dGMP interacts with the positively charged sidechains of the residues R47 and R70 while the purine ring of guanosine settles around the helix α2 and H51 causing large ring-current shifts. The residue H51 could be responsible for selectivity of guanosine or purine-containing bases through favorable π-π stacking interactions. This also explains the significance of the three residues in between the invariant arginine and the highly conserved histidine, as the residues "i" and "i+4" should always be located on the same face of an alpha-helix.

Though our NMR data show that binding strongly impacts the strand β3 as well. As this strand locates too far to be directly involved in dGMP binding, a local conformational change upon dGMP binding could be possible.

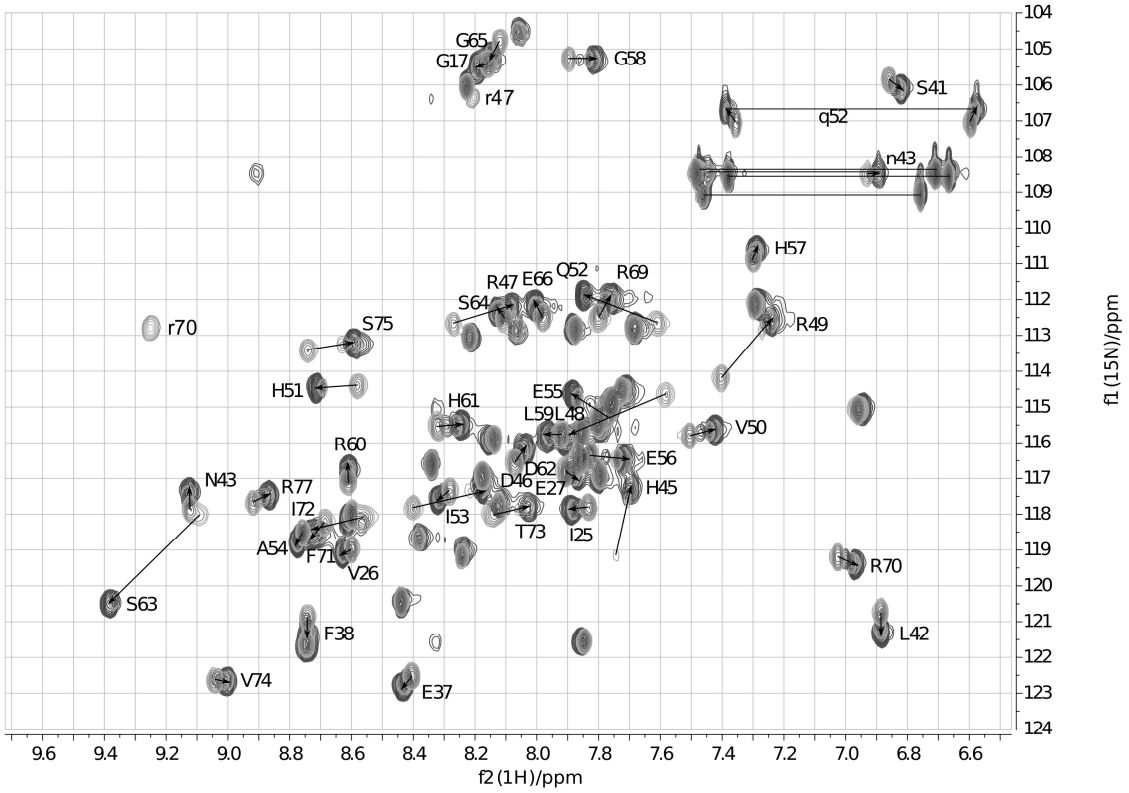

Figure 3. Shifts of amide resonances of the R3H domain signals upon addition of dGMP. [^{1}H, ^{15}N] HSQC spectra at R3H to dGMP ratios of 1:0, 1:0.5, 1:1, 1:2, 1:4, 1:8 are shown. Arrows connecting the peaks at R3H to dGMP ratios of 1:0 and 1:8 are drawn where considerable shifting of signals was observed

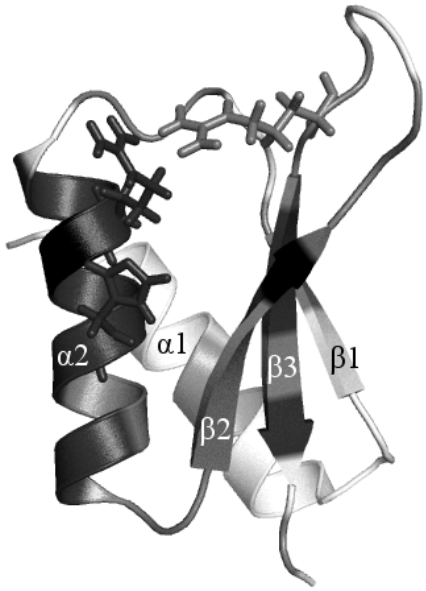

Figure 4. Solution structure of the R3H domain from human Sμbp-2 [2] color coded to reflect the changes in amide ^{1}H or ^{15}N chemical shifts observed in R3H domain complexed with dGMP versus those in free R3H domain (>0.05 ppm ^{1}H or >0.5 ppm ^{15}N, light grey; >0.1 ppm ^{1}H or >1.0 ppm ^{15}N, dark grey; >0.3 ppm ^{1}H or >3.0 ppm ^{15}N, black). The highly conserved R47, H51 and R70 residues are drawn with sticks. Picture produced with PyMOL [4]

Experimental

The R3H domain from human Sμbp-2 with a C-terminal His-tag was produced as described previously [2]. Mononucleotides were purchased from Sigma (USA) and Acros (Belgium) or obtained from Dr. E. Bizdena (Riga Technical University, Latvia).
All NMR spectra were acquired at 25° C using a Varian Unity Inova 600 MHz spectrometer equipped with a triple resonance cold probe and incorporated gradients along the z-axis. WATERGATE [5] solvent suppression technique was used for the suppression of the residual water signal. Chemical shifts were referenced to the residual water signal. Proton 1D spectra were recorded with 64 to 128 scans, an inter-scan delay of 1.0 s, and 8K to 32K data points. 2D [^{1}H, ^{15}N] HSQC spectra were recorded with 8 to 32 scans, an inter-scan delay of 1.0 s, and 1024 x 64 data points. All NMR spectra were processed by double zero filling prior to Fourier transformation. Spectral processing and analysis was performed using MestRe Nova (Mestrelab Research SL) software.
The samples for proton 1D and 2D [^{1}H, ^{15}N] HSQC experiments were prepared with R3H (0.1 or 0.2 mM) in the presence of sodium phosphate (50 mM) buffered 10% D_2O/90% H_2O, pH 7.0. After obtaining a reference spectrum, the ligand was added gradually to obtain the desired protein to ligand molar ratio. Thus for the determination of the dissociation constant (K_D) of the R3H-mononucleotide complex, the 1D solvent suppressed proton spectra at ligand to protein molar ratios of 0.5:1; 1:1; 2:1; 4:1; 8:1 and 15:1 were obtained and the chemical shifts of the protein signals in the methyl group region were monitored. The estimation of K_D was accomplished using non-linear least squares fitting in a home written MS Excel workbook.

Conclusions

Our results show that the R3H domain binds mononucleotide mono-phosphates, and the dissociation constant of the complex is within the micro-molar region. It has a substantial preference for binding with deoxy-ribonucleotides over ribonucleotides, especially for the purine-containing bases. The R3H domain does not bind mononucleotide di-phosphates and tri-phosphates as well as unphosphorylated mononucleotides not present in the DNA and the RNA. The R3H domain has no affinity towards 3'-phosphorylated mononucleotides too. The binding site of deoxy-guanosine mono-phosphate (dGMP) is identified from perturbations of chemical shifts in the 2D [^{1}H, ^{15}N] HSQC spectrum. Our data show that binding of dGMP might occur through electrostatic interactions with the positively charged sidechains of R47 and R70, and π-π stacking interactions with H51.
The obtained results suggest that the R3H domain is involved in DNA binding and could have a preference for binding with guanosine-rich DNA sequences.

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Jaudzems K., Žuļenkovs D., Liepiņš E. Domēna R3H kompleksi ar mononukleotīdiem. *Domēns R3H iepriekš atklāts kā vienojošs sekvences motīvs dažādas izcelsmes organismu proteīnos, kas iepējams saista nukleīnskābes [1]. R3H domēna 3D struktūra šķīdumā iepriekš noteikta ar KMR spektroskopiju [2]. Šajā darbā atspoguļoti mūsu rezultāti par cilvēka Sμbp-2 proteīna R3H domēna saistība pētījumiem ar mononukleotīdiem. Mūsu rezultāti parāda, ka domēns R3H saista mononukleotīdu monofosfātus, veidojot kompleksus ar disociācijas konstanti mikro-molārā rajonā. Saistība ar dezoksiribo-mononukleotīdiem ir ievērojami stiprāka nekā ar ribo-mononukleotīdiem, īpaši to nukleotīdu gadījumā, kas satur purīna atlikumu. R3H domēns nesaistās ar mononukleotīdu difosfātiem un trifosfātiem, kā arī nefosforilētiem nukleozīdiem, kas tāpat nav sastopami DNS un RNS. R3H domēns nesaista arī 3'-fosforilētus mononukleotīdus. Dezoksi-guanozīna monofosfāta saistības vieta uz R3H identificēta pēc R3H signālu nobīdēm 2D [^{1}H, ^{15}N] HSQC spektrā. Iegūtie rezultāti norāda, ka R3H domēns mijiedarbojas ar DNS, un varētu būt selektīvs pret guanozīna bagātām DNS sekvencēm.*

Jaudzems K., Zhulyenkov D., Liepinsh E. R3H domain complexes with mononucleotides. *The R3H domain has previously been identified as a conserved sequence motif in putative nucleic-acid-binding proteins from diverse range of organisms [1].. The 3D solution structure of the R3H domain from human Sμbp-2 has been determined by NMR spectroscopy[2]. In the present paper we report the results obtained from our binding studies of the R3H domain from human Sμbp-2 and mononucleotides. Our results show that the R3H domain binds mononucleotide mono-phosphates, and the dissociation constant of the complex is within the micro-molar region. It has a substantial preference for binding with deoxy-ribonucleotides over ribonucleotides, especially for the purine-containing bases. The R3H domain does not bind mononucleotide di-phosphates and tri-phosphates as well as unphosphorylated mononucleotides not present in the DNA and the RNA. The R3H domain has no affinity towards 3'-phosphorylated mononucleotides too. The binding site of deoxy-guanosine mono-phosphate is identified from perturbations of chemical shifts in the 2D [^{1}H, ^{15}N] HSQC spectrum. The obtained results suggest that the R3H domain is involved in DNA binding and could have a preference for binding with guanosine-rich DNA sequences.*

Яудземс К., Жулеиков Д., Лиепиньш Э. Комплексы доменов с мононуклеотидами. *Домен R3H был ранее идентифицирован как объединяющий мотив последовательности протеинов различного происхождения, которые связываются с нуклеиновыми кислотами [1]. Трехмерная структура домена R3H человеческого Sμbp-2 в водном растворе ранее была определена методом ЯМР спектроскопии [2].*
В настоящей работе представлены данные наших исследований домена R3H человеческого Sμbp-2 полученные на основе изучения связывания домена человеческого Sμbp-2 с мононуклеотидами. Наши результаты показали, что домен R3H связывает монофосфат мононуклеотида с образованием комплексов с константой диссоциации в микромолярном диапазоне. Связь с дезоксирибо-мононуклеотидами заметно сильнее чем с рибомононуклеотидами, особенно в случае нуклеотидов, содержащих пуриновые остатки. Домен R3H не связываеся с дифосфатами и трифосфатам мононуклеотида, а также с нефосфорилированными нуклеозидами, отсутствующими в ДНК и РНК.
Домен R3H не связывается также с 3-фосфорилированными мононуклеотидами. Определено место присоединения дезокси-гуанозино моно фосфата по изменению химических сдвигов в спектре 2D [^{1}H, ^{15}N] HSQC. Полученные результаты подтверждают, что домен R3H участвует в ДНК связывании и является селективным к последовательностям ДНК обогащенным гуанозином .