"Dark Mediators" of Proteins as Revealed by NMR in Water: Residue-selective Anion Bindings that are Masked by Pre-existing Buffer

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Abstract

Ions are commonly believed to impose their effects on proteins by unspecific electrostatic screening. Here, by NMR we reveal that in water sulfate, chloride and thiocyanate are able to bind a well-folded WW domain at distinctive residues and affinities, which is surprisingly masked by the pre-existing buffer. Our study reveals that the specific anion binding is so ubiquitous and consequently no longer negligible in establishing "postreductionist framework" for protein biochemistry.

Inorganic salts are key components of Earth and have been proposed to direct the origin and evolution of life[1]. Ion-specific effects are ubiquitous in nature[2] and critical for protein folding/stability, aggregation as well as enzymatic activity[3-12]. However, except for some cations serving as cofactors of proteins, ion-protein interactions are commonly believed to act by non-specific electrostatic screening at diluted salt concentrations (<200 mM)[2,3,8] and anion-protein interactions are completely absent in the textbook of bioinorganic chemistry[13]. Nevertheless, previously it was found that non-specific electrostatic screening could not completely account for the ion's effects on a variety of global properties of proteins such as stability[3,11], aggregation[6,10], cloud-point temperature and effective charges[8]. To reconcile these fundamental discrepancies, a high-resolution view is required to answer two key questions: 1) whether different ions bind to distinctive set of protein residues; and 2) whether bindings show saturation; and if so, what are their dissociation constants (Kd).

NMR spectroscopy is the only available technique to visualize residue-specific ion-protein interactions. Recently, we revealed that in contrast to the common belief, in water at pH 4.0, 8 anions located in the middle, on the left and right sides of the Hofmeister series were able to bind distinctive sets of residues of the intrinsically-unstructured cytoplasmic domain of ephrin-B2 with very high affinity. In particular, sulfate anion is the tightest binder, with apparent dissociation constants (Kd) of ~1 mM[10]. Our finding disagrees with a previous NMR investigation on the well-folded B1 domain of protein L (ProtL) in 20 mM phosphate buffer at pH 6.0, demonstrating that anion-protein interactions showed no saturation with salt concentrations up to the molar range[12]. Here, we selected the well-folded, 39-residue WW4 as a study system. Previously we have determined its NMR structure and binding with a Nogo-A peptide[14]. Moreover, no evidence exists to suggest the requirement of any anion as its cofactor. Subsequently by use of NMR HSQC titrations[10], we monitored its binding to three physiologically relevant salts (Na_2SO_4, NaCl and NaSCN)[15,16] with salt concentrations up to 200 mM (800 protein molar equivalents), under three solution conditions: 1) in water at pH 6.4; 2) in 20 mM phosphate buffer at pH 6.4; and 3) in water at pH 4.0.

As shown in Figure S1a, no significant difference is found for far-UV CD spectra of WW4 in water at pH 6.4 and 4.0, implying that WW4 has very similar secondary structures at two pHs. Furthermore, similar far-UV CD spectra in the presence of 800 molar equivalents of Na_2SO_4 and NaCl at pH 6.4 (Figure S1b) and 4.0 (Figure S1c) indicate that addition of two salts triggers no significant change of secondary structures. Subsequently under three solution conditions, we acquired series of HSQC spectra of WW4 with progressive addition of Na_2SO_4 (Figure S2), NaCl (Figure S3) and NaSCN

(Figure S4). In the absence of salts, WW4 is well-folded as evident from its large ^{1}H (~2.9 ppm) and ^{15}N (~22 ppm) HSQC spectral dispersions. Furthermore, addition of three salts up to 200 mM leads to no dramatic change of HSQC spectral dispersions, suggesting no significant alternation of tertiary structures. Nevertheless, addition of three salts does induce shifts of distinctive sets of HSQC peaks. Specifically, as shown in Figure 1a, in water at pH 6.4, NaCl induces significant shifts (>0.03 ppm) for only two residues (Arg35 and Asn36); Na_2SO_4 for three (Phe31, Lys32 and Asn36) and NaSCN for nine (Trp9, Glu10, Glu16, Gly17, Asp23, Arg27, Lys32, Arg35 and Asn36). Markedly, the overall shift patterns induced by each salt are similar in water at pH 6.4 and 4.0. We also titrated with sodium phosphate up to 200 mM in water (pH 6.4) and only Glu16 and Asn36 are significantly perturbed (Figure S5). Furthermore, to determine the relative contribution of cations and anions of three salts, we titrated WW4 with $MgSO_4$, KCl and KSCN in water at pH 6.4. Their HSQC shift patterns are very similar to those by their sodium salts respectively (Figures S6-S8), suggesting that the observed HSQC peak shifts here are mostly triggered by the asymmetric binding of anions to WW4 as we previously showed on ephrinB2 at pH 4.0[10]. The most unexpected finding is that the pre-existence of 20 mM sodium phosphate considerably changes the shift patterns by all three salts. In the buffer, some residues, which are not perturbed either by that salt or sodium phosphate separately, suddenly appeared to be significantly perturbed. For example, in the buffer Na_2SO_4 is able to significantly perturb Trp9, Asp23 and Asn25, which are not largely perturbed by Na_2SO_4 alone in water either at pH 6.4 or 4.0.

We fitted all titration tracings with ^{1}H chemical shift differences > 0.03 ppm to obtain the apparent dissociation constants (Kd) as we previously described[10] (Table 1).

Intriguingly, although Na_2SO_4 perturbs much less numbers of residues than NaSCN, it has the strongest binding affinity, with average Kd values of 32.0, 15.7 and 86.3 mM respectively for backbone amide protons under three conditions. NaCl, NaCSN and Na_2HPO_4 have much lower affinity, with average Kd values of ~100 mM even in water. Noticeably, only Na_2SO_4 appears to extensively bind to side-chain amide protons, in particular at pH 4.0, although the binding affinity is approximately 3-fold lower than that of the backbone at the same condition (Table 1). Unbelievably, the pre-existence of 20 mM sodium phosphate buffer significantly reduce the binding affinity of all three salts, as exemplified by titration curves and Kd values of several representative residues (Figure 1b). For Na_2SO_4, although the presence of the buffer leads to an approximately 3-fold affinity reduction for backbone amide protons, titration curves still show saturation to some degree. By contrast, for NaCl and NaSCN, the presence of the buffer renders the titration curves to appear to be almost liner which can not be fitted with good confidence (Figure 1b and Table 1).

We then mapped the binding sites onto the WW4 NMR structure[14] (Figures 2a-i). On the other hand, we determined the exposure degree of WW4 amide protons to solvent by H/D exchange experiments[17] (Table S1). Interestingly, Na_2SO_4, NaCl and Na_2HPO_4 appear to bind only well exposed amide protons (with Kex > 5 h^{-1}) which are located on loops/turns or a short 3_{10}-helix over Pro34-Arg35-Asn36. By a sharp contrast, NaSCN is able to bind well-protected amide protons such as from Trp9, Glu10, Val22 and Asp23 (with Kex < 5 h^{-1}), which are on two central β-strands (Figure 2k). In particular, Glu10 is one of two residues with the most protected amide protons (another is Ile11) with a Kex of 0.97 h^{-1}. Further analysis of electrostatic potential surfaces reveals a surprising picture:

the amide protons interacting with Na_2SO_4, NaCl and Na_2HPO_4 in water are almost all located on the electrostatically-positive regions while NaSCN is able to bind to the amide protons of Trp9, Glu10, Val22 and Asp23 located on electrostatically-negative regions (Figures 2k, l and m). This implies that Na_2SO_4 binding is highly electrostatically-dependent while NaSCN is not. Indeed, the binding affinity of Na_2SO_4 at pH 4.0 has a ~2-fold increase as compared to that at pH 6.4 while no significant difference is found for NaSCN at two pHs. Very surprisingly, the presence of 20 mM sodium phosphate renders Na_2SO_4 to behave like NaSCN, capable of significantly perturbing Trp9 and Asp23 on two core β-strands with well protected amide protons (Figure 2b).

Comparison of the present results with our previous[10] reveals key factors governing anion-protein interactions. Sulfate and chloride are highly-hydrated[18,19], and consequently they only bind well-exposed amide protons driven mostly by electrostatic interactions. The high charge-density renders sulfate to form the most stable hydrogen bonds with amide protons in both structured and unstructured proteins[10]. By contrast, thiocyanate is weakly solvated with low charge density[19]. As such, van der Waals interaction seemingly also plays key roles. This is evident from the fact that thiocyanate is able to bind the largest set of amide protons including some well protected amide protons located on electrostatically-negative patches (Trp9 and Glu10). On the other hand, no significant change has been observed for the binding sites of three salts at pH 6.4 and 4.0, implying that additionally to electrostatic properties, other geometric/dynamic parameters of the protein surface patches are also critical for coordinating anions[20,21].

Remarkably, ~10-fold reduction is observed in terms of the affinity of three salts to the well-folded WW4 as compared with those to an unstructured domain at same pH[10].

This suggests that a well-folded protein is significantly shielded from ion binding. As such, we propose here that other than unspecific electrostatic screening, the specific ion-binding appears to also have a key role in mediating protein aggregation, in particular for the "insoluble proteins" with disrupted tertiary packing[9,22]. Our results also imply an extreme complexity for salts' effects on global protein properties: even only for the binding event, different anions already have differential binding residues and affinities. As global effects are concerned, many other processes are involved and as such other properties of anions such as polarisability will further come into play critical roles.

The reduction of binding affinity by the presence of the buffer may be explained by electrostatic screening or/and competition for binding sites. Nevertheless, in the buffer, the surprising alteration of the perturbation patterns particularly by sulfate implies the non-additive interaction between phosphate and sulfate anions. Previously, it was only found that the interaction between cations and anions is not additive and the underlying mechanism might be extremely complex[23,24]. To the best of our knowledge, here is the first time to report the non-additive interaction for two anions. This finding bears practical implications as phosphate buffers are so extensively used for buffering proteins for functional and NMR studies.

Strikingly, the binding regions by sulfate and thiocyanate on WW4 have significant overlap with its Nogo-A peptide binding regions (Figure S9d)[14]. As proteins have been exposed to various inorganic salts in evolution, in particular during the prebiotic period, it is tempted to hypothesize that the specific ion-binding has been coupled to protein functions. For example, chloride was selected as the dominant anion in the extracellular space (~150 mM) probably because of its relatively inert binding ability.

Recently ion-protein interactions are proposed to play much more important roles than previously expected in mediating various aspects of proteins in the crowding cellular environment where unspecific electrostatic interactions become dominant[4]. The ubiquitous but specific effects of anions on proteins as we decipher here strongly argue that anions may serve as "dark mediators" for proteins. Consequently, without a deep understanding of anion-protein interactions, we will fail to comprehend and then computationally model how proteins function in cells[5].

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Figure legends

Figure 1. NMR HSQC titrations.

a. Residue-specific chemical shift difference (CSD) of amide protons (^{1}H) of WW4 upon addition of three salts (Na_2SO_4, NaCl and NaSCN) at 150 mM (blue bars) and 200 mM (red circles). Residues with significant ^{1}H chemical shift changes (>0.03 ppm) are labeled: red for residues with amide proton H/D exchange rate (Kex) <5 h^{-1} and blue for residues with significant changes only in the presence of 20 mM sodium phosphate buffer (pH 6.4). b. Residue-specific apparent dissociation constants (Kd). Experimental (dots) and fitted (lines) values are shown for the ^{1}H chemical shift changes induced by gradual addition of two salts (Na_2SO_4 and NaSCN). Red is for the data in water (pH 6.4), green for those in water (pH 4.0), and blue for those in 20 mM sodium phosphate buffer (pH 6.4).

Figure 2. Binding sites on WW4.

a-c. WW4 NMR structures with significantly perturbed residues colored in green, by 200 mM Na_2SO_4 in water (pH 6.4), the buffer (pH 6.4) and water (pH 4.0) respectively. d-f. by 200 mM NaSCN in water (pH 6.4), the buffer (pH 6.4) and water (pH 4.0) respectively. g-h. by 200 mM NaCl in water (pH 6.4) and the buffer (pH 6.4) respectively. i. by 200 mM Na_2HPO_4 in water (pH 6.4). Red is used to label residues with significant changes only in the presence of the buffer (pH 6.4). Sticks are used to indicate the side

chain amide protons with significant ^{1}H chemical shift changes (>0.03 ppm). j. Experimental (dots) and fitted (lines) values are shown for the HSQC peak intensities of WW4 residues in H/D exchange experiments. k. WW4 NMR structure colored with H/D exchange rates (Kex): blue for residues with HSQC peaks disappeared in 2 min after the lyophilized sample was dissolved in D_2O; green for residues with Kex >5 h^{-1} and red for residues with Kex <5 h^{-1}. i-m. The electrostatic potential of WW4 at pH 6.4 and 4.0 respectively, which is calculated with APBS (see Supplementary Methods) visualized at the level of the accessible surface of the protein, with blue and red corresponding to positive and negative potential values.

Table 1. Apparent Dissociation Constants (Kd) for Binding of Salts to WW4 Domain

Residue		Na2SO4			NaCl			NaSCN		Na2HPO4
					Backbone					
	pH 6.4	Buffered	pH 4.0	pH 6.4	Buffered	pH 4.0	pH 6.4	Buffered	pH 4.0	pH 6.4
W9		96.8 ±15.3					>250	>250		
E10							126.2 ± 24.4		81.8 ± 10.0	
E16							111.4 ± 20.1	>250	86.7 ± 5.5	79.7 ± 6.2
G17			18.0 ± 2.7				79.4 ± 18.3			
V22									116.7 ± 25.3	
D23		92.0 ± 11.4					135.4 ± 17.5	>250	95.0 ± 5.5	
H24								>250		
N25		76.6 ± 11.7			>250			>250	27.2 ± 3.2	
T26			12.6 ± 1.2						25.3 ± 1.5	
R27							115.0 ± 12.9		95.8 ± 13.1	
F31	30.3 ± 4.3	94.2 ± 6.6	18.9 ± 2.0					>250	150.7 ± 33.0	
K32	18.6 ± 2.8		13.3 ± 1.4				48.2 ± 8.6			
R35				>250			>250	>250		
N36	47.2 ± 4.1	71.9 ± 8.1		94.8 ± 13.9			82.0 ± 12.6	>250		149.1 ± 13.3
Average[b]	**32.0**	**86.3**	**15.7**	**94.8**			**99.7**		**84.9**	**114.4**
					Side Chain					
N1s			62.5 ± 6.5							
N25s	77.4 ± 16.8		38.5 ± 3.5				114.8 ± 17.3	>250	61.0 ± 11.7	
N36s	150.8 ± 28.4	>250	56.3 ± 5.5							
R15s			44.5 ± 7.9							
R27s			55.2 ± 6.1							
R35s			30.2 ± 3.3						98.6 ± 12.3	
Average[b]	**114.1**		**47.9**				**114.8**		**79.8**	

[a] "Buffered" refers to "in 20 mM sodium phosphate (pH 6.4)". [b]In calculating the average values, Kd values > 250 mM are not included.