Design of a Peptide-Based Model Leads for Scavenging Anions

Tridip Sheet and Raja Banerjee

ABSTRACT: Among several peptide-based anion recognition motifs, the “C’NN” motif containing C’s, Nα, and Nα+1 of three consecutive residues is unique in its mode of interaction. Having a spatial geometry of βαα or βαβ, this motif occurs in the N terminus of a helix and often found at the functional interface of a protein, mediating crucial biological significance upon interaction with anion(s). The interaction of anion(s) with chimeric peptide sequences containing the naturally occurring “C’NN” motif (CPS224Ac, CPS226, and CPS228) reported in our previous attempts strongly confirms that the information regarding the interaction is embedded within the local sequences of the motif segment. At these prevailing circumstances, an effort has been pursued to design novel scaffolds based on the “C’NN” motif for achieving better recognition of anion(s). Exploring the existing data set of the “C’NN” motif available in the FSSP database, four novel peptide-based scaffolds have been designed (DS1, DS2, DS3, and DS4), and preliminary screenings have been performed using computational approaches. Our initial work suggests that two (DS1 and DS3) out of the four scaffolds are potential candidates for better anion recognition. By employing biophysical characterization using both qualitative and quantitative measures, in this present study, we report the interaction of sulfate and phosphate ions with these two designed scaffolds, in which there is much better recognition of anions by these scaffolds than the natural sequences, justifying their logical engineering. Our observation strongly suggests that these designed scaffolds are better potential candidates than those of the naturally occurring “C’NN” motif in terms of anion recognition and could be utilized for the scavenging of anion(s) for different purposes.

INTRODUCTION

Protein supersecondary structures often consist of “motifs” that play crucial roles as a functional interface. Several examples of such functional motifs (e.g., “structural P-loop”, phosphate-binding “cup”, nest, motifs responsible for the recognition of nucleotides, their analogues, etc.) are available in the literature, which mediate anion–protein interactions. All such motifs have distinct characteristic features based on their sequence/structural perspective as well as their mode of interactions. Similar to other peptide-based anion recognition motifs, the “C’NN” motif (containing C’s, Nα, and Nα+1 of three consecutive residues found in the FSSP database) is a novel “structural motif for the protein recognition of phosphate ions” but is unique in its mode of interaction, both in terms of structure and sequence. In the structural database (FSSP based on crystal structures), this motif, found in several classes of proteins, is usually present in the loop region preceding a helix with a spatial geometry of βαα or βαβ for the three constituting residues. Its conformational facet (details of conformational landscape) is coupled with its interaction with the anion(s) in such a fascinating way that the interaction extends the length of the anchoring helix by one additional turn at the motif site present at the N terminus of the helix along with the stabilization of the adjoining helix. Although this motif was initially discovered in juxtaposition with the phosphate-binding sites in proteins (protein–nucleic acid interaction), it was reported that in several instances a sulfate ion replaced the phosphate ion during the co-crystallization process with ammonium sulfate.

These reports served as a motivation for us to study the “C’NN” motif in a context-free system to understand whether the recognition of an anion is an intrinsic property of the “C’NN” motif. In our previous attempts, we chose a few naturally occurring “C’NN” motif sequences listed in 104-fold representative structures of the FSSP database. We appended these sequences at the N terminus of a predesigned thermostable model helical peptide (ABGY) as to achieve three chimeric peptide sequences, namely CPS224Ac, CPS226, and CPS228 (Table S1). Using an in silico approach as well as complementary spectroscopic techniques, we provided enough experimental evidence to corroborate that these sequences having a conserved GXX sequence motif are potential candidates to mimic the anion–peptide interaction in a similar fashion as reported for the native protein structures even in a context-free nonproteinaceous environment. However, the nature of the sequence (composed of either

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Table 1. Comparison of the Interaction of Anions (Sulfate and Phosphate) between the Designed Scaffolds (DS1 and DS3) and the Peptides Containing Naturally Occurring "C°NN" Motifs (CPS224Ac and CPS226) in Terms of Conformational Marker [$\theta$, ($\omega$)°/($\phi$)°], and Other Interacting Parameters [Dissociation Constant ($K_d$), Binding Free Energies, Inhibition Constant, and Residence Time] Obtained from CD, Molecular Docking, and MD Simulation Studies, Respectively

| Peptide            | R2 ($\theta_2$/$\pi_2$)° | $\theta$°/($\omega$)° | $K_d$ (mM) | free peptide binding free energies from docking* (kcal/mol) | inhibition constant from docking* (mM) | residence time from MD simulation* (ns) |
|--------------------|-------------------------|----------------------|-----------|-----------------------------------------------------------|----------------------------------------|-----------------------------------------|
| DS1 free peptide   | 0.77                    | 1.16                 | 117       | −3.85                                                     | 1.51                                   | 2.62 ns                                 |
| with sulfate       | 0.85                    | 1.16                 | 117       | −2.91                                                     | 7.22                                   |                                         |
| with phosphate     | 0.84                    | 1.13                 | 166       | −2.91                                                     | 7.22                                   |                                         |
| DS3 free peptide   | 0.84                    | 1.07                 | 60        | −4.50                                                     | 0.49                                   | 4.92 ns                                 |
| with sulfate       | 0.89                    | 1.11                 | 137       | −3.64                                                     | 2.01                                   |                                         |
| with phosphate     | 0.89                    | 1.11                 | 137       | −3.64                                                     | 2.01                                   |                                         |
| CPS224Ac free peptide | 0.75                |                      |           |                                                            |                                        |                                         |
| with sulfate       | 0.79                    | 1.06                 | 181       | −3.34                                                     | 3.54                                   | 400 ps                                  |
| with phosphate     | 0.81                    | 1.08                 | 263       | −2.40                                                     | 17.30                                  |                                         |
| CPS226 free peptide | 0.71                |                      |           |                                                            |                                        |                                         |
| with sulfate       | 0.84                    | 1.55                 | 143       | −3.37                                                     | 3.36                                   |                                         |
| with phosphate     | 0.79                    | 1.38                 | 187       | −2.53                                                     | 13.66                                  |                                         |

*CD data of DS1 and DS3 are recorded at 298 K as mentioned in the text, whereas those of CPS224Ac and CPS226 were recorded at 278 K.17

RESULTS AND DISCUSSION

Mass Spectrometry. The presence of the peptide DS1 (MW 1674) has been confirmed by the appearance of m/z values at 1674.9, 1696.9, and 1712.9 (M + H, M + Na, and M + K added species of DS1, respectively) in the positive mode of MALDI. Similarly, m/z values at 1705.1, 1727.1, and 1743.1 (M + H, M + Na, and M + K added species of DS3, respectively) have established the occurrence of peptide DS3 (MW 1704). The appearance of m/z values at 885.3 and 885.8 in the negative mode (Figure 1), whereas 887.2 and 887.5 in the positive mode (Figure S1), with an isotopic distribution of 0.5, has confirmed the binding of sulfate (SO_4^{2−}) and phosphate (HPO_4^{2−}) ions, respectively, with the peptide DS1. On the other hand, m/z values at 900.3 and 900.8 in the negative mode (Figure 1), whereas 902.2 and 902.1 in the positive mode (Figure S1), with an isotopic distribution of 0.5, clearly demonstrate the binding of sulfate and phosphate ions to the peptide DS3, respectively.

CD Spectroscopy. Interaction with Anions. Figure 2 shows the far-UV CD spectra of the free peptides along with their sulfate- and phosphate-added species recorded at 298 K. The CD spectra of both the peptides (DS1 and DS3) showed two negative maxima at ~205 (π−π°) and 222 (n−π°) nm, representing the characteristic helical geometry of the peptide backbone. From the ratio of ($\theta_{222}/\theta_{198}$), it appears that the helicity of DS3 is little higher than that of DS1 (the R2 value of DS3 is 0.84, whereas that of DS1 is 0.77; Table 1). Upon the addition of anion(s) to the designed peptides, there has been an increase in CD intensities with a slight red shift at around 210 nm, indicating the occurrence of anion-motif interactions within the designed model scaffolds (Table 1).

A study of the values obtained from the molecular docking and MD simulations (

hydrophilic or hydrophobic residues) and the anion play a crucial role in stabilizing the interaction. The results strongly validate the intrinsic affinity of the naturally occurring "C°NN" motifs for anion(s) (for both the sulfate and phosphate ions) through "locally" mediated interactions, in which the relative affinity for the sulfate ion predominates over the phosphate ions. The quantitative aspect of the comparative interaction was estimated using the binding free energies derived from molecular docking experiments and further validated from the dissociation constant ($K_d$) values obtained from the circular dichroism (CD) spectra. As the information regarding the recognition of an anion is embedded within the "local sequence" involving the naturally occurring "C°NN" motif segment and the recognition occurs in a context-free manner, an attempt has been made here to utilize this consensus as a guideline for designing "peptide-based model scaffolds" toward a better recognition of anions compared to sequences having a naturally occurring "C°NN" motif and which can be employed in various applications (e.g., peptide-mediated passive movement of hydrophilic anions (sulfate and phosphate) in the lipid bilayer of cell membranes). In our recent publication, based on the natural sequences of the "C°NN" motif segment from the S1-fold-representative structures enlisted in the FSSP database, we have modeled a series of anion recognition templates (DS1: Gly-Lys-Ser; DS2: Gly-Gly-Ser; DS3: Ser-Lys-Ser, and DS4: Ser-Gly-Ser). In silico experiments (molecular docking and molecular dynamics (MD) simulations) provide enough evidence of better efficiency in the recognition of anions by these designed templates, especially two sequences, namely DS1: Gly-Lys-Ser and DS3: Ser-Lys-Ser, in comparison to the naturally occurring sequences (CPS224Ac, CPS226, and CPS228). This comparison (Table 1) has been made in terms of the binding free energies obtained from the molecular docking experiments and the retention time of the anion to the motif segment in MD simulations. At this juncture, to validate the results obtained from the molecular docking and the MD simulation studies, we have conducted biophysical experiments with the synthetic peptides containing these designed motif scaffolds (e.g., DS1 and DS3) in order to provide both qualitative and quantitative measures regarding their anion recognition. Such studies in a context-free system will not only provide the justification for the logical engineering of the designed model scaffolds toward recognizing anion(s) in a better way but also serve as a guide to a new trend in peptidomimetics for employing peptides as a potential candidate for the scavenging of anion(s) having harmful effects.

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enhancement of CD signals at both the $\pi - \pi^*$ transition and especially the n-$\pi^*$ transition along with an $\sim 1$ nm bathochromic shift of the $\pi - \pi^*$ transition ($\sim 205$ nm). This observation has been consistent for both the peptides as well as for both the anions (Figure 2a,c,e,g). Upon the addition of both the sulfate and phosphate ions, the $R_2$ value for both the peptides increases and attains a limiting value. For DS1, it is $\sim 0.85$ (for sulfate 0.85 and for phosphate 0.84), whereas for DS3, it is $\sim 0.89$ (for both sulfate and phosphate) (Table 1), indicating the greater interaction of both the anions with DS3. Such an observation has been consistent with the molecular docking results (Table 1), which predicted greater binding free energies associated with DS3 in comparison to DS1. As the enhancement of the CD signal is not always consistent with the actual helix stabilization, one should rely more on the $R_2$ values that suggest that DS3 can act as a better potential scaffold over DS1 toward the recognition of anion(s), which is in accordance with the results obtained from the computational experiments.

Quantitative Estimation of Interaction. The selective preference for the recognition of a specific anion (sulfate ion over the phosphate ion) by both the peptides (DS1 and DS3) can further be established from the respective calculated $K_d$ values (Figure 2b,d,f,h) based on the magnitude of $[\theta]_{222}$ as a function of concentration of the added anion at 298 K, as recorded during the measurement of the CD spectra. For the peptide DS1, the calculated $K_d$ value for the sulfate ion is $\sim 117$ $\mu$M, whereas for the phosphate ion the value is around $\sim 166$ $\mu$M. Similar to our observation with DS1, a comparable trend of
affinity toward the sulfate ion over the phosphate ion is also observed for DS3. For the sulfate and phosphate ions, the calculated $K_d$ values with DS3 are found to be $\sim 60$ and $\sim 137 \mu M$, respectively. Although because of the small changes in ellipticity, the binding affinities estimated based on the CD experiments are not very sensitive and may not be quantitatively precise, the overall findings emphasize two features of interaction. First, the selective preference for the sulfate ion over the phosphate ion by both of the designed model scaffolds (DS1 and DS3) is on par with our previous observations (Table 1) for the peptides containing the naturally occurring “C’NN” motif.16,17 Second, DS3 is found to be more effective in recognition of a specific anion (sulfate/phosphate) than DS1 and others. Both observations, which are aligned with the $R_2$
values achieved from the CD experiments, substantiate the results obtained using the computational approaches for these designed scaffolds (DS1 and DS3), that is, the binding free energy values from the molecular docking experiments (Table 1).18 Furthermore, this preferential recognition of an anion by the designed scaffolds, especially by DS3, over the natural sequence is really in accordance with the higher retention (residence) time (Table 1) of the sulfate ion with the designed scaffold (for DS1: 2.62 ns and DS3: 4.92 ns) in comparison to that of the sulfate ion with CPS224Ac (0.4 ns), as found in the MD simulations with an explicit aqueous solvent environment.16,18 In a nutshell, the comparison of the respective anion interactions between the designed scaffolds and the naturally occurring sequences (Table 1; at 298 K for the designed scaffolds, whereas at 278 K for the peptides with naturally occurring sequences17) led us to conclude and confirm that peptide sequences with the designed model scaffolds (DS1 and DS3), and especially peptide DS3, are superior in terms of the recognition of anions (sulfate and phosphate ions) than the peptides containing the naturally occurring “C^N” motif (CPS224Ac, CPS226, and CPS228) scaffold.15–17 Such an observation may open an avenue, whereby these designed scaffolds (DS1 and DS3, especially DS3) would be utilized as scavengers for sulfate/phosphate ions or harmful anions like arsenate having similar stereoelectronic properties to the sulfate/phosphate ions.

\( ^1H \) Nuclear Magnetic Resonance. The results obtained from MS and CD clearly corroborate that both DS1 and DS3 show better recognition of the anion(s) through enhanced binding in comparison to the naturally occurring sequences. However, the biophysical tools, MS and CD, can portray only the average of all of the related changes in conformation of the peptide backbone upon the addition of the anion. In order to investigate the effect of anion(s) at the residue level, \( ^1H \) nuclear magnetic resonance (NMR) experiments have been carried out on both the peptide scaffolds (DS1 and DS3). This not only can describe the details of recognition of an anion by the individual residue(s) of the scaffold but also point toward the associated changes in conformation, if any, at the point of interaction upon the addition of the anion. One-dimensional (1D) and two-dimensional (2D) \( ^1H \) NMR experiments have been performed at 278 and 298 K with both the designed peptides (DS1 and DS3) in a fully aqueous solution with and without the addition of sulfate ions. As the directional deviation of the observed chemical shift values of NH and CaH of a particular residue from the respective chemical shift values at the random coil (rc) situation can enlighten the nature of the secondary structure of the peptide backbone (the upfield shift of ∼0.3 ppm for NH and ∼0.35 ppm for CaH indicates 100% helix),21–23 the NH and CaH chemical shift values of all 18 amino acid residues of DS1 and DS3 have been individually recorded from the total correlation spectroscopy (TOCSY) experiments at 278 K (Table S2) for both the sulfate-free and sulfate-added states. Although the HN chemical shift values are sensitive to the environment and make the quantitative conclusions a little difficult to assess, the difference of the chemical shift values (∆δ = δ\text{anion added} − δ\text{free}) of both NH and CaH in the presence of an anion and with respect to the anion-free sample can substantiate that in both the cases (DS1 and DS3) the interaction of the anion is localized at the N-terminal site of the peptides containing the designed scaffold based on the “C^N” motif (Figures 3a,b and S2), as reported in the literature and observed in our previous studies with naturally occurring “C^N” motif sequences.3,15,17 Both the individual peptides, DS1 and DS3, are predominantly helical at 278 K, having an average upfield shift of ∼0.15 ppm for CaH (although all the residues starting from Ala1 show an upfield shift compared to their respective rc chemical shift values, the major deviation was noticed from Ser4 onward; Figure 3c). However, upon the addition of sulfate ions to the peptide(s) (at a 3:1 molar ratio of sulfate: peptide), an appreciable downfield shift (+\( ∆\delta \)) is observed for NH of K3 and S4 (for both DS1 and DS3), whereas an appreciable upfield shift (−\( ∆\delta \)) is observed for CaH from residue 1 to residue 5 for both DS1 and DS3 (Figure 3a,b). The relative changes for other residues, especially Ala7 onward, are negligible (Table S2). Upon the addition of sulfate ions, the further upfield shift of the CaH at the N-terminal residues (\( ∆\delta \) having negative values), as
observed, corroborates the induction and stabilization of the helical population at this segment. However, the observed downfield shift of the main-chain NH (Δδ having positive values) of Lys3 and Ser4 under the same situation apparently contradicts the concept of stabilization of the helical population (as similar to CaH, an upfield shift for NH is expected for the formation of a helix).22,23 Actually, the observed downfield shift identifies that the NH groups of Lys3 and Ser4 interact with the oxygen atom(s) of the sulfate ion through H-bond interactions even in an isolated context-free condition. Hence, the overall downfield shift of these main-chain NH groups is actually an average of a small upfield shift, for the enhancement of helicity (supported by the upfield shift of CaH), and a downfield shift for the H-bond interaction with the SO4\(^2\)- ion. Furthermore, upon the addition of the sulfate ion, the observed lowering of \(^{1}J_{\text{NαN}}\) values within the helical range (\(^{1}J_{\text{NαN}} < 6\) Hz represents a helical conformation \(^{22,24}\)) of these N-terminal residues (residue 2 to residue 5) of both peptides DS1 and DS3 (for free DS1, Ala1: 4.84, Gly2: 5.89, Lys3: 6.42, Ser4: 5.75, and Ala5: 3.54; and with sulfate-added DS1, Ala1: 5.11, Gly2: 5.88, Lys3: 6.13, Ser4: 5.45, and Ala5: 2.39; for DS3, Ala1: 5.04, Ser2: 5.88, Lys3: 5.88, Ser4: 5.4, and Ala5: 3.98; and with sulfate-added DS3, Ala1: 5.08, Ser2: 5.75, Lys3: 5.58, Ser4: 5.33, and Ala5: 3.68) confirms the recognition of the sulfate ion along with the anion-induced enhancement of the helical population of the peptides at this segment.

**Nonsequential Nuclear Overhauser Effect Cross-Peaks.**

The presence of contiguous NN (\(i, i+1\)) and αN (\(i, i+3\)) nuclear Overhauser effect (NOE) cross-peaks is a characteristic measure of a regular helical (α/310) signature in a protein/peptide.24 Although both NN (\(i, i+1\)) and αN (\(i, i+3\)) NOE cross-peaks are very important parameters for identifying helices, we will concentrate our discussion only on the NN (\(i, i+1\)) cross-peaks, as because of the spectral overlap at the αN region, unambiguous assignment of αN (\(i, i+3\)) cross-peaks have not been possible. The helical backbone of the peptides (DS1 and DS3) has been characterized by the appearance of sequential NN (\(i, i+1\)) NOE cross-peaks almost throughout the sequences (Figure 4), although all the cross-peaks are not of the same intensity. In the absence of sulfate ions, the intensities of the NN (\(i, i+1\)) NOE cross-peaks at the N terminal are (among K3/S4, S4/A5, and A5/B6) weak, whereas from the middle of the sequence onward, toward the C terminal, the cross-peaks have either moderate or strong intensities (Figure 4a,c). These observations suggest that in the absence of the sulfate ion, the first few residues of the N terminus of both DS1 and DS3 (based on the careful screening of the FSSP database) have comparatively lower helical content than the rest, which may be because of the lack of strongly helicogenic Aib (B) residue; however, this helps in understanding the effect of anions on the motif segment. Upon the addition of the sulfate ion to the peptides, it has been observed that the intensities of the NN (\(i, i+1\)) NOE cross-peaks at the N-terminal residues (G2/K3, K3/S4, S4/A5, and A5/B6 of DS1; S2/K3, K3/S4, S4/A5, and A5/B6 of DS3) have been appreciably enhanced along with the appearance of a new NOE cross-peak between S4 and B6 in DS3 (Figure 4b,d), whereas there has been no appreciable changes in the intensities of the cross-peaks from B6/A7 onward. Such evidence has revealed two aspects: first, the interaction with the sulfate ion has been confined only to the residues of the N terminus of the sequence of the respective peptides (DS1 and DS3), and second, there has been an anion (sulfate)-induced augmentation of helicity at the anion-interacting site of the peptide sequence. This is consistent with our previous findings exploiting molecular docking and MD\(^{18}\) and is on par with the analysis of the protein database by Denessiouk et al.\(^{7}\) Both the in silico and biophysical experiments (especially NMR) clearly emphasize that information regarding the recognition of the anion (sulfate) ion is embedded within the designed anion recognition scaffold (G−K−S of DS1 and S−K−S of DS3) of the peptides, which is context-independent and even overruled the presence of several Lys residues in the sequence. Such a sulfate ion-induced conformational switching toward helical conformation at the designed anion-binding scaffolds accom-

**Figure 4.** Sequential NN NOE cross-peaks in fully aqueous condition measured at 278 K: (a) DS1, (b) DS1 with SO4\(^2-\), (c) DS3, and (d) DS3 with SO4\(^2-\).
panied by the extension of the length of the original helix by one added turn toward its N terminus is highly consistent with the “C^NN” structural motif in proteins proposed by Denessenki et al.\(^3\)

**Calculation of Fraction Helicity.** All the biophysical techniques (MS, CD, and NMR) undoubtedly confirm the interaction of anions (sulfate/phosphate) with the designed scaffolds (DS1 and DS3) along with the anion-induced augmentation of helicity. However, the amount of induced helicity of the overall peptide, or residue-wise, can be quantified by the calculation of fractional helicity (\(f_H\)).

From the recorded CD spectra of the short peptides considered to exist as an ensemble of different conformational states, the calculation of fractional helicity (\(f_H\)), which is considered to be directly proportional to the \(\pi^*\) transition (\([\theta]_{222}\)), can be obtained by following eq 1, where the contributions of different folded states \((i)\) having unequal limiting values of \([\theta]_{222}\) are taken into consideration.12

\[
f_H = \sum f_i = \sum \frac{\theta_{\text{obs}}^{(i)} - \theta_{\text{U}}^{(i)}}{\theta_{\text{H}}^{(i)} - \theta_{\text{U}}^{(i)}}
\]

For simplicity, eq 1 can be rewritten as eq 2, considering it to be a two-state phenomenon [helix-(folded) and coil (unfolded) state]

\[
f_H = \frac{\theta_{\text{obs}}^{(i)} - \theta_{\text{U}}^{(i)}}{\theta_{\text{H}}^{(i)} - \theta_{\text{U}}^{(i)}}
\]

[where \(H\) denotes the \(\alpha\)-helix (folded) and \(C\) denotes the coiled (unfolded) state], where, for the coiled state at 222 nm, a zero contribution may be assumed (\(\theta_{\text{U}}^{(i)} \approx 0\)). Thus, the ratio of the enhancement of the negative ellipticity at 222 nm (\([\theta]_{222}\)) of the individual peptides upon the addition of the anion with respect to that of the anion-free peptide \([\theta_{\text{obs}}^{(i)} - \theta_{\text{U}}^{(i)}]/\theta_{\text{H}}^{(i)} - \theta_{\text{U}}^{(i)}\)] would provide the quantitative estimation of enhancement of fractional helicity (\(\Delta f_H\)) of the peptides upon the addition of anions (as for 100% \(\alpha\)-helix, \(\theta_{\text{H}}^{(i)}\) is constant). At 298 K, it is found that upon the addition of the anions, the enhancement of the fractional helicity (\(\Delta f_H\)) of peptides containing the designed scaffolds (DS1 and DS3) is \(10\% - 15\%\) only (the enhancement of \(f_H\) for DS1 with sulfate and phosphate was 15 and 12%, respectively, whereas for DS3, the enhancement of \(f_H\) with sulfate and phosphate was 7 and 11%, respectively). Furthermore, the fractional helicity (\(f_H\)) can be obtained from the CD secondary chemical shift values from the NMR experiments using the equation: \(f_H = \Delta \delta_{\text{observed}}/\Delta \delta_{100\% \text{ helix}}\) (where \(\Delta \delta_{100\% \text{ helix}} \approx -0.35 \text{ ppm}\)). However, the upfield shifting of \(-0.35\) ppm, as expected for the helical backbone in proteins, may be an overestimate for the solvent-exposed state of a short monomeric state.25

It is observed that at 278 K, for Lys3 and Ser4 of the peptides DS1 and DS3, a very little enhancement of such cross-peaks (\(I_{i1}/I_{i1}\)) \((I_{i1}/I_{i1})^{1/6} = d_{\text{obs}}/(d_{\text{obs}} + 1) = 2.9(3.5f_H + 2.4(1 - f_H))\).12

These observations in terms of the enhancement of fractional helicity (\(f_H\)) of the designed scaffolds apparently move in a reverse direction to our previous observations, where peptides containing the naturally occurring “C^NN” motif (CPS224Ac, CPS226, and CPS228)\(^17\) showed a higher enhancement of fractional helicity upon the addition of anions. However, comparison of the dissociation constant \((K_d)\) values, which measure the binding affinity, reveals that for the designed scaffold there have been threefold decrease of the \(K_d\) value at 298 K with respect to that for the sequence containing the naturally occurring motif sequence at 278 K (60 \(\mu\)M for DS3 with sulfate at 298 K in contrast to 181 \(\mu\)M for CPS224Ac with sulfate at a temperature of 278 K (Table 1)). This strongly suggests that the interactions of the anions with the designed model scaffolds are considerably stronger even at higher temperatures (additional 20 K) compared to the naturally occurring “C^NN” motif sequence and justifies the much higher retention time (4.92 ns) of the sulfate ion with DS3 in the MD simulations.18 Such an appealing contradicting observation can be explained from the fact that the N-terminal sequences of the peptides DS1 and DS3 attain an appreciable amount of helicity compared to that of naturally occurring sequences, as revealed from the comparison of the CSI (chemical shift index) of CoH of the designed scaffold with the naturally occurring sequence (Figure 3c). This can further be established by the comparison of the observed \(R_2\) values of the free peptides and those with added anions (at 298 K, \(R_2\) value of DS1: 0.77; DS1 sulfate, 0.85; DS1 phosphate, 0.84, and \(R_2\) value of DS3: 0.84; DS3 sulfate, 0.89; DS3 phosphate, 0.89, whereas at 278 K, \(R_2\) value of CPS224Ac: 0.75; CPS224Ac sulfate, 0.79; CPS224Ac phosphate, 0.81, and \(R_2\) value of CPS226: 0.74; CPS226 sulfate, 0.84; CPS226 phosphate 0.79\(^17\)). Such a higher \(R_2\) value even at a high temperature (298 K) for a DS series firmly designates that the anion recognition N-terminal sequences of DS1 and DS3 are themselves comparatively more helical than the sequences with naturally occurring amino acids, as the model anchored helix ABGY is same for all of the peptides. Such a higher helicity in the designed scaffold justifies the enhancement of fractional helicity upon the addition of anions is comparatively less for the designed sequence (limiting value of the anion-added \(R_2\) in all cases = 0.85).

As the anion–peptide interaction is locally mediated and the anion-induced augmentation of helicity is cooperative in nature, it can be assumed that for less helical sequences (CPS224Ac and CPS226), an appreciable part of the total interaction free energy is utilized to attain the helicity required for the recognition of anions, whereas peptides (DS1 and DS3) with a higher helical nature can interact spontaneously and strongly with the anions, which is reflected in their respective lower \(K_d\) values.

## MATERIALS AND METHODS

In order to pursue biophysical characterization of the designed model scaffolds, two model scaffolds (Gly-Lys-Ser for DS1 and Ser-Lys-Ser for DS3) have been chosen and appended at the N terminus of the predesigned thermally stable helix, ABGY.12,13,27

Rationalization of designing the sequences has been described in detail in our recent publication.18 A single alanine residue before the motif segment has been inserted at the N terminus of the chimeric peptides to make it equivalent to the three naturally occurring sequences (CPS224Ac, CPS226, and CPS228); the detailed sequences of the two peptides are as follows:

**DS1:** Ac-Ala-Gly-Lys-Ser-Ala-Ala-Lys-Ala-Aib-Gly-Gly-Tyr-CONH₂

**DS3:** Ac-Ala-Ser-Lys-Ser-Ala-Ala-Lys-Ala-Aib-Gly-Gly-Tyr-CONH₂

These crude synthetic peptides have been procured from USV Ltd., synthesized in solid phase using the standard Fmoc protocol. Reverse-phase HPLC technique (using a gradient of 0–60% CH₃CN/H₂O) has been employed for the purification of the peptides, in Waters 2487 equipped with a dual λ...
Mass Spectrometry. Binding of anions(s) has been directly checked by using both MALDI and ESI-FTMS. At the outset, the molecular mass of the peptides DS1 and DS3 (MW of DS1 1674, MW of DS3 1704) has been checked in the positive mode of MALDI-MS (Autoflex III TOF/TOF 200 using 1:1 H₂O/acetonitrile with 0.1% HCOOH). Then, both the positive and negative modes of ESI-FTMS (Apex Ultra 70, Bruker Daltonics direct infusion mode using 1:1 H₂O/CH₃CN with 0.1% NH₃) have been deployed to identify the anion-bound (sulfate ion added as Na₂SO₄ and phosphate ion added as Na₂HPO₄) species of both DS1 and DS3.

CD Spectroscopy. Far-UV (190–250 nm) CD spectra of the designed peptide sequences have been recorded at room temperature (298 K) in order to monitor the conformational changes, if any, resulting upon interaction with the anion(s). The CD spectra of the peptides in fully aqueous state (before and after the addition of anions) have been recorded in a Jasco J-815 CD spectropolarimeter. For recording the CD spectra, the concentrations of the peptide solutions have been kept at 50–75 μM (measured from Tyr absorbance at 275 nm, ε = 1450 cm⁻² M⁻¹), whereas the concentrations of the anion(s) have been varied from 0.5 to 10 times of the peptide concentration used for the study. The recorded CD signals have been normalized and reported as mean residual ellipticity (deg cm² dmol⁻¹).

To estimate the relative affinity of the anions (both sulfate and phosphate) toward the peptides DS1 and DS3, the dissociation constant (Kₐ) has been measured at room temperature, considering the recorded CD intensity of nα transition at 222 nm ([θ]ₐ₂₂) as a function of concentration of individually added anions (sulfate and phosphate). From the obtained scatter plot of [θ]ₐ₂₂ values as a function of concentration of the added anions, Kₐ has been calculated by fitting the data points in a user-defined equation (from the concept of Michaelis–Menten equation)

\[ y = A + \frac{b}{K_\text{d} + x} \]  

where “y” denotes the recorded CD values ([θ]ₐ₂₂) as a function of concentration of added anions; “A” represents the initial CD value ([θ]₀) of the anion-free peptide; “b” represents the maximum CD value ([θ]ₚ) of the peptide obtained because of the addition of anions (sulfate/phosphate); “K_\text{d}” is the dissociation constant, and “x” indicates the concentration of anions (sulfate/phosphate). “K_\text{d}”, has been calculated using the software OriginPro 8.

¹H NMR Studies. ¹H NMR experiments (both 1D and 2D) have been recorded in purely aqueous conditions with 10% D₂O (v/v) at 278 and 298 K in Bruker DRX 700 MHz spectrometers using standard protocols. The 2D ¹H NMR experiments [TOCSY, NOESY, and ROESY (rotating-frame Overhauser effect spectroscopy) (with a mixing time of 250 ms)] have been carried out to get the details of the residue-wise conformational landscape of the peptide(s) upon the addition of anions. The concentration of the peptide samples has been kept at ~2 mM, and the sulfate ion has been added at varying concentrations. TSP is used as the internal standard for the calibration of the chemical shift values. The programs Sparky 3.113 and TopSpin 4.0.7 (Bruker BioSpin) are used to analyze the 2D ¹H NMR data.

CONCLUSIONS

Based on the consensus obtained regarding the intrinsic affinity of the naturally occurring “C”NN motive for anion(s) in a context-free nonproteinaceous isolated system, an attempt has been made toward designing in vitro “model scaffolds” for novel binding sites for better recognition of anions. During the design of the model scaffolds based on the naturally occurring sequences found in the FSSP database, emphasis has been given to the motif–anion interaction mediated through “local” interactions, and the presence of polar amino acid in the motif sequence prefers anion recognition. The interaction of the four designed model scaffolds (DS1, DS2, DS3, and DS4) with the anion(s) using computational techniques (molecular docking and MD simulation) was reported recently. Out of these four designed model scaffolds, the two scaffolds DS1 and DS3 which showed a stronger affinity toward anion(s) (sulfate and phosphate) led us to ascertain the fact in a real-life experiment. Using complementary spectroscopic techniques, it has been established here that even without any tertiary effect, the designed model scaffolds DS1 and DS3 can recognize the anions (both sulfate and phosphate) in a fashion similar to the naturally occurring “C”NN motif but in a much better way and especially for DS3, as revealed by its respective Kd value. The mode of interaction is found to be in appreciable agreement with the results obtained using computational tools. The existence of a higher helical content along with an augmentation of the helical conformation, although small in magnitude, at the designed scaffold segment ascertains its conformational privilege toward the recognition of anions. The confirmation of the intrinsic affinity of these designed motif sequences toward anions like sulfate/phosphate in a context-free system opens an avenue for exploring a new trend in peptidomimetics as a potential candidate for the scavenging of harmful anion(s) having similar stereochemical constraints with sulfate/phosphate, which can further be justified by our recent attempt using arsenate as an anion to trigger the interaction.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.9b04180.

Details of peptide sequences containing the naturally occurring “C”NN motif segment used for earlier studies; NH and CαH chemical shift values of DS1 and DS3 along with their sulfate-added states in a fully aqueous condition at 5 °C obtained from ¹H NMR, ESI–MS data in positive mode showing the binding of anions to the peptides: DS1 with SO₄⁻; DS1 with HPO₄²⁻; DS3 with SO₄⁻; and DS3 with HPO₄⁻ and the isotopic distribution having a difference of 0.5 emphasizing the doubly charged species of the anion-bound peptides; and comparative 1D NMR spectra of anion-added and free peptides showing the effect of SO₄⁻ ion to the peptide–NH chemical shift values of the N-terminal residues: DS1 with SO₄⁻; DS1; DS3 with SO₄⁻; and DS3 at 278 K (PDF)

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Notes

The authors declare no competing financial interest.

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