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How Do Halogen Substituents Contribute to Protein-Binding Interactions? A Thermodynamic Study of Peptide Ligands with Diverse Aryl Halides

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The large inventory of known alkyl and aryl halides, of both manmade and natural origin, attests to the fact that useful and often unique properties are enjoyed by such organic structures. The presence of fluorine, chlorine, bromine and iodine is especially notable when considering biologically active substances, many of which derive benefit from the presence of one or more halogen substituents.[1,2] In the case of a biomolecular interaction—frequently that of a protein with a small molecule or peptide ligand—one of the accrued benefits is often an increase in binding affinity. But underlying such empirical observation is an incomplete understanding of the actual energetic contributions that allow a halogenated compound to enhance a molecular association.

Much of our understanding about the biomolecular recognition properties of halogen functionality derives from model systems, such as organic receptors or peptide scaffolds.[3,4] Conspicuously absent, however, are studies that systematically examine the influence of halogen substituents in the thermodynamic context of an actual protein–ligand interaction.[5,6] Here, we present the first report in which the free energy, enthalpy and entropy changes of a protein–ligand binding interaction have been measured as a function of varying halogen identity and position. Using isothermal titration calorimetry (ITC), we determined the thermodynamic profile of a series of aryl halide-bearing peptide ligands for a small modular protein, the third PDZ domain (PDZ3) of a mammalian neuronal protein, postsynaptic density-95 (PSD-95).

Our previous efforts at ligand design for PDZ3 have yielded a variety of native and modified peptides of both linear[7,8] and cyclic[9,10] form that bind the protein with low micromolar K_d values. Our desire to incorporate halogens within the existing framework of one of our established linear inhibitors was intended not only to allow for a study of the fundamental binding parameters, but also to determine whether such substitution could drive the development of improved PDZ domain-directed cellular probes.[11] In addition to possible increases in affinity, halogenation of small molecule therapeutics has been shown to improve the ability to bind and cross lipid membranes.[12] As applied specifically to peptides, incorporation of halide functionality has been linked to enhanced blood–brain barrier permeability.[13]

A previous binding study of PDZ3[7] with a series of peptides based on KKETEV, demonstrated that the P_{-1} position glutamate could be replaced without significant change in affinity by several different residues, including tryptophan (Figure 1).

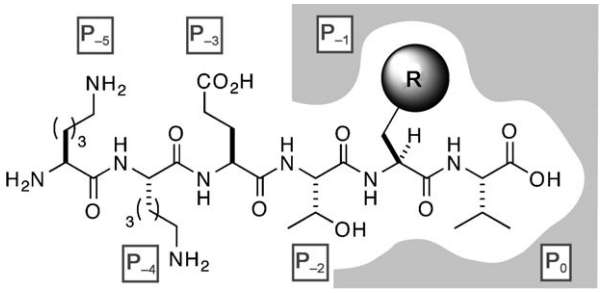

Figure 1. Positionally-labeled diagram of KKETXV bound to PDZ3.

An X-ray crystal structure of PDZ3 bound to KKETWV (PDB ID: 1TP5) showed that the P_{-1} side chain occupies a relatively well-defined region on the protein. The affinity of this association ($K_d = 2.8$ μM), in conjunction with the observation that the indole moiety appears comfortably nestled within a concavity of the protein surface, prompted us to investigate whether an additional aromatic functionality can be favorably accommodated.

Initial replacement of Trp at P_{-1} with Phe yielded KKETFV (**1**), which exhibited a twofold improved affinity of $K_d = 1.4$ μM (Table 1). This successful indole-to-phenyl transition prompted us to examine further the nature of the aromatic presence at P_{-1} and how side-chain perturbation could positively affect the

Table 1. Thermodynamic binding parameters for KKETFV (**1**) and substituted halide KKETXV ligands with PDZ3 of PSD-95.

Ligand[a]	K_d [μM]	ΔG [kcal mol^{-1}]	ΔH [kcal mol^{-1}]	$T\Delta S$ [kcal mol^{-1}]
1	1.4 (±0.1)	−8.0 (±0.1)	−6.9 (±0.2)	1.1 (±0.2)
2	1.2 (±0.2)	−8.1 (±0.1)	−2.9 (±0.5)	5.2 (±0.4)
3	0.53 (±0.02)	−8.6 (±0.1)	−4.3 (±0.1)	4.3 (±0.1)
4	0.57 (±0.1)	−8.5 (±0.1)	−4.4 (±0.1)	4.1 (±0.2)
5	0.38 (±0.03)	−8.8 (±0.1)	−4.4 (±0.1)	4.4 (±0.1)
6	7.6 (±0.4)	−7.0 (±0.1)	−2.1 (±0.1)	4.9 (±0.1)
7	2.1 (±0.1)	−7.7 (±0.1)	−2.5 (±0.1)	5.2 (±0.1)
8	1.8 (±0.2)	−7.8 (±0.1)	−3.2 (±0.1)	4.6 (±0.1)
9	0.44 (±0.01)	−8.7 (±0.1)	−3.6 (±0.1)	5.1 (±0.1)
10	0.28 (±0.01)	−8.9 (±0.1)	−4.5 (±0.1)	4.4 (±0.1)

[a] All values are the average of at least two independent titrations. Reported error ranges represent arithmetic deviation from the average.

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binding interaction. Instead of replacement with different ring structures, however, this afforded us the opportunity to probe a fundamental question that has been largely ignored in most protein–ligand binding studies: the thermodynamic consequences of varying the pattern of halogen substitution on a benzene ring.

Our plan was to design, synthesize and calorimetrically analyze a series of halogenated analogues of the KKETFV ligand in order to observe the effect of substitution on the free energy, enthalpy and entropy parameters upon binding to protein. This was accomplished by systematic variation in both the position of the halogen as well as its identity at two different stages. The first stage involved fixing the substitution at the *para* position of the phenyl ring and varying the halide. In the second stage, the halogen identity was kept constant and different positions around the ring were studied with single and double substitutions. The use of four different halides—singly and multiply—at different positions, could lead to a large combinatorial population of compounds. For this initial inquiry we chose ten representative peptides to prepare and evaluate (Figure 2).

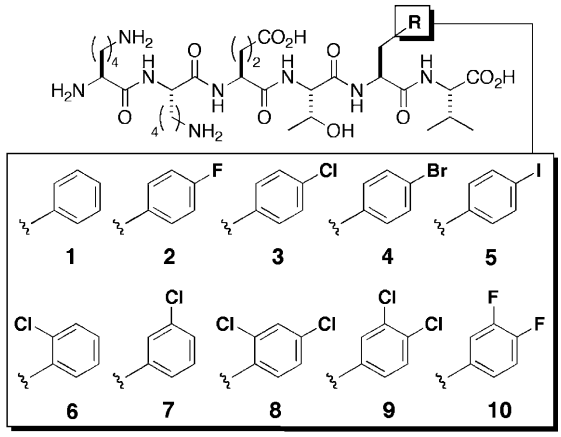

Figure 2. Hexapeptide ligands for PDZ3 with variably halogenated phenyl side chains at position P_{-1}.

Compared to the nonhalogenated parent **1**, each of the *para*-substituted analogues exhibited an improvement in the entropy of binding (**2**–**5**; Table 1). The small incremental increases in peptide affinity from **1** to **5** is loosely correlated with the increasing van der Waals radii of the atoms at the *para*-position (H: 1.20 Å, F: 1.47 Å, Cl: 1.77 Å, Br: 1.92 Å, I: 2.06 Å; halogen values reflect the effective radii for phenyl substitution).[14] Aside from the fluorine analogue **2**, in which entropy was the major contributor to binding, the chlorine, bromine and iodine analogues received equal contributions from ΔH and $T\Delta S$. The largest improvement in affinity measured approached a fourfold enhancement when hydrogen was replaced with iodine. One plausible explanation for the increased $T\Delta S$ values is to evoke a classical hydrophobic effect, in which dehydration of ordered (low entropy) waters is enhanced.

For the next series of compounds (**6**–**9**), we chose to further explore chlorine substitution, which represented a compromise between affinity (better than fluorine) and size/mass (smaller than bromine or iodine). The position of substitution was found to influence binding strength, perhaps due to unfavorable steric hindrance effects that lead to suboptimal binding interactions. A 14-fold diminishment was observed when chlorine was moved from the *para* to the *ortho* position. This was due to a notable drop in enthalpy, whereas $T\Delta S$ did not deviate significantly. The *ortho* analogue **6** exhibited the weakest affinity of the entire series; this was attributable to the least favorable ΔH value. The *meta*-positioned chloride experienced a reduced drop in affinity, again due to a less favorable change in enthalpy.

Upon addition of the second chlorine at the *para* position, it was possible to "rescue" the binding of the singly-substituted *ortho* and *meta* analogues. The **6**→**8** and **7**→**9** transitions resulted in four- and fivefold improvements in affinity, respectively. The doubly-substituted **9** in fact exceeded the affinity of the singly *para*-chlorinated ligand itself. Dissecting the component thermodynamic values, 3,4-dichloro **9** was able to maintain the favorable entropy change of the *meta* analogue **7**, but without the corresponding loss of enthalpy. In other words, it appears that the dichlorinated analogue **9** has managed in part to evade enthalpy–entropy compensation, due to the presence of the second chloride.

In the case of the single or double chlorinated substitutions that involve the *ortho* or *meta* positions, the issue of degeneracy could be a consideration. Although the phenyl ring might be expected to undergo rapid rotation, there might be energetically preferred rotamers, which would not impact symmetrical analogues, such as **3**.

Given the demonstrated potency of **9**, we decided to prepare the corresponding 3,4-difluoro analogue, **10**. This was based on the rationale that since fluorinated compounds are disproportionately represented among all the halogens in synthetic therapeutic agents, any future development of these peptide ligands towards use as cellular probes could benefit from such substitution.[15–17] Ligand **10** exhibited the highest affinity of the entire series, with a dissociation constant of 0.28 μM. This represents a fivefold improvement in affinity over the original KKETFV peptide, and an almost tenfold improvement over the initial parent peptide KKETEV.

In summary, the first report of thermodynamically characterized halogenated protein–binding ligands has been presented, in which a series of peptides was systematically varied in terms of halogen identity and position. While these measurements pertain to a single binding pocket within one protein, they do provide insight into the nature of the binding interaction as influenced by a halogenated aromatic moiety. Although generalizations will have to await similar reports for other protein–ligand systems, our results suggest that a prudent addition to peptide ligand discovery programs is the inclusion of halogenated phenylalanine analogues. The potential gain in affinity is particularly attractive considering the ease with which such halogenated amino acid residues can be incorporated into the chemical syntheses of individual sequences or libraries.

Experimental Section

Experimental procedures are provided in the Supporting Information.

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