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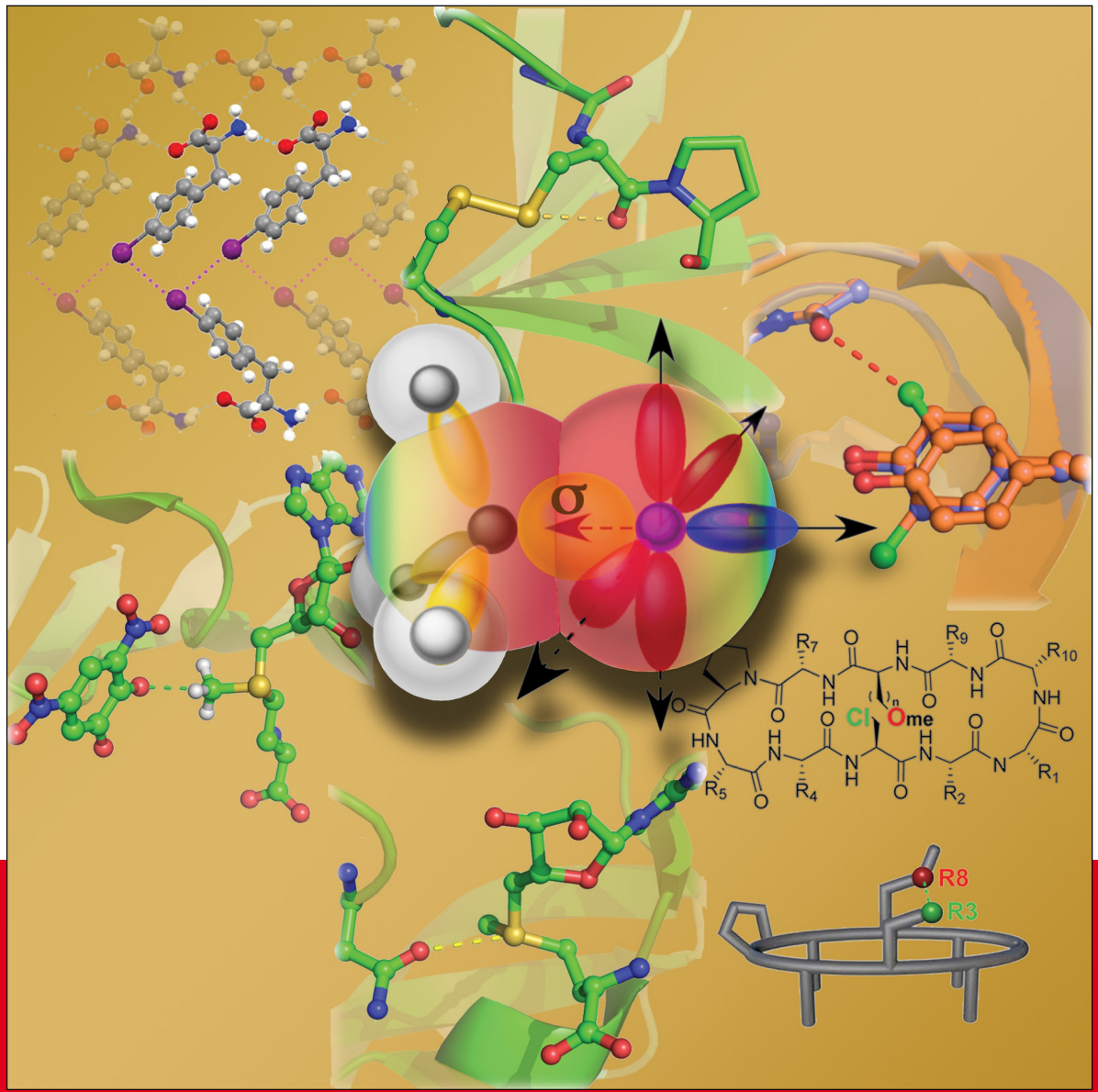

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Front Cover:
P. Shing Ho et al.
Non-classical Non-covalent σ-Hole Interactions in Protein Structure and Function: Concepts for Potential Protein Engineering Applications

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The σ-hole theory describes the anisotropic distribution of electrostatic potential of organic substituents that participate in a broad range of non-classical noncovalent (*nc*NC) interactions found in chemistry and biochemistry. This review explores how halogen bonds, chalcogen bonds and tetrel bonds can affect the structures, assemblies, and potential functions of peptides and proteins. These three types of *nc*NC interactions have energies that are comparable to the H-bond and, therefore, serve as important concepts in molecular recognition and potential tools for biomolecular design. More information can be found in the Review by P. Shing Ho et al.

*M. G. Walker, C. G. Mendez, P. S. Ho**

Special Issue

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CHEMISTRY AN ASIAN JOURNAL Review

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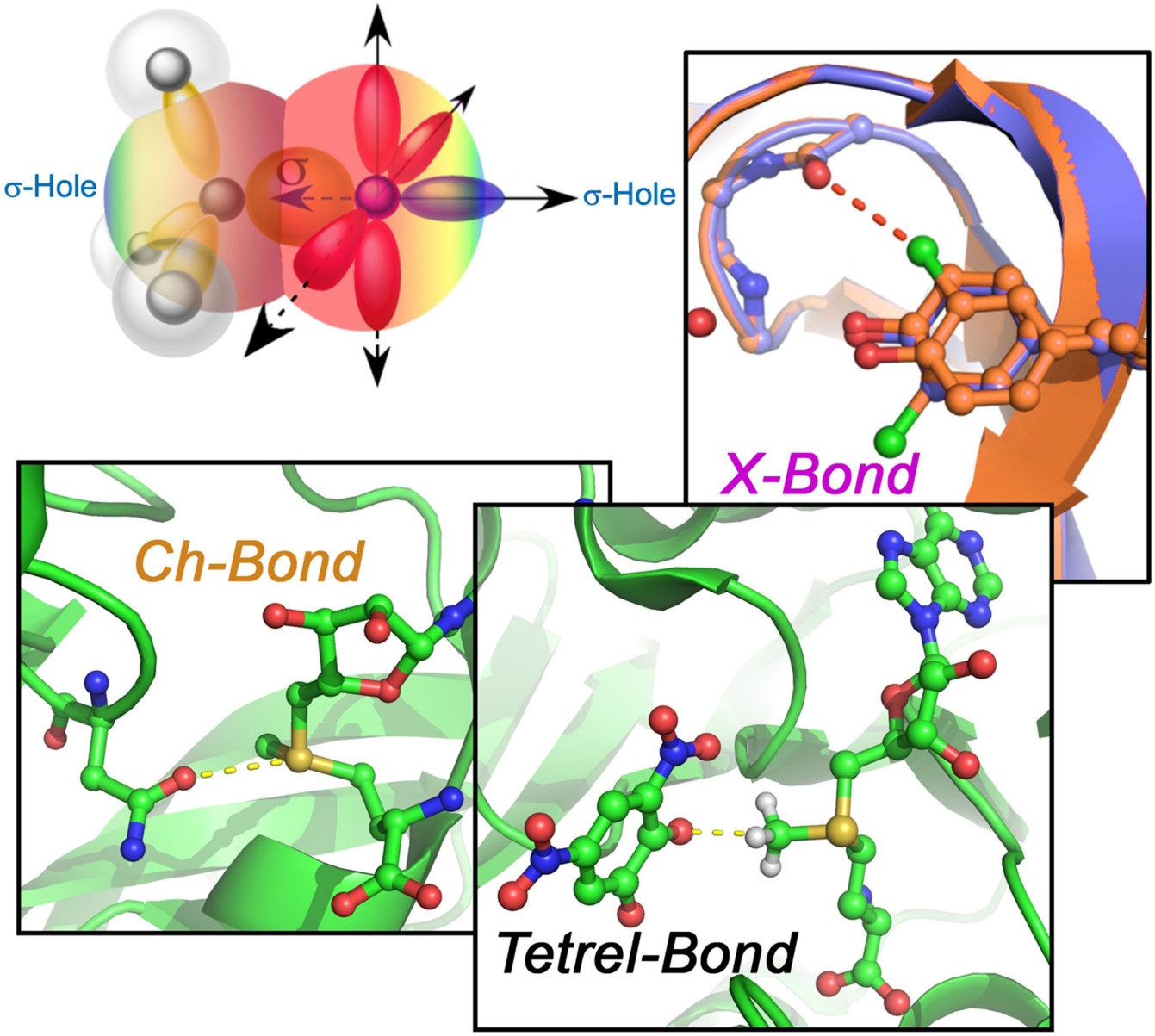
Special Collection

Non-classical Non-covalent σ-Hole Interactions in Protein Structure and Function: Concepts for Potential Protein Engineering Applications

Margaret G. Walker[+], C. Gustavo Mendez[+], and P. Shing Ho*[a]



Abstract: The structures and associated functions of biological molecules are driven by noncovalent interactions, which have classically been dominated by the hydrogen bond (H-bond). Introduction of the σ-hole concept to describe the anisotropic distribution of electrostatic potential of covalently bonded elements from across the periodic table has opened a broad range of nonclassical noncovalent (*nc*NC) interactions for applications in chemistry and biochemistry. Here, we review how halogen bonds, chalcogen bonds and tetrel bonds, as they are found naturally or introduced synthetically, affect the structures, assemblies, and potential functions of peptides and proteins. This review intentionally focuses on examples that introduce or support principles of stability, assembly and catalysis that can potentially guide the design of new functional proteins. These three types of *nc*NC interactions have energies that are comparable to the H-bond and, therefore, are now significant concepts in molecular recognition and design. However, the recently described H-bond enhanced X-bond shows how synergism among *nc*NC interactions can be exploited as potential means to broaden the range of their applications to affect protein structures and functions.

Please provide academic titles (Prof., Dr.) for all authors.

1. Introduction

The folding of a protein is driven by non-covalent (NC) interactions, with the hydrogen bond (H-bond) being classically the dominant determinant of its functional three-dimensional conformation.[1] However, there is now a greater appreciation that non-classical NC (*nc*NC) interactions involving elements from groups IV to VII of the periodic table can play significant roles in defining the structures and functions of molecular systems at various levels in chemistry,[2] which can by extension be applied to the future design of new functional proteins.

A general model describing the class of *nc*NC interactions is the σ-hole theory (Figure 1).[4] This model posits that when the valence electrons of an atom's p-orbital forms a covalent bond with another atom, this orbital becomes depopulated in the direction diametrically opposed to the associated σ-molecular orbital. The result is an electropositive crown called the σ-hole, which sits directly opposite the covalent bond. The electropositive potential of the σ-hole is accentuated by electron withdrawing groups. Such *nc*NC interactions have now been given bonding names specific to their groupings in the periodic table: tetrel bonds, pnictogen bonds, chalcogen bonds, and halogen bonds (Figure 2) but have more generally been referred to as "σ-hole bonds".[5] Even the H-bond has at times been placed under this broader classification, if we consider its 1 s orbital to be similarly polarized when a hydrogen is chemically bonded to any other atom.[6] Each of these *nc*NC interactions pair its σ-hole (analogous to the H-bond donor) with an electron-rich acceptor, typically an oxygen, nitrogen, or delocalized electron system (such as an aromatic ring). We should note here, however, that the σ-hole describes only the electrostatic component of such interactions. For at least the halogen bond, additional components, including charge-transfer (its initial conception[7]), and dispersion,[8] as well as electrostatics[9] have been supported by various levels of computational theory. It is likely that these concepts will be consistent for describing the various physical contributions to *nc*NC interactions across the group IV to VII elements.[10]

In chemistry, these *nc*NC interactions have been shown to facilitate the assembly of supramolecular complexes (including crystals and liquid crystals), the recognition of ions and ligands, and the catalysis of organic reactions.[2e] The study of *nc*NC interactions in biology have not been as extensive as in small organic and inorganic molecular systems but have been found to primarily be important as recognition elements in ligands/inhibitors against protein targets.[2f,11]

As the field begins to mature, there is a growing mountain of studies on how various *nc*NC interactions are found in a multitude of biomolecular and biomimetic systems. The intent of this review, however, is not to be a comprehensive summation of all occurrences of *nc*NC interactions (see Jena, *et al.*,[12] for a more complete summary of various *nc*NC interactions found in proteins system) but explicitly on exploring the applications of *nc*NC interactions that are emerging as being potential design elements to engineer functional proteins. In particular, we will focus on how halogen, chalcogen, and tetrel bonding are applied to understand and/or manipulate the assembly, stability, and function of peptide and protein systems, along with their role in the recognition of functional cofactors. For each of these *nc*NC interactions, we start by discussing studies that survey the Protein Data Bank (PDB[13]) for their occurrence, followed by a discussion of the energies of such interactions, and conclude with examples of where such interactions have been found or engineered to control the structure, stability, and/or function of a peptide/protein system. These discussions initiate with the halogen bond, since its role in biology has been most extensively studied.

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This manuscript is part of a special collection on Halogen Bonding.

2. The Halogen Bond

The halogen bond (X-bond, for short) is now well recognized[14] and is finding utility across a broad range of chemical applications.[15] The X-bond was first recognized as an important *nc*NC interaction in biology through a survey of the PBD.[11a] This initial PDB survey showed that X-bonds were primarily important in recognition of halogenated ligands and inhibitors to protein targets, including the hormone thyroxine to its receptors[16] and agonists and antagonists against cancer related protein kinases[17] (Figure 3). The primary X-bond acceptor in such cases, similar to H-bonds, is the carbonyl oxygen of the peptide backbone of the protein, likely because of its ubiquity in such systems. The σ-hole model predicts X-bonding potentials to be dependent on the polarizability of the donor halogen and, consequently, correlated with the size of the halogen. The predicted order of X-bond strength would thus be $I > Br > Cl \gg F$ (F is seldom seen as X-bond donors in biological systems). The energies of biological X-bonds have been estimated through a unique assay system[18] that takes advantage of the similarity between H- and X-bonds to compete one against the other in a four-stranded DNA Holliday junction.[19] The resulting X-bond energies range from +0.8 kcal/mol (F) to −0.15 kcal/mol (Cl) to −4.2 kcal/mol (Br) and to −6.5 kcal/mol (I).[20] These fundamental concepts of biological X-bonds will be foundational for any design element in protein engineering.

Subsequent surveys further revealed the amphipathic nature of the halogen in serving as both X-bond donor and H-bond acceptor,[21] the orthogonality between H- and X-bond donors when both are paired to the same acceptor[22] (Figure 4b), and how water can serve as a bridge between X-bond donors and receptors.[23] Thus, the X-bond plays an important function in understanding and designing therapeutic agents against clinically important protein targets. The biomedical role of X-bonds has been reviewed extensively elsewhere[17b,24] and, therefore, will not be discussed further here. In this review, we will focus on studies that explore the effects of *nc*NC interactions that of halogenated systems that affect the structure, stability, and/or function of peptides and proteins.

2.1 Halogen bonds in the assembly of amino acids and peptides

The fundamental building block of proteins is the amino acid. Naturally halogenated amino acids have been found to play significant roles in affecting protein structure and function across a broad range of organisms. For example, halogenated tyrosine (Y) and tryptophan (W) in marine organisms have been found to facilitate protein crosslinking,[26] while chloro- and bromotyrosines resulting from oxidative halogenation are markers for asthma in humans.[27]

The assembly of amino acids into proteins requires formation of peptide bonds, which is mimicked, in its simplest form, as *N*-methylacetamide (NMA). Vasylyeva, *et al.*,[28] showed that cocrystals of NMA with dihalotetrafluorobenzene forms H-bonded layers (reminiscent of interactions that stabilize regular structures in proteins), with each NMA layer bridged by a pair of perpendicular X-bonds from individual aromatic ligands (Figure 5a). The concept of orthogonality relating X- and H-bonds, initially conceptualized from surveys of proteins,[22] thus extends even within simple peptide mimics.

Studies by Bertolani, *et al.*,[29] show that isolated iodophenylalanine (^{I}F) amino acids crystallize with the backbone amides forming H-bonded layers (Figure 5b). These layers are connected through a series of I···I X-bonds in which the electropositive σ-hole of one iodine points towards the electronegative waist of the second. Similarly, *para*-iodophenylalanine–phenylalanine (^{I}F–F) dipeptide was found to crystallize as β-sheet like complexes, with I···I X-bonds bridging across the H-bonded sheets.[30] Both the mono and di-iodinated phenylalanyl dipeptides were seen to form nanotube-like

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Gustavo Mendez received his bachelor's degree in Biochemistry and studied pulmonary inflammation under Dr. Kenyon at the University of California Davis. He then worked in a winery as a lab technician, winemaker, and vineyard farmer. Currently he is a PhD student at Colorado State University in Dr. P. Shing Ho's lab where he is studying halogen bonds in enzymatic systems. Outside of lab he enjoys cooking and the outdoors.

P. Shing Ho earned his Ph.D. degree with Brian M. Hoffman at Northwestern University studying mechanisms of electron transport in hemoproteins. He was an American Cancer Society postdoctoral fellow with Alexander Rich at the Massachusetts Institute of Technology, after which he started his academic career at Oregon State University. In 2007, he became Chair of Biochemistry & Molecular Biology at Colorado State University. In 2017, he served as a Program Director with the National Science Foundation. He has earned recognition for his studies on DNA structures and halogen bonds in biology and as an inspirational educator.

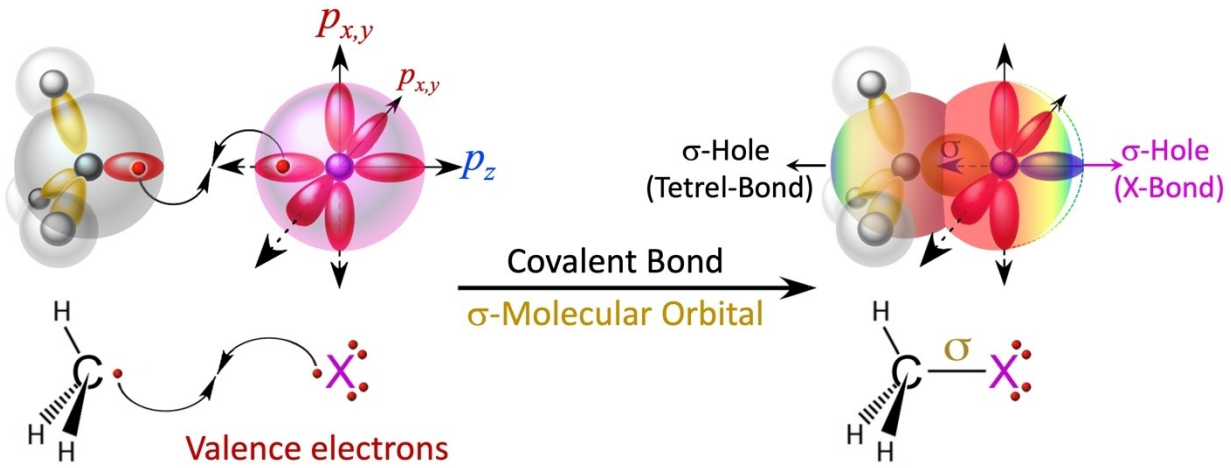


Figure 1. Schematic of the σ-hole model to describe halogen and tetrel bonding. In this model, pairing an electron from the valence p_z-orbital of a halogen (X) with that of, for example, the carbon of a methyl group to form a single covalent bond results a σ-molecular orbital (σ-MO). We note that only the p-orbital valence electrons are shown in this figure. This σ-MO subsumes the electron from the halogen p_z-orbital, leading to an electropositive crown and flattening of the van der Waals radius (the σ-hole) but leaving an electronegative waist around the halogen substituent. This σ-hole provides an electrostatic basis for the halogen bond. In similar fashion, a σ-hole is created on the surface of the carbon of the methyl group opposite the σ-MO, which in turn forms the electrostatic basis for a tetrel bond. Adapted from Ho.[3]

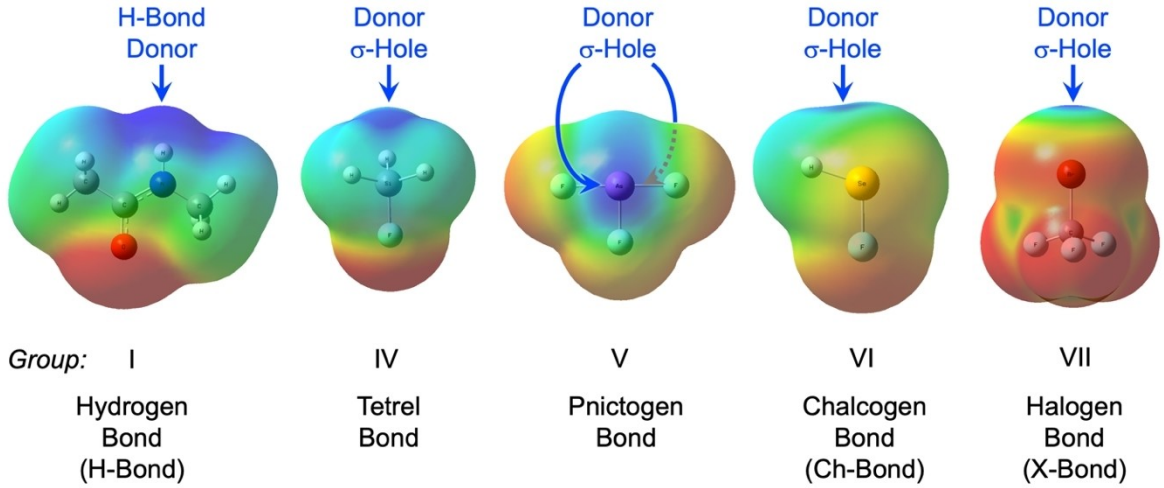


Figure 2. Examples of σ-hole leading to electrostatic "bonding" interactions for various elemental groups. Electrostatic potential (ESP) maps were calculated for a H-bond donor in *N*-methylacetimide (NMA) as a model peptide bonds of proteins, for a tetrel bond donor in $FSiH_3$ from group IV, for a pnictogen bond donor in F_3As from group V, for a chalcogen bond (Ch-bond) donor in FSeH from group VI, and for halogen bond (X-bond) donor in F_3CBr from group VII. Positive electrostatic potentials are shown as blue surfaces while negative electrostatic potentials are in red. Adapted from Czarny, *et al.*.[2f]

macrostructures in solutions of DMSO, with red-shifts in the infrared spectra in DMSO solvated solids or films supporting formation of weak X-bonds in the solid state. Thus, even in the simplest cases, X-bonds facilitate assembly of halogenated amino acids and dipeptides into extended complexes.

A synthetic peptide construct containing eight unnatural α/L-sulfono-γ-amino acids (halogenated with Cl, Br, or I at the *para*-position of the phenyl ring, Figure 6a) was seen by Teng, *et al.*,[31] to self-assemble into right-handed 4_{13}-type helical (4_1 symmetry) "foldamer" structures (Figure 6b), with sets of helices stacked in near continuous alternating parallel and perpendicular arrangements. X-bonds from the halogen of the sulfono side chain to the center of the phenyl ring of the sulfono side chain across pairs of adjacent parallel-aligned helices help hold the assemblies together (Figure 6b inset), with QM calculated energies ranging from 2.5 kcal/mol to 2.8 kcal/mol. Close halogen–halogen interactions were also seen linking perpendicularly packed helices but these synthons did not appear to be consistent with standard X-bond geometries in proteins.[14,17b]

X-bonding can play a role in the assembly of amyloid-related peptides. The DFNKF pentapeptide is a core motif that triggers fibrillation of human calcitonin polypeptides. Bertolani, *et al.*, saw that halogenation of the phenylalanine (F) residues in this pentapeptide promotes the formation of hydrogels, which are seen to result from formation of fibrillar networks.[29] The dependence of fibrillation of the halopeptides follows the trend I > Br > Cl, which supports the involvement of X-bonds in the process (the unhalogenated peptide did not form hydrogels

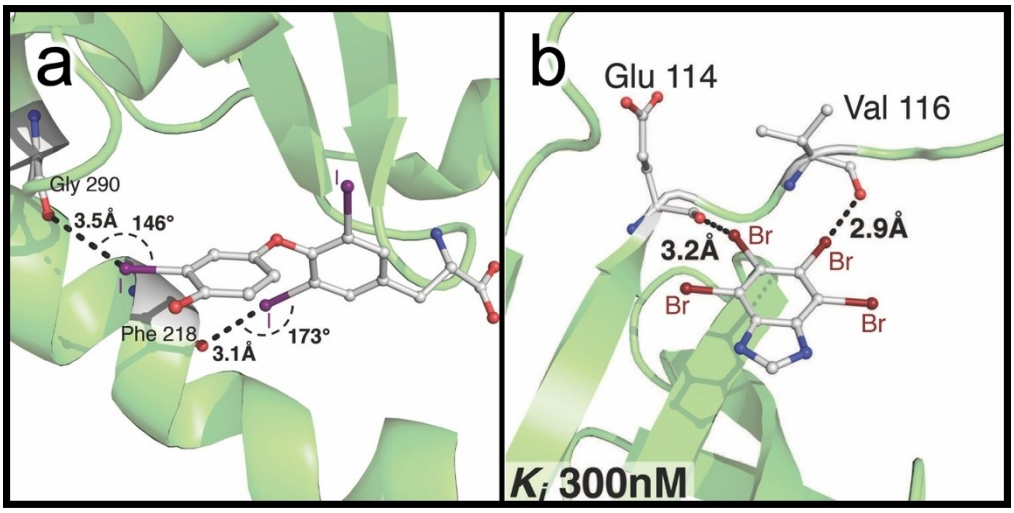


Figure 3. Examples of X-bonds in protein-ligand complexes. a. The hormone 3,5,3′-triiodothyroxine in the binding site of human thyroid hormone receptor. The distances and angles that define the geometries for two X-bonds involved in recognition of the iodinated hormone are labeled. b. Two X-bonds that facilitate binding of the tetrabromobenzimidazole inhibitor to cyclin kinase CK2 are shown, which contribute to the near 600-fold reduction in its inhibitory effect (as reflected in the K_i in the nanomolar range) on the enzyme target. Adapted from Scholfield, *et al.*.[17b]

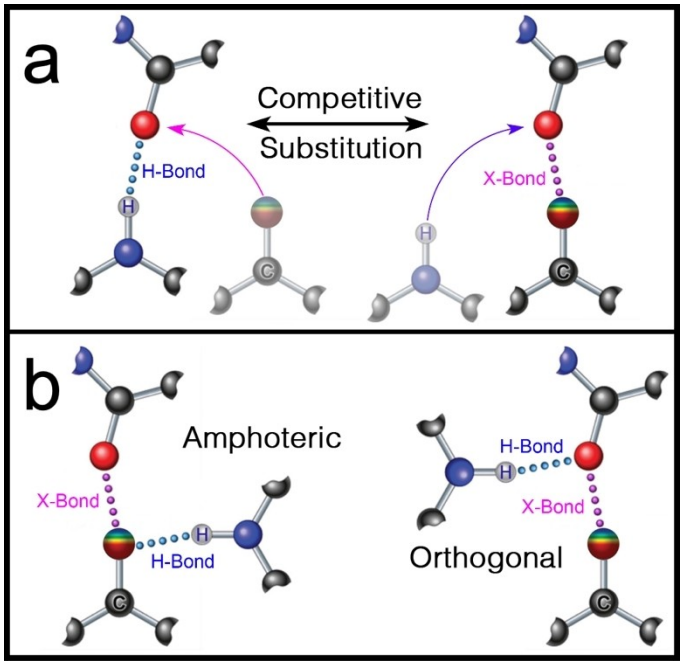


Figure 4. Relationships between X- and H-bonds in protein systems. a. The commonality in acceptors between H- and X-bonds indicates that they can compete and/or substitute for each other. b. The amphipathic nature of a halogen substituent (left)[25] allows it to serve as both an X-bond donor and H-bond acceptor simultaneously. In contrast, when an H-bond and a X-bond donor share the same acceptor atoms (right), the interaction is an orthogonal relationship[22] in respect to their perpendicular geometry and the independence of their interaction energies. Adapted from Rowe and Ho.[21b]

under similar experimental conditions). A follow-up crystallographic study by this group found that waters were the X-bond acceptors in these fibers, thereby suggesting that the role of the halogen is to structure the solvent at the wet interface of the hydrogels and potentially promote other NC (including C–H··· π and π-π stacking) interactions.[33]

2.2 Halogen bonds affecting structure and function of peptides and proteins

In humans, oxidative halogenation of tyrosines in proteins by myeloperoxidase or eosinophile peroxidase[34] have been associated with aging, neurodegenerative disease and cancer.[35] A recent study showed that a single halogenated tyrosine (^{X}Y) at the interface between the *N*- and *C*-termini can affect the ability of tubulin-like proteins to undergo GTP-dependent self-organize and polymerize,[36] although X-bonding was apparently not a factor. This raises the questions of how halogenation affects the structure, stability, and function of proteins and whether X-bonds can affect these properties.

Danelius, *et al.*[37] studied the structures of a series of cyclic peptide and found that an intramolecular Cl···O X-bond across the peptide (from a chloroalanine at the third residue to a methylates serine at position 8) helped stabilize a β-hairpin foldamer over conformation (Figure 7). The cyclic peptide was seen by solution-state NMR to be an intrinsically dynamic system. In the presence of the Cl···O X-bond, the peptide was seen to have a 74% probability of folding into the β-hairpin foldamer, while that probability was reduced to 58% when the Cl is replaced by an H-bonding OH and to only 29% when replaced by a non-interacting CH_3 group. A QM calculation indicated that the Cl··O X-bond was weak, with the stabilizing potential being only ~1.5 kcal/mol. This cyclic peptide system, therefore, was presented as a means to evaluate the effect and potential energy of weak *nc*NC interactions such as the Cl···O X-bond in a peptide/protein system.

Halogenated amino acids can be incorporated into proteins through site-specific incorporation of unnatural amino acids.[38]

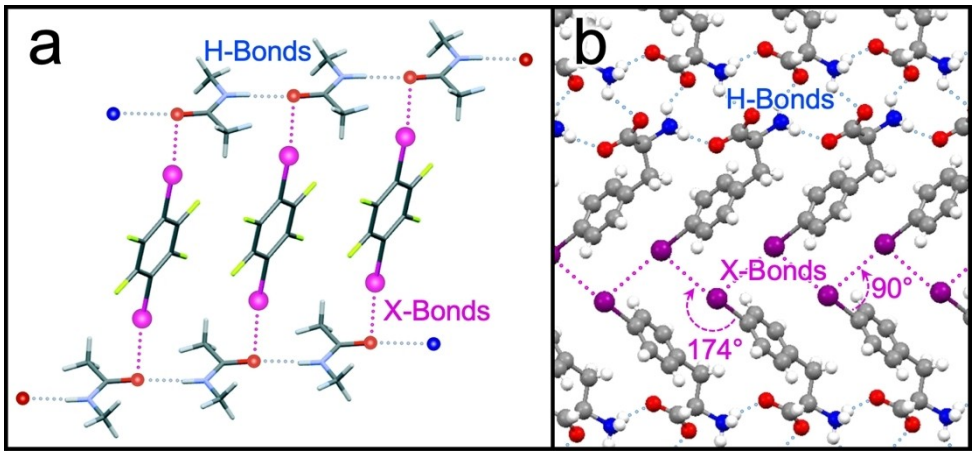


Figure 5. X-bonds bridging assemblies of an H-bonded peptide mimic (**a**) and H-bonded iodophenylalanine (**b**). **a**. X-bonds (magenta dots) from diiodotetrafluorobenzene bridge the antiparallel arrays of H-bonded (blue dots) antiparallel *N*-methylacetimide (NMA) chains. Adapted from Vasyleva, *et al.*.[28] **b**. Crystal structure of *p*-iodophenylalanine shows chains assembled through N–H···O H-bonds between the amino and carboxylate groups, with each set of arrays linked through I···I X-bonds. Labeled are the near linear C–I···I angle aligning the electropositive σ-hole of the donor to acceptor and the nearly perpendicular C–I···I angle for the electronegative waist of the acceptor to the donor. Data from Bertolani, *et al.*.[29]

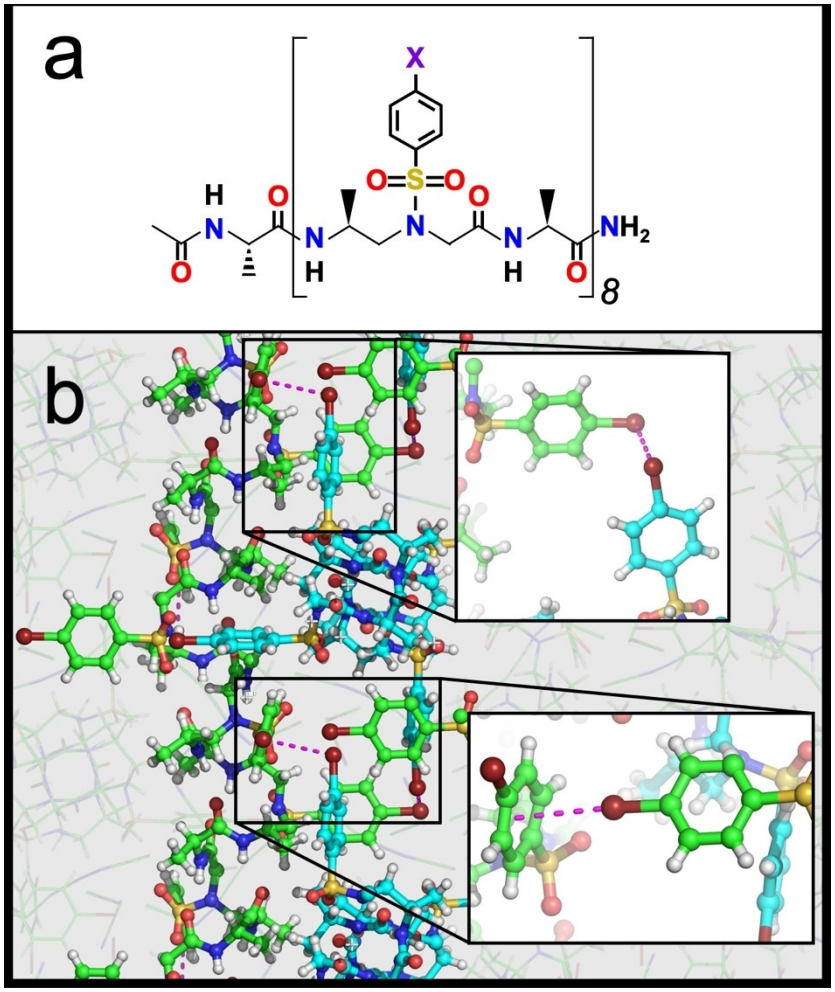


Figure 6. Peptide foldamers assembled from *para*-halo-α/L-sulfono-γ-amino acids. a. Sequence of the synthetic peptide containing eight repeats of the halogenated unnatural amino acid. b. Foldamer structure showing one set of 4_{13} helices packed against neighboring parallel (green) and perpendicular (cyan) oriented helices. A type I I···I contact between parallel and perpendicular helices is shown in the top inset, while an I···π X-bond between parallel helices is shown in the bottom inset. Data from Teng, *et al.*,[31] adapted from Ho and Anderson.[32]

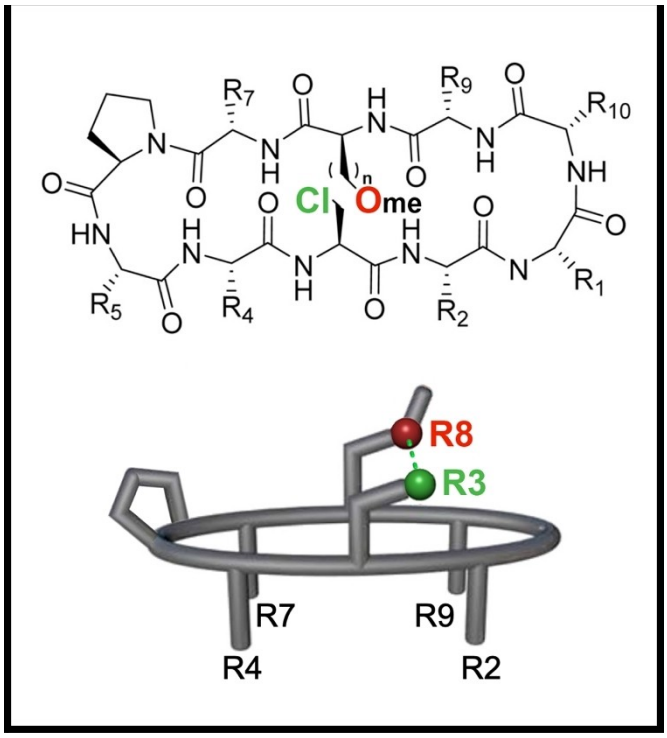


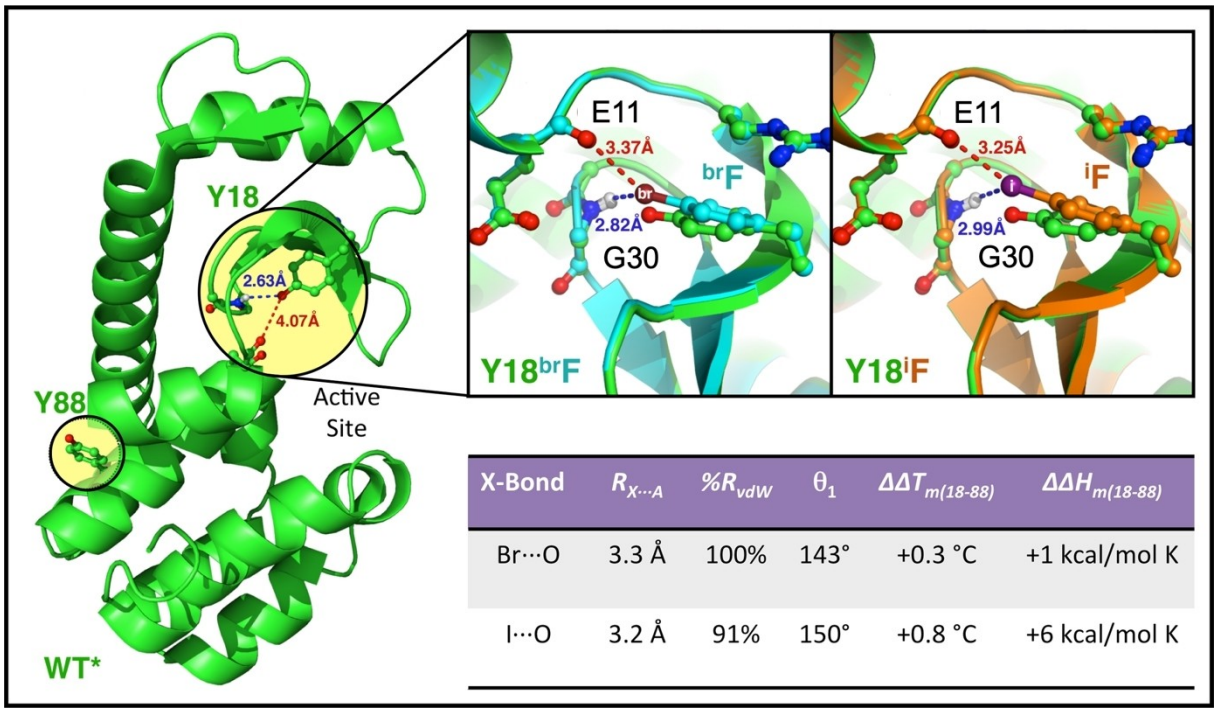
Figure 7. NMR structure of a cyclic decapeptide held in a β-hairpin conformation by an X-bond. The chemical schematic of the cyclic decapeptide is shown on top, while a cartoon of its β-hairpin structure determined by solution-state NMR is shown on the bottom. The chlorine (green sphere) of residue 3 sidechain (R3) is shown X-bonded (green dotted line) to the oxygen (red sphere) of the methylserine residue at position 8 (R8). Adapted from Danelius, *et al.*.[37]

One application of halogenation is to facilitate phasing of X-ray diffraction data to help solve the single crystal structures of proteins, with the argument that such modifications would have little effect on the protein itself. Indeed, the first example of such a strategy was seen with the incorporation of an iodophenylalanine (^{I}F) a hydrophobic pocket of T4 lysozyme (a classic model enzyme to study protein folding), with no X-bond acceptors within 4 Å of the iodine.[39] More recently, the halogenated amino acids have been incorporated in order to introduce X-bonding as a design element to affect the stability and activity of proteins and enzymes.

In our own laboratory, we asked the question of whether an X-bond can be introduced to replace an H-bond to stabilize the structure of T4 lysozyme.[40] Our first set of studies focused on tyrosine at position 18 (Y18, Figure 8), which sits on a β-strand adjacent to the active-site of the enzyme. Y18 accepts an H-bond from the NH of the peptide bond at glycine (G30) and is bridged to the peptide oxygen of the catalytic glutamic acid E11[41] by a series of water molecules. The structure of the protein in which Y18 is replaced to either a bromo- or iodophenylalanine (Y18^{br}F or Y18^{I}F mutant constructs) showed that the halogens formed X-bonds to the backbone of E11, while maintaining the H-bond from G30 (Figure 8 inset). The solvent exposed amino acid Y88 served as a control for the effect of substituting the hydrophilic OH group with a either Br or I substituent atoms. In all cases, substitution of the OH with a halogen (whether at Y18 or Y88) reduced the overall stability of the protein as reflected in the lower melting temperature (T_m) and enthalpy of melting ($\triangle H_m$) determined by differential scanning calorimetry (DSC), indicating that the OH of Y18 is critical to the stability of T4 lysozyme (Figure 8). However, the in comparing these DSC thermal parameters among the mutants at the two positions, the halogens in the X-bonding site of Y18 increased the T_m by 0.3° to 0.8 °C and $\triangle H_m$ by +1 to +6 kcal/mol relative to the analogous mutants at the solvent exposed Y88 position. Thus, we showed that introducing a halogen into a solvent exposed position is destabilizing (consistent with the effect of exposing a hydrophobic group, in this case a halogen atom) but the ability to form an intramolecular X-bond rescues this loss in stability by upwards of 6 kcal/mol.

Recognizing the critical nature of the interactions of Y18, we next asked whether an X-bond can be introduced to augment the stability of T4 lysozyme.[42] For this study, we replaced Y18 with a *meta*-halotyrosine (Y18^{mX}Y mutants, where X is Cl, Br, or I). The crystal structures of these constructs showed that this pocket did not fully accommodate the halogens (Figure 9). The chlorine of the ^{mCl}Y18 side chain was seen to be 54% sitting inside the pocket and X-bonded to the carbonyl oxygen of glycine G28, while 46% of this side chain was rotated outside and not forming any *nc*NC intramolecular interactions (Figure 9a). The halogen of the ^{mBr}Y18 construct was 22% X-bonded inside and 78% outside, while that of ^{mI}Y18 was 100% out (Figure 9b), indicating that this pocket in T4 lysozyme is very rigid and can only partially accommodate the smaller Cl halogen substituent. Even then, however, ^{mCl}Y18 was seen to increase the T_m by 1 °C and the $\triangle H_m$ by 3 kcal/mol relative to the wildtype enzyme, indicating that this partial X-bond seen in the structure resulted in a more stable protein. Furthermore, the chlorinated mutant showed ~15% increased enzymatic activity compared to the wildtype at elevated temperatures. A similar but smaller increase in activity was also observed for the brominated construct, suggesting that as the temperature increased, the protein pocket in which ^{mX}Y18 resides becomes more accommodating to the halogen substituents, thereby allowing more of the side chain to rotate to form a stabilizing X-bond.

We noticed, however, in the ^{mCl}Y18 T4 lysozyme studies that the energy associated with the Cl···O X-bond was significantly larger than the chlorine X-bonding energies seen in any biomolecular system so far. A Cl···O energy of ~3 kcal/mol is greater than the 0.15 kcal/mol determined by competition with H-bonds in our prior DNA junction systems[18,20] or the ~1.5 kcal/mol from the cyclic peptide system described above[37] and is more similar to what is expected for a Br···O X-bond. This anomalously large stabilizing energy led us to propose that the X-bonding potential of the chlorine was enhanced by the adjacent OH substituent of the tyrosyl side chain (Figure 9c). Hydroxyl groups are known to be electron donating substituents and we would expect that the X-bonding potential of a halogen adjacent to an OH would be reduced. However, QM calculations indicate that when the hydroxyl is rotated such that the OH can form an H-bond to the electronegative waist of the halogen, the electropositive σ-hole



X-Bond	$R_{X\cdots A}$	$\%R_{vdW}$	θ_1	$\Delta\Delta T_{m(18\text{-}88)}$	$\Delta\Delta H_{m(18\text{-}88)}$
Br···O	3.3 Å	100%	143°	+0.3 °C	+1 kcal/mol K
I···O	3.2 Å	91%	150°	+0.8 °C	+6 kcal/mol K

Figure 8. T4 lysozyme constructs with tyrosines at residue 18 (Y18) or 88 (Y88) replaced by halophenylalanines (^{X}F). The structure of the wildtype T4 lysozyme (WT*) is shown as a cartoon, with the active site labeled and tyrosine to be mutated (Y18 and Y88) shown in ball and stick models and highlighted with yellow circles. The left inset compares details of the crystal structures of the brominated Y18^{br}F construct (cyan) against WT* (green). The Br···O X-bond to glutamate11 (E11) is shown as red dots and its distance labeled, while the H-bond from the amino group of glycine 30 (G30) is shown as blue dots and its distance labeled. The right inset compares details of the crystal structures of the iodinated Y18^{i}F construct (orange) against WT* (green). The I···O X-bond to E11 is shown as red dots and its distance labeled, while the H-bond from the amino group of G30 is shown as blue dots and its distance labeled. The table lists for the brominated and iodinated constructs the distance between the halogen X-bond donor and oxygen acceptor ($R_{X\cdots A}$), the associated percent of the sum of the van der Waals radii for the two interacting atoms ($\%R_{vdW}$), the X-bond angle from donor to acceptor (θ_1), the difference in DSC measured melting temperature between the mutant at the Y18 *versus* Y88 position ($\triangle\triangle T_{m(18\text{–}88)}$), and the difference in DSC measured melting enthalpies between the mutant at the Y18 *versus* Y88 position ($\triangle\triangle H_{m(18\text{–}88)}$). Data from Scholfield, *et al.*.[40]

becomes significantly enlarged due to polarization effects, thereby increasing both the stabilizing potential and the angular range afforded the halogen as an X-bond donor. We called this an H-bond enhanced X-bond, or HBeXB for short.

A similar effect was observed at nearly the same time by Berryman's group in a small molecule sensing system. In their studies, the affinity for halide ions of a bidentate anion receptor increased when an NH_2 substituent is added to form H-bonds to the two iodine X-bond donors of the iodopyridinium groups.[43] Surveys of the Cambridge Structural Database (CSD) and the PDB indicates that the requirements for HBeXB interactions are prevalent in both small and large molecule systems.[44] There is some indication from QM studies that with multiple enhancing H-bonds, even fluorine can serve as a potent X-bond donor.[45] Thus, the concept of the synergistic HBeXB seems to be general across chemistry and biochemistry and greatly expands the range of energies and geometries available for molecular design applications compared to X-bonds alone.

3. The Chalcogen Bond

In biology, chalcogen bonds (Ch-bonds) primarily involve either S or Se as donors and O, N, or S as acceptors.[11c] Unlike the X-bond, the atoms of Ch-bond donors have typically two covalent bonds, which means that there will potentially be two σ-holes, each aligned opposite of their associated covalent bonds.[2d,46] Surveys for Ch-bonds would thus be complicated by potential interactions of acceptors with an additional electropositive surface, but also possible steric clashes with adjacent substituent groups. A survey of protein-ligand complexes in the PDB by Kriz *et al.*, showed that nearly a quarter (23%) of ligands containing S or Se showed geometries consistent with Ch-bonding.[11c] Biswal, *et al.*'s comprehensive survey of S/Se donors to O acceptors in the PDB found that Ch-bonds are dominated by S···O interactions in turn structural element of proteins (although all secondary structures of proteins were represented).[47] The survey had identified primarily intermolecular Ch-bonds in protein-ligand complexes, but one interesting intramolecular S···O was found within the Cys558 residue (Figure 10a). Quantum mechanical calculations indicated that the interaction energies of Ch-bonds selected from the survey could range from −0.7 to −14.1 kcal/mol. As with the X-bond, the focus of this discussion will not be on Ch-bonds associated

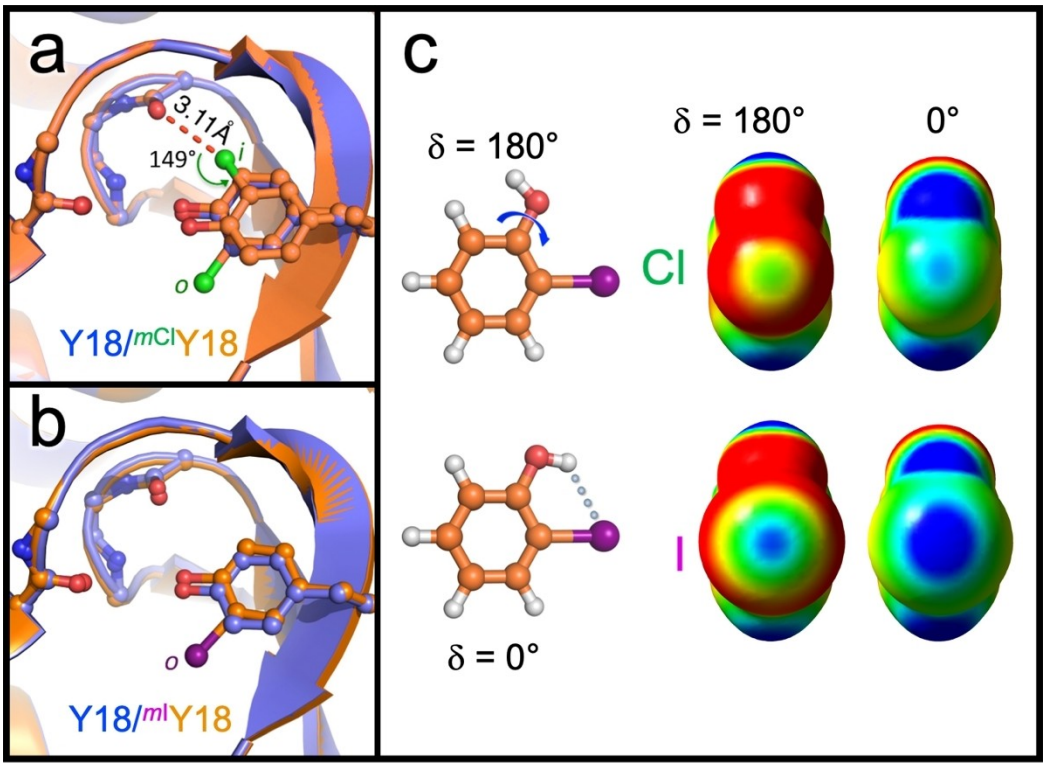


Figure 9. Comparison of the details of T4 lysozyme structure with tyrosine at position 18 (Y18) replaced by *meta*-halotyrosine (^{mX}Y18). a. Structure of the chlorinated ^{mCl}Y18 mutant (orange) compared to WT* (blue). The sidechain of ^{mCl}Y18 can be oriented to point the chlorine into the pocket (*i*) to form an X-bond (distance and θ_1-angle as labeled) or out of the pocket (*o*) with no X-bond interaction with the protein. b. Structure of the iodinated ^{mI}Y18 mutant (orange) compared to WT* (blue). The sidechain of ^{mI}Y18 was seen to be oriented only to place the iodine out of the pocket (*o*) with no X-bond interaction with the protein. c. Effect of rotating the hydrogen of the hydroxyl group in the ^{mX}Y18 sidechain either away (δ-angle = 180°) or towards (δ-angle = 0°) the halogen substituent. In the δ = 0° orientation, the OH serves as an H-bond donor to the electronegative waist of the halogen, which in turn enhances the σ-hole of the halogen (Cl top and I bottom) and consequently its potential to serve as an X-bond donor. Adapted from Carlsson, *et al.*.[42]

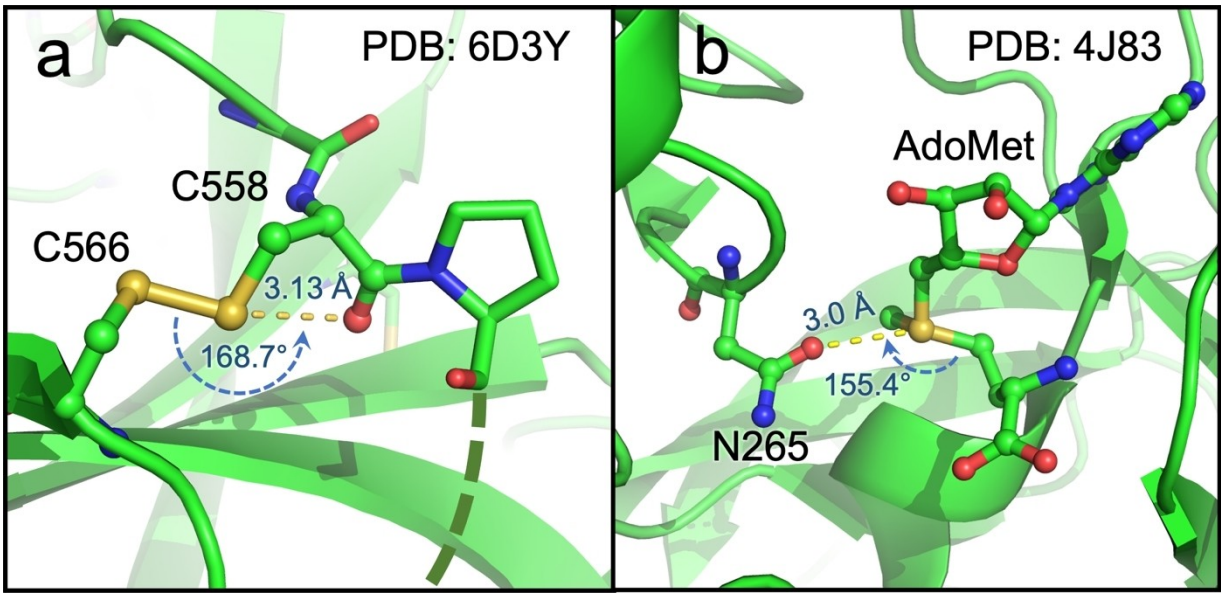


Figure 10. Chalcogen (Ch-bonds) in proteins. a. An intramolecular chalcogen bond (yellow dots) was observed from the sidechain sulfur to the peptide carbonyl oxygen of cysteine Cys588. The σ-hole on this sulfur is established by the covalent S–S disulfide bond to cysteine C566. Data from Swedberg, *et al.*.[48] (PDB code; 6D3Y). b. A chalcogen bond (yellow dots) is highlighted between the sulfur of AdoMet and carbocyclic oxygen of Asparagine at position 265 (N265) in histone-lysine N-methyltransferase Set 7/9. Data from Horowitz, *et al.*,[49] (PDB code 4J83).

with the design of protein inhibitors (of which there are several examples) but on one that is integral to the protein's natural function.

A particularly interesting class of proteins where *nc*NC interaction, including Ch-bonds may play an important role in function are the *S*-adenosylmethionine (AdoMet)-dependent methyltransferases (MTases). AdoMet-dependent MTases are enzymes (ubiquitous across all phyla of life) that add methyl groups from AdoMet to small molecules in the biosynthesis of secondary metabolites, and to nucleic acids and proteins to control cellular functions. The SET domain containing lysine methyltransferase (SET7/9) specifically methylates a lysine on the histone 3 protein, which is a marker for transcriptional activation.[50] Fick, *et al.*[51] found that the oxygen of the asparagine265 (N265) side chain of SET7/9 is positioned 3.0 Å from the with the sulfur of the bound AdoMet cofactor, consistent with an S···O Ch-bond (Figure 10b). QM calculation on models that mimic this interaction resulted in an overall (–S–CH_3)···O interaction energy of ~16 kcal/mol, although it was not possible to disentangle the contributions of the S···O Ch-bond from the C–H···O H-bond. In order to determine the role of this Ch-bond, the authors replaced N265 with an alanine (N265 A mutant). Removing the amide of the side chain resulted in ~10-fold reduction in the affinity of the enzyme for AdoMet and ~7-fold reduction in the catalytic rate but, again, it would be difficult to distinguish the contributions to the binding of each of the two classes of *nc*NC interactions. One possible approach may be to study the system with a selenium analog of AdoMet, which should significantly increase its potential as a Ch-bond donor but not the H-bonding potential of the methyl group. We will see in the next section how tetrel bonds may play a catalytic role in this same class of enzymes.

4. The Tetrel Bond

Although tetrel bonding in chemistry can involve any of the group IV elements (C, Si, Ge, Sn, or P), the primary donor for this class of interactions in proteins are the carbons of methyl groups. The identification of tetrel bonds to methyl groups would be complicated by potential weak C–H···Acceptor H-bonds and, therefore, care must be taken to distinguish among the two competing interactions. Mooibroek's 2019 survey of tetrel bonds published found nearly 2,800 such *nc*NC interactions in the PDB.[52] The survey showed that oxygen dominated over sulfur and aromatic acceptors, with water O representing over half the interactions (57%), followed by amide O (~24%), carboxylic O (~15%), thiol S (4%) and aromatic rings (<1%). QM calculations on interaction energies representing tetrel bond type interactions are weak (ranging from −0.8 kcal/mol to −3.7 kcal/mol) but not insignificant.

As with the Ch-bond, proteins where tetrel bonds may be particularly important are the AdoMet-dependent MTases. In this case, however, it is not the sulfur of AdoMet involved in the *nc*NC interaction but the methyl group that is being transferred, with the tetrel bond proposed as stabilizing a state that precedes the S_N2 transition state of the transfer reaction.[53] Although carbons of methyl groups would not typically form strong tetrel bonds, the positively charged sulfonium of the AdoMet cation could strengthen the *nc*NC interaction through polarization.[49,54] A 2018 survey by Trievel and Schiener[55] of the PDB for tetrel bonds (Figure 11) specifically in structures of AdoMet MTases found 20 unique examples of the *nc*NC interaction between the CH_3 of the AdoMet cofactor and the oxygen of the water solvent or a ligand in the active site (one example was to a chlorine of a ligand). The tetrel bond lengths

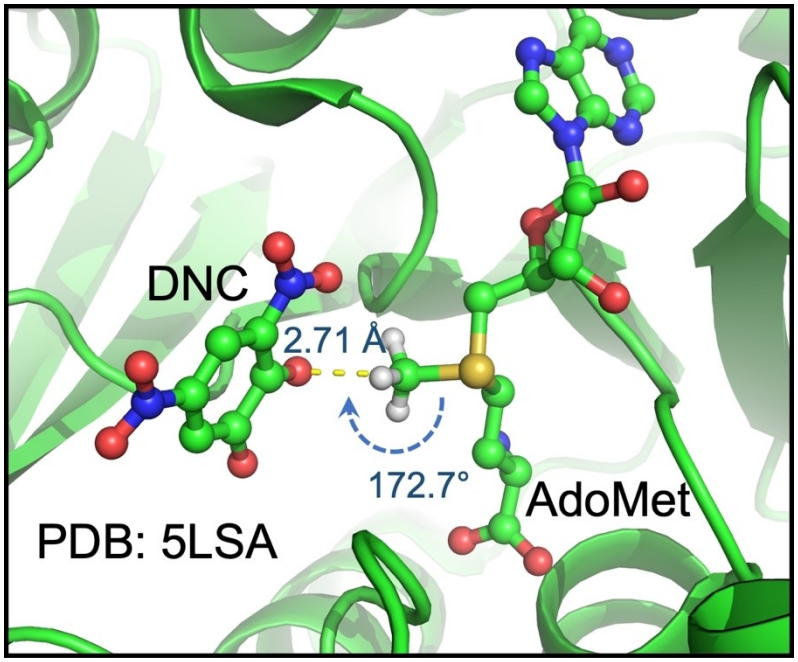


Figure 11. Tetrel bond in methyltransferase. A tetrel bond interaction (yellow dots) is shown between the methyl group of the AdoMet cofactor in human catechol O-methyltransferase and the ligand 3,5-dinitrocatechol (DNC). (PDB code 5LSA).

ranged from 78% to 106% of the sum of the van der Waals radii of the carbon tetrel bond donor to O/Cl acceptor (average = 90.1 ± 7.5%) for AdoMet to ligands and 95.7% to 101% (average = 98.1 ± 1.8%) for interactions to water. The S–C···(O/Cl) angles ranged from 160° to 176° (average = 169.0° ± 5.0°). QM calculations on model systems mimicking four structures from survey showed that interaction energies CH_3···(O/Cl) ranges from −5.2 kcal/mol to −9.0 kcal/mol for neutral acceptors and potentially to −65.7 kcal/mol for an anionic oxygen acceptor. A nonbonding orbital analysis of these energies indicate that the contribution from the tetrel bond 1.6 to 8-times greater than that from the competing C–H···(O/Cl) H-bonds of the methyl group.

5. Conclusions and Perspectives

We had previously argued that to be considered biologically relevant, an *nc*NC interaction must 1) affect a biological function, 2) affect the structures/stabilities of the biological molecule that defines the function, and 3) have energies that are biologically relevant (*i.e.*, similar to that of H-bonds).[2f] The discussion in this review demonstrates that X-bonds, Ch-bonds, and tetrel bonds all fit these criteria and all are fairly well represented in biological systems, as seen from surveys of the PDB. These surveys further point us in multiple directions for how and where such *nc*NC interactions can play significant roles in biology. The most obvious applications of these interactions would be towards inhibitor and drug design against therapeutical protein targets. This could include the design of small molecule ligands as well as peptides that bind specifically to target proteins. The intentional focus of the current review, however, is in *nc*NC interactions that could serve as design elements applicable to engineering proteins that are more stable and/or with additional functions. An exciting area of development of *nc*NC interactions in chemistry is towards the design of new catalysts for organic reactions.[56] We see here that tetrel bonds may play a significant role already in catalyzing SAM dependent methyl transfer.[49,53,55] Thus, it we can now imagine how various *nc*NC interactions can be used to design new enzymatic catalysts.

Finally, we currently have a good understanding for how the energies of each type of *nc*NC interaction is defined directly by the element type (with the σ-hole being more electropositive as we go down the column of each elemental group) as well as the electron withdrawing potential of whatever our donor atom is attached. Such effects are now readily estimated from higher level QM calculations. In addition, classical molecular mechanics/dynamics simulations can now start to model at least the X-bond in proteins and other biological systems by incorporating corrections to the typical spherically uniform forcefield parameters,[57] including the positive extra point approach[58] and the forcefield for biological halogen bonds.[59] The concept that the synergistic HBeXB can as much as double the potential energy of an X-bond,[42,44] however, indicates that *nc*NC interactions can be strongly affected by its environment, potentially through synergistic coupling among different types of non-covalent interactions, not just H-bonds. Thus, to fully model and apply X-, Ch-, and tetrel bonds in protein engineering applications, we must better understand how each are affected by the ubiquitous H-bond and by each of the other *nc*NC interactions in the environments of proteins.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are openly available in RCSB Protein Data Bank at https://doi.org/10.1093/nar/gkaa1038, reference number [REF]. ■■Dear Author, if additional Research Data for your paper is openly available in a public repository please provide the missing information about the public repository here (repository name, repository DOI and reference number). In case there is no public repository please let us know.■■

Keywords: Noncovalent interactions · halogen bond · chalcogen bond · tetrel bond · peptides · protein modifications

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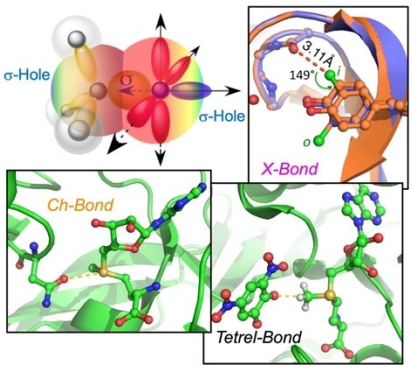
REVIEW

The nonclassical noncovalent interactions (halogen bonding, chalcogen bonding and tetrel bonding) are reviewed in the context of their contributions to the structure and associated functions of peptides and proteins.



Non-classical non-covalent sigma-hole interactions in protein structure and function: Concepts for potential #ProteinEngineering applications. Review by P. Shing Ho and co-workers @ColoradoStateU

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