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Incorporating Target Heterogeneity in Drug Design

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Abstract Traditionally, structure-based drug design has been predicated on the idea of the lock-and-key hypothesis, i.e., the ideal drug should have a structure that complements the target site structurally and energetically. The implementation of this idea has lead to the development of drug molecules that are conformationally constrained and pre-shaped to the geometry of the selected target. The main drawback of this strategy is that conformationally constrained molecules cannot accommodate to variability in the target and, therefore, lose significant binding affinity even in the presence of small changes in the target site. There are three common situations that lead to binding site heterogeneity: (1) genetic diversity; (2) drug resistant mutations; and (3) binding site dynamics. The development of drugs that effectively deal with target heterogeneity requires the introduction of certain degree of flexibility. However, flexibility cannot be introduced indiscriminately because it would lead to a loss of binding affinity and specificity. Recently, structure-based thermodynamic strategies aimed at developing adaptative ligands that target heterogeneous sites have been proposed. In this article, these strategies are discussed within the context of the development of second generation HIV-1 protease inhibitors. J. Cell. Biochem. Suppl. 37: 82–88, 2001.

Key words: drug design; drug resistance; genomic diversity; HIV; AIDS

Genetic Diversity

The recent availability of genomic information for different organisms, including several pathogens, has revealed the existence of nucleotide polymorphisms that manifest themselves as amino acid polymorphisms in the encoded proteins. These polymorphisms have been observed in different proteins including some identified as targets for drug development. In the case of HIV-1, several subtypes with different geographic distribution are responsible for the AIDS epidemic. The B subtype is prevalent in the United States and Western Europe, while the A and C subtypes predominate in Africa, where 70% of the 36 million infected individuals have been identified. The A, B, and C HIV-1 subtypes show genomic variations on the order of 10–30% [Benson et al., 1998]. Interestingly, these variations are not uniform throughout the entire genome, and seem to be more pronounced in matrix and envelope proteins. An analysis of the HIV-1 sequences in GenBank indicates that relative to the B subtype, the reverse transcriptase enzyme exhibits 7–9% amino acid polymorphisms, the protease between 5 and 10%, the integrase between 7 and 9%, gp120 between 18 and 23%, gp41 between 22 and 27%, and the matrix protein p17 between 22 and 27%. In the case of the protease molecule, one of the main target of existing antiretroviral therapies, amino acid polymorphisms between subtypes do not occur inside the binding site, but are able to affect the binding affinity of inhibitors currently in clinical use [Velazquez-Campoy et al., 2001b]

Drug Resistant Mutations

One of the most serious side effects associated with the therapy of HIV-1 infection is the appearance of viral strains that exhibit resistance to protease inhibitors [Ho et al., 1994; Kaplan et al., 1994; Condra et al., 1995; Hong et al., 1996; Ala et al., 1997, 1998]. The loss of sensitivity to protease inhibitors usually occurs because the resistant viral strains encode for protease molecules containing specific amino acid mutations that lower the affinity for the inhibitors, yet maintain sufficient affinity for the substrate. For some mutations, the affinity towards the inhibitor might decrease by up to three orders of magnitude while the K_m for the

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substrate only changes by less than one order of magnitude [Baldwin et al., 1995; Lin et al., 1995; Hong et al., 1996]. In many cases, resistance-causing mutations alter the geometry of the binding pocket without changing its chemical character (e.g., mutations like I84V, V82F, I47V, I50V change the spatial arrangement, shape or volume of the binding pocket without changing its polarity or hydrophobicity). Conformationally constrained inhibitors cannot adapt to these changes and as a result lose van der Waals and other stabilizing interactions, resulting in a diminished binding affinity. The loss of van der Waals interactions have been noticed in crystal structures of inhibitor complexes of mutant proteases (see for example [Baldwin et al., 1995; Hong et al., 1996; Ala et al., 1997, 1998]. The substrate, on the other hand, is a linear peptide with a higher flexibility and a better capacity to adapt to a distorted binding pocket. It, therefore, does not lose as many interactions and its affinity is not affected as much.

Binding Site Dynamics

Binding sites are usually characterized by the presence of regions with low structural stability [Luque and Freire, 2000]. In many cases, the low stability regions are loops that become stable and cover a significant portion of the ligand molecule upon binding. This arrangement maximizes the number of contacts between ligand and protein and simultaneously buries a significant surface area from the solvent. A recent survey of 36 protein/ligand complexes [Luque and Freire, 2000] indicates that small ligands ($MW < 800$) bury $80 \pm 13\%$ of their surface area upon binding. The burial of that large proportion of their area is an indication that ligands are not only attached to the protein surface, but also covered by parts of the protein. Under these conditions, an important component of the binding energetics is given by the energy required to structurally stabilize those protein regions that are structurally unstable prior to binding. This contribution needs to be considered even if the binding reaction is not coupled to a major conformational change. Within this context, mutations that affect the stability of the protein and alter the energetics associated with the protein rearrangement upon binding can influence the binding constant even if they are outside the binding site [Velazquez-Campoy et al., 2001b].

Adaptative Ligands

One of the major challenges for drug design in post-genomic medicine is the development of drug molecules that are efficient against heterogeneous targets. These molecules should be able to adapt to target variability while simultaneously displaying high binding affinity and specificity. The process of designing adaptative ligands requires a different approach to the one traditionally used against a single target defined by a set of atomic coordinates. Against these targets, the most important drug design paradigm is derived from the classic lock–key hypothesis of enzyme specificity originally advanced by Emil Fisher in 1890 [for a review, see Koshland, 1994]. The design paradigm is usually referred to as the "shape complementarity principle" and essentially involves the synthesis of a conformationally constrained molecule pre-shaped to the geometry of the target binding site. A molecule that is pre-shaped to the target and conformationally constrained provides specificity and simultaneously enhances the binding affinity; however, it fails if the target changes (drug resistance) or if presented against multiple variants of the same target (genomic diversity). Adaptative ligands should contain flexible elements that permit accommodation to variable regions in the target family while simultaneously establishing strong interactions with conserved structural elements.

TARGET SPECIFICATION

Traditionally, protein targets for drug development have been described in terms of atomic coordinates that specify their three-dimensional (3-D) structure. The description of heterogeneous targets requires additional parameters. The effects of amino acid polymorphisms can be described in terms of residue-level functions that describe chemical variability, structural stability, mutation probability, and binding site plasticity. Figure 1 illustrates this approach with the structure of the HIV-1 protease (pdb file 1HHP). Panel A shows a standard CPK representation of the molecule in terms of the atom types and position coordinates provided in the pdb file. The binding site is seen as a large cavity near the center of the molecule. Panels B, C, and D illustrate different types of target variability mapped into the 3-D structure of the protease. In panel B, the structure has been colored in terms of the residue-level stability computed according

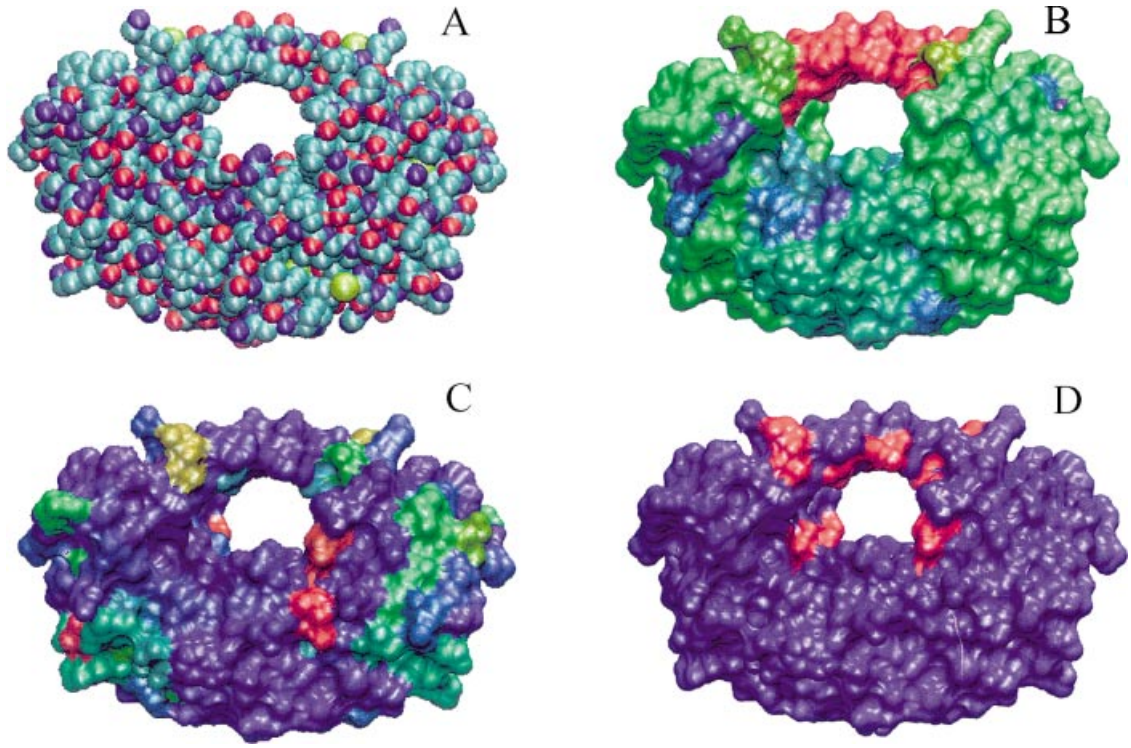

Fig. 1. Structure of the HIV-1 protease. **A**: van der Waals representation color-coded by atom type; (**B**) surface representation showing the distribution of the structural stability according to the COREX algorithm (blue = highest stability, red = lowest stability); (**C**) surface representation illustrating the mutation probability or polymorphism map for 1,408 samples from North America deposited in GenBank (red = highest variability, blue = lowest variability); and (**D**) surface representation highlighting the primary mutations (in red) associated with drug-resistance to five clinical inhibitors in antiretroviral therapy (indinavir, saquinavir, ritonavir, nelfinavir, and amprenavir).

to the COREX algorithm [Hilser and Freire, 1996; Todd et al., 1998]. The central part of the molecule, including the dimer interface plus adjacent areas, and the catalytic portion of the binding pocket (shown in blue), define the most stable part of the protease, while the region corresponding to the flaps (shown in red) defines the least stable part of the molecule. In Panel C, the protein has been colored in terms of the mutation probability (red = highest) calculated from 1,408 protease genes isolated in North America and deposited in GenBank. Because there are structural and functional constraints that select against certain mutations, the mutation probability is not randomly distributed within the protein structure. Even drug-resistant mutations need to maintain a certain level of chemical functionality and be able to bind and hydrolize the substrate molecule. In addition, some sites only allow conservative mutations. Finally, in Panel D, the protease has been colored in terms of the location of the primary resistance mutations (red) associated with the use of the inhibitors Indinavir, Nelfinavir, Ritonavir, Saquinavir, and Amprenavir in antiretroviral therapies. Genomic and thermodynamic information like the one shown in panels B–D are used in the specification of a blueprint for the design of adaptative ligands. This blueprint provides local information about the diversity existing at each location within the binding site and permits estimation of the type of functional group and flexibility required in corresponding regions of the ligand molecule. Design decisions about functional groups, rotatable bonds, conformational constraints, etc. will affect the binding affinity by influencing different components of the Gibbs energy of binding.

MOLECULAR ORIGIN OF THE BINDING AFFINITY

From a thermodynamic point of view, the binding affinity, K_a, is defined in terms of the free energy of binding:

$$K_a = e^{-\Delta G/RT}$$

where R is the gas constant and T is the absolute temperature. The free energy of binding is in turn defined by the enthalpy (ΔH) and entropy (ΔS) changes:

$$\Delta G = \Delta H - T\Delta S$$

therefore,

$$K_a = e^{-(\Delta H - T\Delta S)/RT}$$

$$K_a = e^{-\Delta H/RT} \times e^{\Delta S/R}$$

It is evident that the binding affinity can be optimized by making ΔH more negative, ΔS more positive or by a combination of both. Even though many combinations of ΔH and ΔS values will elicit the same binding affinity (i.e., the same ΔG and therefore, the same K_a), the properties and the response of these compounds to changes in the environment or in the protein target are not the same. The binding enthalpy primarily reflects the strength of the interactions of the ligand with the target protein (e.g., van der Waals, hydrogen bonds, etc.). The entropy change, on the other hand, mainly reflects two contributions: changes in solvation entropy and changes in conformational entropy. Upon binding, desolvation occurs, water is released and a gain in solvent entropy is observed. This gain is particularly important for hydrophobic groups. At the same time, the drug (and certain groups in the protein) loses conformational freedom resulting in a negative change in conformational entropy. Accordingly, from a thermodynamic point of view, there are three important factors responsible for improving binding affinity: (1) Improving ligand protein interactions over those with the solvent in order to obtain a favorable (negative) enthalpy change; (2) Making the ligand more hydrophobic in order to make the solvation entropy large and positive; and, (3) Pre-shaping the ligand to the geometry of the binding site in order to minimize the loss of conformational entropy upon binding.

THERMODYNAMIC SIGNATURES EXPECTED FROM CURRENT DESIGN PARADIGMS

The design paradigm derived from the lock–key hypothesis is usually referred to as the "shape complementarity principle" and essentially aims at developing conformationally constrained ligands pre-shaped to the geometry of the target binding site. A molecule that is pre-shaped to the target and conformationally constrained provides specificity and simultaneously enhances the binding affinity. One rotatable bond that becomes immobilized upon binding carries a Gibbs energy penalty close to 0.5 kcal/mol due to the loss of conformational entropy [DAquino et al., 2000]. Everything else being equal, a conformationally constrained molecule has a higher binding affinity because it does not carry that entropy penalty. Organic and medicinal chemists have been able to successfully implement this strategy and design conformationally constrained molecules against a variety of targets.

Shape complementarity, however, does not guarantee binding. For binding to occur, a favorable Gibbs energy is required. Since the effective binding energetics is the difference between the magnitude of the drug/target interactions and the interactions with the solvent, it is always possible to generate a significant binding affinity by making the interactions of the drug with the solvent unfavorable, i.e., by increasing the hydrophobicity of the drug. Consequently, a common strategy has been to design highly hydrophobic conformationally-constrained ligands, characteristics both that contribute to a large positive binding entropy and define, to a significant extent, the binding thermodynamics of these molecules.

Perhaps, the most significant example is given by the first-generation of HIV-1 protease inhibitors (Indinavir, Nelfinavir, Ritonavir, Saquinavir). The binding of these inhibitors is either enthalpically unfavorable or characterized by only a slightly favorable enthalpy. In all cases the dominant driving force for binding is a large positive entropy change that originates primarily from a large positive solvation entropy due to the burial of a large hydrophobic surface upon binding, and a small loss of conformational entropy. Figure 2 shows the different energetic contributions to the binding affinity of these inhibitors [Todd et al., 2000].

All of these inhibitors are susceptible to the resistant mutation V82F/I84V [Todd et al., 2000]. This double mutation is located at the edges of the active site, distorting its wild type geometry without changing its polarity or chemical composition. A calorimetric analysis of the effects of this mutation on inhibitor binding reveals that ΔG becomes less favorable and that the decrease in affinity is due to a combination of a more unfavorable binding

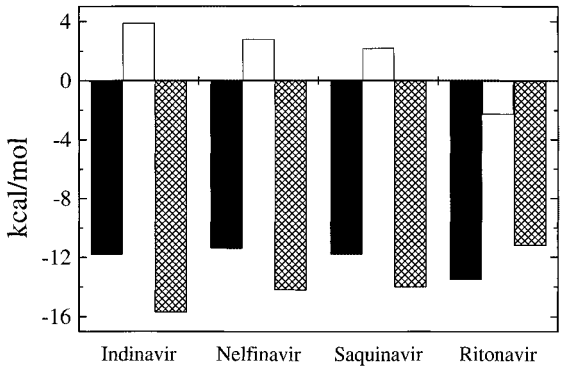

Fig. 2. Dissection of the binding energetics ($\Delta G = \Delta H - T\Delta S$) of Indinavir, Nelfinavir, Saquinivir, and Ritonavir to the wild-type HIV-1 protease. In this figure, solid bars represent ΔG, white bars ΔH, and crossed-hatched bars $-T\Delta S$. All thermodynamic data were obtained at 25°C in in 10 mM acetate, pH 5, 2% DMSO.

enthalpy and a less favorable binding entropy [Todd et al., 2000]. The positive increase in the binding enthalpy reflects in part the inability of these compounds to adapt to a geometrically distorted binding site. The decrease in entropy, on the other hand, is consistent with a less complete desolvation of the inhibitor when bound to the mutant protein.

SECOND-GENERATION HIV-1 PROTEASE INHIBITORS

HIV-1 protease inhibitors that maintain efficiency against common resistance mutations have been identified [Yoshimura et al., 1999; Xie et al., 2000]. These inhibitors are characterized by extremely high binding affinities against the wild type protease, and by experiencing only a mild decrease in their binding affinity against existing protease-resistant mutations. Extremely high binding affinity is achieved by combining favorable enthalpy and entropy changes. KNI-764 (AG-1776), for example, binds to the protease with a K_d of 32 pM [Velazquez-Campoy et al., 2001a]. The corresponding Gibbs energy of binding is −14.3 kcal/mol of which −7.6 kcal/mol is contributed by ΔH and −6.7 kcal/mol by ΔS. This even enthalpy/entropy balance should be contrasted with the one observed for first-generation inhibitors. Indinavir, for example, binds to the protease with a Gibbs energy of −12 kcal/mol; however, ΔS needs to contribute −16 kcal/mol in order to offset the unfavorable ΔH of 4 kcal/mol. For all first-generation inhibitors, the entropic contribution to the Gibbs energy of binding ranges between −16 and −11 kcal/mol compared to −6.7 kcal/mol for KNI-764. The binding affinity of KNI-764 is about 70-fold higher than that of Indinavir and Saquinavir, 140-fold higher than Nelfinavir, and 4-fold higher than Ritonavir measured under identical conditions. Because enthalpically favorable ligands like KNI-764 do not require extreme binding entropies, they can afford the presence of certain flexible elements and still exhibit extremely high binding affinities.

The binding of KNI-764 to the V82F/I84V resistant mutant protease is also characterized by a favorable enthalpy change. However, the binding enthalpy to the mutant is 2.3 kcal/mol less favorable than to the wild type. The resistant mutation V82F/I84V lowers the binding affinity of KNI-764 by a factor of 26, which is equivalent to a decrease in the Gibbs energy of binding of 1.9 kcal/mol. It appears that the unfavorable effect of the mutation is restricted to the binding enthalpy while the binding entropy contribution to ΔG becomes slightly more favorable (−0.4 kcal/mol). The efficacy of KNI-764 against the V82F/I84V resistant mutant can be explained by a combination of two factors. The first factor is its extremely high binding affinity towards the wild type protease, and the second factor is a relatively mild effect of the resistant mutation on the binding affinity. As a result, even after a 26-fold decrease in binding affinity, KNI-764 still binds to the resistant mutant with a higher binding affinity than Saquinavir, Nelfinavir, and Indinavir do to the wild type.

TESTING THE FLEXIBILITY HYPOTHESIS IN THE DESIGN OF ADAPTATIVE LIGANDS

The extremely high affinity of KNI-764 is accounted for by the additive effects of favorable enthalpy and entropy changes, while the low susceptibility to the V82F/I84V resistant mutation can be rationalized in terms of inhibitor flexibility in the P2′ region that permits accommodation to the binding site distortions created by the mutation. The resistant mutant makes the binding enthalpy of KNI-764 less favorable, consistent with diminished inhibitor/target interactions. Contrary to first-generation inhibitors, the entropy change becomes slightly more favorable with the resistant mutant. This result suggests that a mild response to the mutation

can be achieved when an inhibitor is flexible enough to bury a comparable or even larger surface area when bound to the mutant or wild type even if the mutation diminishes the strength of its interactions with the target.

In order to test the validity of this hypothesis, isothermal titration calorimetry (ITC) experiments were performed with a second inhibitor (KNI-577) identical to KNI-764 except that it lacks flexible elements in the P2′ region (Fig. 3). As shown in Figure 3, KNI-764 has a methylbenzylamide group containing two rotatable bonds between the amide and the phenyl ring in the P2′ region, while KNI-577 has a symmetric t-butylamide group at that position with only one rotatable bond. The additional rotatable bond in KNI-764 permits the methylbenzylamide group to adopt different orientations and present different interaction surfaces to the protease. Figure 3 summarizes the binding thermodynamics of these compounds to the wild type HIV-1 protease. The binding affinity of KNI-577 is about 1 kcal/mol weaker due to a less favorable binding enthalpy. As expected, the binding entropy of KNI-577 is more favorable since it loses fewer degrees of freedom upon binding. The major difference between these two compounds becomes evident when confronted to the V82F/I84V resistant mutant.

Figure 4 shows the differential effect of the resistant mutation on the thermodynamic parameters for KNI-577 and KNI-764. While the binding affinity of KNI-764 decreases by a factor of 26 against the resistant mutant ($\Delta\Delta G = 1.9$ kcal/mol), the binding affinity of KNI-577 decreases by a factor of 257 ($\Delta\Delta G = 3.3$ kcal/mol); i.e., the effect of the mutation is ten times more pronounced on KNI-577. For both compounds, the loss of ΔH is very similar (2.3 and 2.6 kcal/mol). The effect on ΔS, however, is completely different. For KNI-764, the entropic contribution to the Gibbs energy becomes more favorable (−0.4 kcal/mol) whereas, for KNI-577 becomes less favorable (0.7 kcal/mol). These results are consistent with the hypothesis that the presence of flexible elements at a critical point permit a ligand to compensate the loss of interactions with the target by burying a comparable or even larger surface area from the solvent. A rigid ligand, on the other hand, may lose comparable interactions, but lacks the ability to rearrange itself and hide efficiently from the solvent.

FUTURE PROSPECTS

The realization that protein molecules identified as targets for drug development may carry amino acid polymorphisms resulting from genetic diversity or drug resistant mutations questions the validity of the lock–key hypothesis as an accurate paradigm for drug design in post-genomic medicine. New strategies for drug design should be aimed at developing adaptative ligands that efficiently bind to a family of targets rather than to individual members. A quantitative description of target diversity

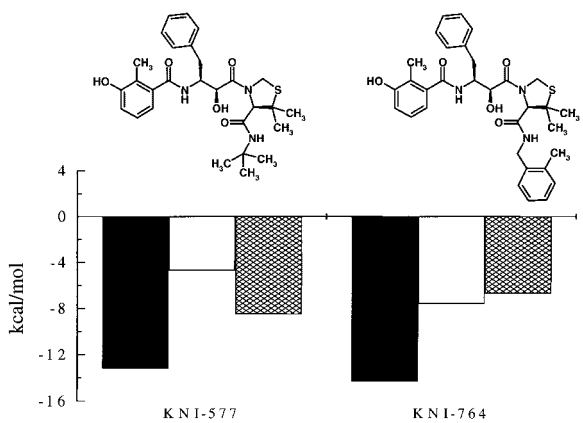

Fig. 3. Dissection of the binding energetics ($\Delta G = \Delta H - T\Delta S$) of KNI-577 and KNI-764 to the wild-type HIV-1 protease. In this figure, solid bars represent ΔG, white bars ΔH, and crossed-hatched bars $-T\Delta S$. The chemical structures of KNI-577 (left) and KNI-764 (right) are shown on top of the data. All thermodynamic data were obtained at 25°C in in 10 mM acetate, pH 5, 2% DMSO.

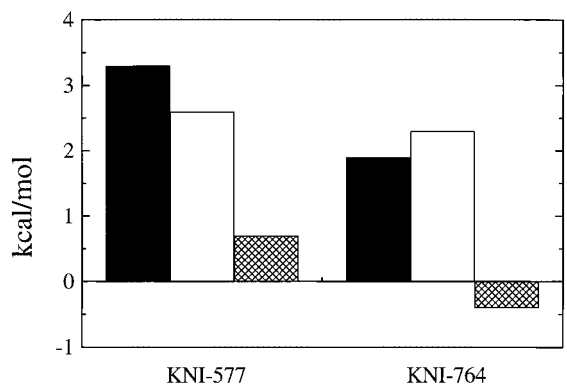

Fig. 4. Thermodynamic dissection of the effect of the active-site double-mutation V82F/I84V in the HIV-1 protease on the inhibitor binding energetics ($\Delta\Delta G = \Delta\Delta H - T\Delta\Delta S$). In this figure solid bars represent $\Delta\Delta G$, white bars $\Delta\Delta H$, and crossed-hatched bars $-T\Delta\Delta S$. All thermodynamic data were obtained at 25°C in in 10 mM acetate, pH 5, 2% DMSO.

needs to be effectively incorporated into the atomic specification of a binding site, since this information provides the necessary guidelines for ligand design. Ligands that are able to adapt to target heterogeneity have distinct thermodynamic signatures, suggesting a critical role for structure-based thermodynamic algorithms in future design strategies.

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