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Crystallization to obtain protein–ligand complexes for structure-aided drug design

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The use of X-ray crystallography to derive three-dimensional structures for structure-aided drug design (SADD) is a common activity in drug discovery today. In this process, the structures of inhibitors or other ligands of interest complexed with their macromolecular target are solved and the structural information is used iteratively to design new molecules. The ability to form cocrystal complexes between a target protein and a ligand is essential to this process and therefore is of considerable interest to anyone practicing in this field. In the course of obtaining the necessary ligand–protein crystals, even with crystallization conditions well established for a protein of interest, obtaining co-structures with inhibitors either through cocrystallization or soaking is too often not successful. There are numerous potential reasons for this lack of success and this article outlines a number of possible factors that may be involved and discusses considerations that should be taken into account when designing successful experiments to obtain iterative costructures.

1. Introduction

Structure-aided drug design (SADD) requires crystals of proteins complexed with ligands that are candidates for development as medicines. With X-ray crystallography these complexes are used to determine a three-dimensional model of the protein–ligand interaction at a molecular level. This information can then help guide a rational design process in the development of the lead compound to become a medicinal candidate. Typically, an iterative process requiring structures with dozens of different compounds often of different structural classes is involved.

Others (McNae *et al.*, 2005; Stura & Chen, 1992) have reviewed some aspects of soaking small ligands into protein crystals, but while the literature contains many examples of proteins complexed with substrates, inhibitors and other ligands, reports focusing on special considerations for obtaining ligand-complex crystals, especially with drug-development compounds, are lacking. It is a common experience that even when conditions are well established for the growth of crystals of a protein of interest, obtaining co-structures with new inhibitors can be difficult. Here, we outline soaking and cocrystallization scenarios and discuss the considerations that should be taken into account when designing experiments to obtain iterative co-structures. In this review, the terms ligand and inhibitor are used inter-

changeably to describe any small ligand of interest bound to the protein for the purpose of structure-aided drug design.

2. Soaking *versus* cocrystallization

Cocrystallization and soaking are two common approaches used in seeking cocrystal structures with inhibitors of interest. In the soaking approach, the compound is incubated with preformed crystals of the target protein. This method commonly employs crystals of a form of the protein that has been crystallized in the absence of any added inhibitor (apocrystal form), but may also involve displacement of an originally cocrystallized inhibitor with a second one. The soaked compound is expected to bind at a functional binding site of the protein, such as the enzyme active site or a functional regulatory site. In the cocrystallization approach, the inhibitor is combined with the protein prior to crystallization and the complex is crystallized. This process is then repeated for each new inhibitor.

When compared with small-molecule crystals, protein crystals are loosely packed, typically containing from 30 to 80% solvent (Matthews, 1968, 1974). The network of lattice interactions determine the size and configuration of channels traversing the crystal, which average typically from 20 to 100 Å in diameter (Vilenchik *et al.*, 1998). These channels contain the bulk solvent bathing the crystals in addition to a shell of 'bound water' interacting with the protein molecules and provide considerable access for small ligands to the protein molecules in the lattice. Soaking inhibitors into preformed crystals is often used in iterative SADD and can provide certain advantages. The ability to stockpile a large number of crystals of known structure and diffraction quality and then soak them in compounds of interest can provide speed, convenience and reproducibility. Importantly, the crystals used in these experiments must be compatible with inhibitor binding. For example, if inhibitor binding induces conformational changes in the protein that are not compatible with the crystal lattice-packing interactions, crystal cracking or dissolution may occur and the crystals become of no use for diffraction experiments. Alternatively, if lattice packing in the apocrystal form includes interactions that inhibit access to the binding site, the crystals may also not be of use for soaking studies. Where possible, it is useful to obtain multiple crystal forms so that any limitations of one form may be overcome in a second packing arrangement.

A variation of the soaking method is to cocrystallize with an initial inhibitor bound in the site of interest and then to soak a new inhibitor into that site. In this case, the relative binding affinity and solubility of the new compound being soaked in *versus* the bound compound being displaced should be considered as discussed below. Again, crystal lattice interactions must be compatible with any protein solubility or conformational changes that occur during the binding of a new structurally different compound.

If, in the preformed crystal, steric hindrance or incompatible conformational changes upon inhibitor binding occur and if a new crystal form compatible with binding cannot be found, soaking may not be successful. In addition, in some cases there may be concern that the binding mode observed in a soaked structure may not accurately represent the solution binding mode (Zhu *et al.*, 1999; Hiller *et al.*, 2006). Adding an inhibitor to the target protein to form a complex in solution prior to crystallization (cocrystallization) may circumvent these issues and should be considered. One should keep in mind that in cocrystallization the solubility and/or conformation of each new complex formed may differ from that of the apoenzyme or of other inhibitor complexes. This may lead to an inability to grow crystals of a given complex even when apoenzyme crystals or other complexes were successful. When this is the case, the individual protein-inhibitor complex should be screened for crystallization conditions as a unique crystallization problem and a crystal form compatible with the inhibitor-bound complex may be found.

3. Protein–ligand binding

In the process of crystallization, it is well accepted that the homogeneity of the sample being crystallized can be of critical importance. With impure or heterogeneous protein preparations, crystals may not be possible or incorporation of defects into the crystal lattice during growth may lead to poor crystal quality (Van der Laan *et al.*, 1989; McPherson, 1999; Moreno *et al.*, 2005; Carter, 1988). In the crystallization of a ligand–protein complex, we necessarily introduce some heterogeneity in solution, since we have both free protein and protein–ligand complex present at some ratio. Whether this heterogeneity negatively affects crystal growth will depend on the ability of each form of the protein to incorporate into the lattice. Studies with turkey egg-white lysozyme (TEWL) contaminated with various levels of either the closely related hen egg-white lysozyme (HEWL) or RNAse A revealed that the HEWL incorporated into the TEWL crystals while RNAse A did not (Abergel *et al.*, 1991; Hirschler *et al.*, 1998). In the general case, if the unliganded protein does not act as an impurity, interfering with crystal lattice formation by the ligand-bound protein, crystals of complex may grow normally from the mixture. However, there is also the possibility that both the apoprotein and ligand–protein complex can incorporate into the crystal at some level. The impact of this incorporation on crystallization will depend on whether that incorporation has a negative effect on the lattice. In any event, starting the cocrystallization experiment with maximum ligand–protein occupancy (and therefore homogeneity) should provide the best chance of growing high-quality ligand–protein crystals.

When free ligand (L) is added to its protein receptor (P) binding occurs and an equilibrium is established between the protein, the ligand and ligand–protein complex (PL). A dissociation constant, K_d, can be defined as

$$K_d = \frac{[P][L]}{[PL]}. \quad (1)$$

Fractional saturation Y can be defined as

$$Y = \frac{[\mathrm{PL}]}{[\mathrm{P}] + [\mathrm{PL}]} \quad (2)$$

and is simply the amount of protein bound with ligand divided by the total protein. Substituting from the equation for K_d, this can be re-expressed as

$$Y = \frac{[\mathrm{L}]}{K_d + [\mathrm{L}]}. \quad (3)$$

This form of the fractional saturation equation emphasizes that the fraction of protein in the ligand-bound form, Y, will be affected by the ratio of [L] to K_d. The affinity and concentration of ligand added, as well as ligand solubility, will influence the ratio of these components at equilibrium. For 90% occupancy, the amount of ligand added must be greater than the amount of protein so that free ligand [L] at equilibrium is not depleted to less than about $10 \times K_d$. In practice, in order to crystallize from a solution where the protein–ligand complex is well populated, [L] should be greater than $10 \times K_d$, or even greater if possible, and the total ligand added should be in excess of protein. Ratios of ligand to protein of up to 10:1 or more are commonly used, but very large excesses should be avoided owing to the possibility of ligand binding to secondary lower affinity sites or inhibition of crystallization. In many cases, a measured K_d or K_i is not available but the dose of compound giving 50% inhibition (IC_{50}) will be. With knowledge of the assay conditions (substrate and enzyme concentrations), this value can be a good substitute. With a lack of even IC_{50}, as may be the case for small fragments, a calculated K_i such as that from Ludi score (Böhm, 1994) could be considered.

A number of methods are available to analyze ligand–protein binding in solution. Isothermal titration calorimetry (Freire, 2004), differential scanning calorimetry (DSC), surface plasmon resonance (Huber, 2005; Neumann *et al.*, 2005) and NMR-based experiments (Muchmore & Hajduk, 2003) may all provide valuable information about protein–ligand complexes. A recent report describes the use of Raman spectroscopy to monitor the progress of ligand binding into single crystals *in situ* in the crystal-growth drop (Carey & Dong, 2004). Using one or more of these methods, it is possible then to confirm ligand–protein binding and to estimate binding affinity. This can be performed simply to confirm binding, to assess binding with expected affinity under conditions similar to those used for crystallization or to rank order compounds for crystallization experiments.

In cases such as those recently reported for 'fragment screening by X-ray crystallography' (Hartshorn *et al.*, 2005; Gill *et al.*, 2005; Nienaber *et al.*, 2000), random libraries of compounds with relatively weak affinity (*e.g.* micromolar to millimolar) are soaked into preformed crystals or cocrystallized. Owing to the weak affinity, the concentration of ligand (and therefore ligand solubility) may have to be tens of millimolar or higher in order to observe crystallographic occupancy (Hartshorn *et al.*, 2005; Nienaber *et al.*, 2000). For example, in choosing compounds for their fragment libraries, Hartshorn and coworkers avoided compounds unlikely to be soluble under conditions used for crystallographic screening and used concentrations ranging from 25 to 200 mM for soaking (Hartshorn *et al.*, 2005). The requirement for millimolar solubility suggests that weak binding compounds obtained from medicinal chemistry synthetic efforts, where aqueous solubility is often low (Lipinski, 2004; Aveed, 2001), may be problematic in soaking or cocrystallization experiments.

4. Compound solubility

Since ligand concentration is one of the critical factors in the attainment of ligand–protein occupancy, it is worth considering the solubility properties of medicinal chemistry compounds. The compounds typically produced in medicinal chemistry efforts have low aqueous solubility when compared with physiological ligands. For example, when a cohort of lead optimization compounds were tested in a kinetic solubility assay, 40% had aqueous (buffer pH 7) solubility less than 20 μg ml^{-1} (<40 μM for MW = 500; Lipinski, 2004). In a simplistic sense, compound solubility is determined by solvation energy and crystal lattice disruption, so the solid-state form as well as lipophilicity of a compound can have a major impact on its solubility. Low-energy crystalline forms can be difficult to solublize even in DMSO. Neat DMSO, however, may be an effective solvent even for 'greasy' amorphous compounds and should be tried when poor compound aqueous solubility is suspected (Lipinski, 2004). Concentrations at or above 5% DMSO have been found to improve compound solubility, while those below that level are generally not much better than pure aqueous (Aveed, 2001; Lipinski, 2004). The 5% (or so) DMSO concentration is often achieved by dissolving in neat DMSO and then diluting to lower DMSO concentration. This may improve the kinetics of solvation since dissolving in low DMSO concentration aqueous solutions may be a very slow kinetic process. In addition, crystallization solutions often contain polyethylene glycols (PEGs), alcohols or other components that may improve compound solubility (Gusiakov *et al.*, 1968; Aminabhavi *et al.*, 2003); therefore, even with very low measured aqueous solubility in hand one should not rule out a good result. If measured aqueous solubility is not available, clogP, the logarithm of a compound's partition coefficient between *n*-octanol and water, is a well established measure of a compound's hydrophilicity. A number of websites have free online tools for the calculation of clogP (*e.g.* http://www.organic-chemistry.org). In over 50 000 turbidometric solubility assays, 75% of compounds with clogP greater than 5 had experimental solubility in pH 7 phosphate buffer of less than 5 μg ml^{-1} (10 μM for MW = 500; Lipinski, 2004). In any case, it should be recognized that in order to achieve the required ligand solubility in the aqueous milieu of crystallization, the inherent solubility of the ligand in that environment must be equal to or greater than the concentration required for occupancy.

5. Soaking and binding equilibrium

An important assumption in using co-structures obtained from X-ray diffraction experiments is that the ligand-binding mode and affinity in the crystal is unaffected relative to solution binding. Wu and coworkers have shown that this assumption is true for binding of a dipeptide ligand to preformed cyclophilin crystals (Wu *et al.*, 2001). In these experiments, the dipeptide, with a K_d of 23 m*M*, was soaked for 17–27 h at varying concentrations of dipeptide up to 125 m*M*. The binding of ligand was estimated by crystallographic refinement of the fractional occupancy at the ligand-binding site. Using this method, a crystal binding affinity, K_c, was calculated and found to be 27 m*M*, in excellent agreement with the solution K_d of 23 m*M*. Occupancy of the crystal soaked at 125 m*M* dipeptide was 81.2%, also in excellent agreement with $Y = 82\%$ predicted by the equilibrium equation (3).

While the equilibrium occupancy in soaking will be similar if not identical to that in solution (cocrystallization), the time required to reach that equilibrium may vary. In the case of cyclophilin crystal soaking described above, times as short as 60 s led to the same occupancy as 17 h, as long as ligand concentration was adequate. In some cases, short soaking times of only minutes are required to obtain well occupied electron density for an inhibitor, while in other cases hours to days are required to obtain complete ligand occupancy (McNae *et al.*, 2005; Wu *et al.*, 2001; Jackson *et al.*, 2005). The reasons for the extended time requirement to achieve full occupancy in some cases are not completely understood but some possibilities are discussed below. The study by Wu and coworkers suggested that at least 25–30% of the binding sites may need to be occupied before ligand density is interpretable in electron-density maps (Wu *et al.*, 2001). Reaching this level of occupancy upon soaking, which must occur from the surface toward the interior of the crystal, may require longer times, depending on the nature of the bulk-solvent channels through the crystal lattice and other considerations. As was pointed out earlier, a ligand concentration [L] of $10 \times K_d$ or higher is desirable to ensure good occupancy of PL complex when cocrystallizing. However, when soaking into a preformed crystal the reported requirement for only 25–30% occupancy may allow successful electron-density interpretation in cases where [L] is somewhat less than $10 \times K_d$.

As discussed above, when ligand solubility is too low in the crystal mother liquor, increasing the level of PEG or alcohol already present or adding additional solvents such as DMSO may improve the ligand solubility. Increasing precipitants like PEG are often tolerated and may even stabilize crystals for soaking while at the same time increasing the solubility of some ligands. Some crystals can withstand concentrations of DMSO up to 20% or more. Finally, crystals may be stabilized by cross-linking (Quiocho & Richards, 1964; Stura *et al.*, 1983; Lusty, 1999), thereby allowing higher levels of solvent to be used for ligand solubility. More recently, a gentle vapor-diffusion method for cross-linking crystals was described (Lusty, 1999). Crystals cross-linked by this method were found to be more resistant to lattice disorder induced by cryocooling, but also are likely to be more stable in other situations where crystals undergo mechanical stress such as soaking to form a protein–ligand complex.

In solution, where both ligand and protein diffuse freely, ligands collide with their target sites with rates (k_{on}) in the range 10^7–10^8 s^{-1} M^{-1}. In the preformed crystal, however, this rate may be decreased owing to the fact that the protein in the crystal cannot diffuse freely and that inhibitor must diffuse through the bulk-solvent-filled channels in the crystal lattice to active sites in the protein. In a non-stirred system such as the crystal-soaking experiment, this diffusion will be driven primarily by the ligand-concentration gradient from the surface to the interior of the crystal (McNae *et al.*, 2005). Owing to its chemical properties, the protein surface in the lattice channels can also provide opportunities for ligand-binding interactions. Studies with cross-linked protein crystals, used for chromatographic resolution of molecules of various size and chemical type, confirm that not only the size of the channels but also the chemical make-up of the protein crystal can affect separation of molecules (Vilenchik *et al.*, 1998). Depending on its chemical properties, the ligand may interact through polar or hydrophobic interactions and even size-exclusion mechanisms in the channels; thus, diffusion may be slowed down as the ligand binds and dissociates its way to the interior of the crystal. In addition, in many cases the bulk solvent in a crystallization drop may contain viscous polymers such as polyethylene glycol up to 25% or more by volume that can affect diffusion rates. Finally, it has been recognized that some lead compounds found by high-throughput screening (HTS) and by virtual screening (Abagyan & Totrov, 2001), as well as medicinal compounds, aggregate at micromolar concentrations in aqueous solution (McGovern *et al.*, 2002; Seidler *et al.*, 2003; Feng *et al.*, 2005). In one study (Feng *et al.*, 2005), approximately 20% of about 300 randomly chosen compounds from HTS-like chemical libraries were found to be aggregators at 30 μ*M*. Analysis of the aggregation can be performed on a light-scattering instrument, such as those typically found in most crystallization laboratories (Seidler *et al.*, 2003). The particles range from 30 to 400 nm (McGovern *et al.*, 2002), making them unlikely to pass through lattice channels owing to their size. With such barriers to free diffusion, it is not surprising that soaking times required to achieve good occupancy may vary.

6. Lattice compatibility

Even when compounds for cocrystallization or soaking have been carefully chosen for solubility and potency, they may not result in successful co-structures. There are a number of ways, in addition to the effect of bulk-solvent channels, that the protein interactions in a given crystal lattice may have an effect on the ability to soak or cocrystallize.

One of the inherent requirements for the functionality of proteins is the ability to undergo conformational change when, for example, substrate or other ligands bind. These changes may occur at all levels of protein organization and can be small

or relatively large. The protein–protein contacts that occur to form the protein crystal lattice are similar to those that mediate oligomer protein–protein interactions in solution (Janin & Rodier, 1995); however, they are generally smaller and involve fewer hydrophobic interactions (Carugo & Argos, 1997; Dasgupta *et al.*, 1997). Compared with those in small-molecule crystals, these contacts are fewer and weaker, leading to the high solvent content and fragility of protein crystals (Dasgupta *et al.*, 1997; McPherson, 1999). Consequently, removal of just one or a few of these contacts owing to ligand-induced conformational change may have major effects on the lattice, disrupting the crystal, leading to a different packing arrangement or disallowing the occurrence of crystallization. Numerous reports of new or improved crystal forms obtained through protein surface engineering support the concept that minor changes to the surface of a protein molecule can result in major effects on the crystal lattice (Price & Nagai, 1995; Derewenda, 2004).

It is not uncommon for a given protein and its ligand complex or different inhibitor–protein complexes of the same protein to produce different crystal forms and require different crystallization conditions (Nagar *et al.*, 2002; Wu & Pai, 2002; Pauly *et al.*, 2003; Morikawa & Matsushima, 1989). Conformational changes upon inhibitor binding may change the availability of specific surface residues for protein packing or may change the shape of the protein surface, resulting in steric interference of required lattice interactions. These surface changes may also affect the solubility of the complex, in turn affecting its crystallization requirements. If protein crystal-packing interactions involve regions at or near the active site or if the bound inhibitor itself participates in a lattice interaction, binding of the ligand may be incompatible and crystals may not form or different crystal forms may result from each inhibitor complex.

Small proteins or those with a shallow active site exposed on the surface may be especially prone to involvement of the ligand in crystal lattice interactions. Parge and colleagues found ligands synthesized as part of their structure-aided drug-design program for FKBP12 to mediate crystal packing, leading to different crystal forms for each complex (Parge *et al.*, 1997). Analysis of these ligand-mediated crystal contacts identified a moiety that could be attached to a variety of ligands to produce crystals of FKBP–ligand complexes on demand.

Crystal-packing interactions required in an apoprotein crystal may also complicate obtaining inhibitor-bound crystals. An example of crystal-packing differences between an apoenzyme and an enzyme–inhibitor complex has been reported for different crystal forms obtained for fibroblast collagenase MMP-1 (Hassell *et al.*, 1994; Lovejoy *et al.*, 1994). The authors found two different apocrystal forms, each with the N-terminus of the protein involved in a crystal contact, while crystals of the MMP-1 inhibitor complex did not have this interaction. The apoprotein crystals only grew from protein that had autocatalytically lost two amino acids from the N-terminus. The truncated N-terminus was inserted into the active site of a symmetry-related molecule, making interactions with active-site residues. The inhibitor-complex crystals had a free disordered N-terminus, with the inhibitor making the same interactions with the active-site residues. Binding of inhibitor to the apocrystal forms was presumably not possible owing to the crystal packing at the active site.

Of course, there are also many examples of robust crystals, obtained either by cocrystallization or soaking, compatible with binding a diverse array of inhibitors (Pandit *et al.*, 2000; Wright *et al.*, 2002; Rath *et al.*, 2000; Anderson *et al.*, 2003) As discussed above, the ability of such a system to bind inhibitors without negative effect on crystallization will be dependent on the packing interactions involved in the crystal lattice, the occurrence of any detrimental conformational changes upon inhibitor binding and the properties of the inhibitor.

7. Chemical environment of the co-structure experiment

Binding affinity measurements, whether K_d, K_i or IC_{50}, are generally obtained in a chemical environment that is optimal or at least within the usual physiological range for ligand binding to the protein. Conditions for crystallization, on the other hand, may sometimes occur well outside the physiological or optimal range. One factor that can influence both ligand binding and solubility is the ionization state of functional groups on the ligands as well as at the binding site. Consequently, the pH of the crystallization mother liquor can have a major effect on the binding affinity and hence the ability to successfully obtain structures of those inhibitors bound to the protein. Some preformed crystals are stable over a range of pH and can be pre-equilibrated to an appropriate pH prior to addition of ligand (Kuo & Seaton, 1989). Cross-linking of the crystal lattice, as mentioned earlier, may improve crystal stability in such experiments. In the case of soaking, in addition to the effects of the chemical environment on ligand binding at the active site, it should also be considered that proteins are weak ion exchangers with variable isoelectric points, so pH variations in the mother liquor may also affect the kinetics of achieving binding-site occupancy. Finally, it should be remembered that some enzymes are good biocatalysts capable of performing many reactions leading to the transformation of xenobiotics. As a result, one may soak or cocrystallize with a given inhibitor and find something else in the maps (Borbulevych *et al.*, 2004).

The presence of ions or additives in the crystallization solution that may compete for binding at or near the active site can also preclude or affect inhibitor binding. Crystal structures obtained from crystals grown with high levels of sulfate or phosphate salts sometimes have ATP-binding sites occupied, for example, with inorganic phosphate or sulfate. If inhibitors take advantage of this portion of the active site, these crystals may not be compatible with SADD studies. Crystals of aldose reductase (AR) grown in the presence of citrate were found to contain this anion bound in the active site, leading to the recognition of an 'anion-binding pocket' within the active site of the enzyme (Harrison *et al.*, 1994). This pocket is accessed by many aldose reductase (AR) inhibitors being developed

as medicines (Oka *et al.*, 2000; Urzhumtsev *et al.*, 1997). Cacodylate, a common buffer in crystallization trials, was also shown to bind in this site. While potent AR inhibitors were able to compete out binding in co-structures of these complexes, it should be remembered that high levels of even weak binding additives, crystallization components or cryo-protectants such as PEG, ethylene glycol or glycerol can preclude or displace binding of the ligand of interest. This is especially important to consider with weak binding inhibitors.

The competitive kinetic nature of reversible inhibitor binding can also be critical to know in designing proper soaking or cocrystallization experiments. For example, if a compound is an uncompetitive inhibitor, therefore requiring substrate for binding to the enzyme, it will be critical to include substrate in the experimental setup. For non-competitive or allosteric inhibitors, knowledge of inhibitor kinetics can alert the crystallographer to look carefully beyond the active site for electron density. There are a number of cases where potent inhibitors discovered by high-throughput screening have been found not to bind at the active site, but instead inhibit by binding at novel allosteric sites (Rath *et al.*, 2000; DeDecker, 2000; Wright *et al.*, 2002; Love *et al.*, 2003)

8. Conclusions

Even with well established growth conditions for a single-crystal form, obtaining structures of inhibitors or other ligands of interest in complex with target proteins for SADD is not always straightforward. Consideration of the protein–ligand binding equilibrium, ligand solubility, limitations imposed by and on the crystal lattice, factors affecting the kinetics of binding in the preformed crystal and optimal chemical conditions for ligand–protein binding and inhibition are all critical to successful relevant co-structures.

Thorough knowledge of the physical and chemical properties of the ligand/inhibitor being studied including solubility, affinity, inhibition kinetics and tendency to aggregate may help explain lack of success in obtaining some protein–ligand co-structures. If necessary, improvement of ligand solubility through addition of compatible solvents in the experiment should also be investigated. Searching for and following up on multiple crystal forms, when possible, may help overcome lattice incompatibility issues in both soaking and cocrystallization scenarios. Gentle cross-linking procedures are available that may stabilize the crystal lattice to perturbations to allow soaking at higher DMSO concentrations, for example. Finally, attention should be paid to the requirements of the chemical environment for ligand binding and presence of possible competitive ligands with respect to the crystallization or soaking solution.

A consideration of these factors during the design of experiments or if initial experiments fail should lead to strategies that increase chances of obtaining the protein–ligand complex crystals that are so critical to SADD.

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