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Crystallization of protein–ligand complexes

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Obtaining diffraction-quality crystals has long been a bottleneck in solving the three-dimensional structures of proteins. Often proteins may be stabilized when they are complexed with a substrate, nucleic acid, cofactor or small molecule. These ligands, on the other hand, have the potential to induce significant conformational changes to the protein and *ab initio* screening may be required to find a new crystal form. This paper presents an overview of strategies in the following areas for obtaining crystals of protein–ligand complexes: (i) co-expression of the protein with the ligands of interest, (ii) use of the ligands during protein purification, (iii) cocrystallization and (iv) soaks.

1. Introduction

One of the first questions that arises when we tackle the problem of growing crystals of protein–ligand complexes is 'when do we add the ligand to the protein?' Adding the ligand during protein expression may enable us to obtain soluble protein. If the ligand is added during certain steps of protein purification, during the entire protein purification or during the final concentration, the protein may be stabilized and aggregation problems lessened or eliminated. If the protein is stable, is it better to cocrystallize the ligand with the purified protein? In instances where protein supply is limited, can we soak the ligands into pre-existing crystals without disrupting the crystal lattices and destroying the crystals? It is impossible to predict which route will be successful, so systematic testing is required to determine which method will work best for your crystals.

2. Co-expression with ligands of interest

When the protein is expressed with ligands of interest, we have seen increased levels of protein expression and increased amounts of soluble protein. These are often dependent upon the ligand, the potency of the ligand, the solubility of the ligand and the DMSO concentrations used and compound availability. In several cases, the introduction of additional mutations significantly affected the protein expression in the presence of ligands.

Obtaining sufficient quantities of purified steroid nuclear receptor ligand-binding domain (LBD) was critical for the eventual structure determination of progesterone receptor (PR), androgen receptor (AR), glucocorticoid receptor (GR) and mineralocorticoid receptor (MR). In all of these cases, co-

expression with a high-affinity ligand was key to obtaining protein for structural studies (Williams & Sigler, 1998; Matias *et al.*, 2000; Sack *et al.*, 2001; He *et al.*, 2004; Madauss *et al.*, 2004; Fig. 1).

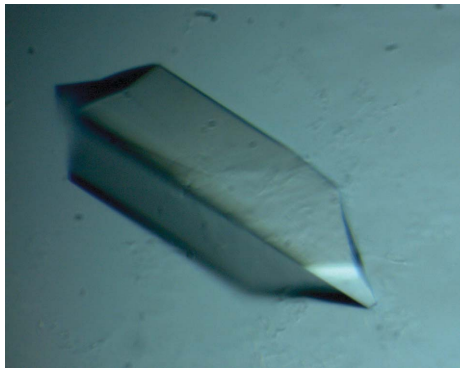

Figure 1
Androgen receptor. Incorporation of a high-affinity ligand during protein expression led to a system that routinely gives sub-2 Å structures (He *et al.*, 2004).

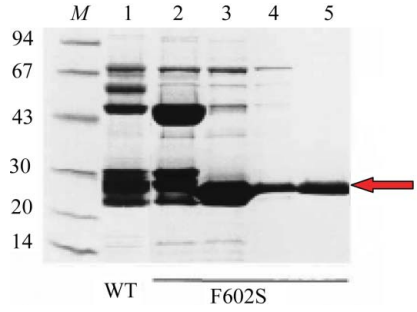

Figure 2
Effect of the F602S mutation on GR expression. Comparison of the protein expression of wild-type GR (lane 1) and F602S GR (lane 2) in the presence of 10 μ*M* dexamethasone. The proteins shown are the soluble fractions from the Ni^{2+} column. Lanes 3–5 show the purification of the F602S LBD (lane 3, sample after thrombin digestion; lane 4, Ni^{2+} column flowthrough of the thrombin-digested material; lane 5, final purified protein; Bledsoe *et al.*, 2002).

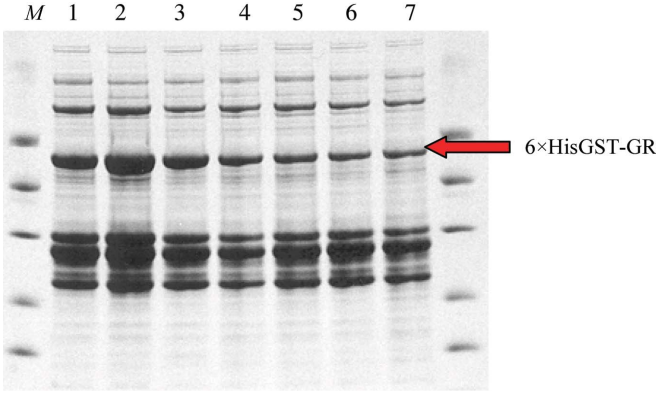

Figure 3
Effect of ligand on GR LBD expression. The red arrow denotes GR LBD expressed with seven different ligands. In some cases, the addition of a methyl group to the ligand could make the difference in increased expression levels.

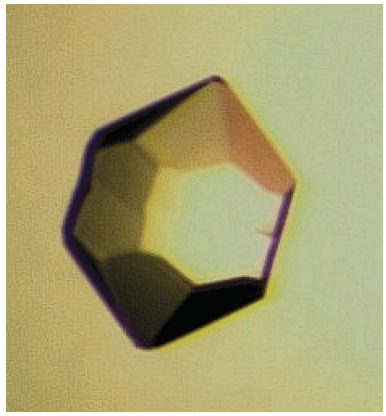

Figure 4
GR LBD complexed with a ligand. Growth of diffraction-quality GR crystals depended on the presence of a high-affinity ligand during protein expression, the F602S mutation and the type of ligand.

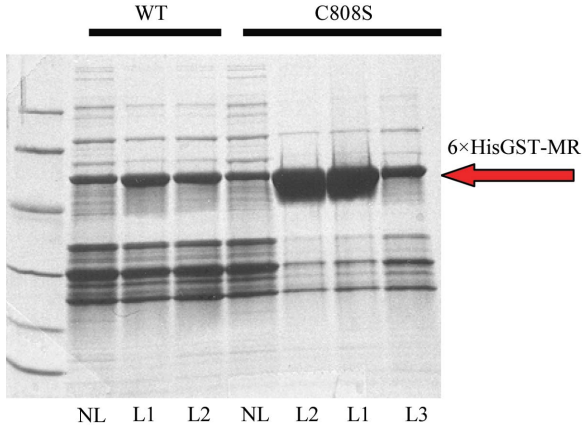

Figure 5
Effect of the C808S mutation and ligand type on MR expression. Although the C808S mutation is equivalent to the GR F602S mutation, it has a more pronounced effect on GST-MR LBD expression. This mutation produced increased expression of MR in the presence of a variety of ligands (L1, ligand 1; L2, ligand 2; L3, ligand 3). High levels of protein expression still require the presence of ligand during cell growth.

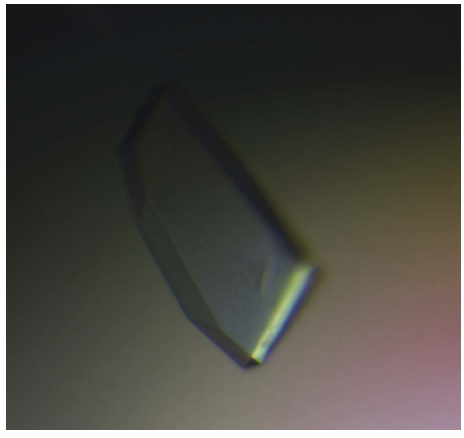

Figure 6
Mineralocorticoid receptor. The MR C808S mutant expressed well in the presence of potent compounds and its structure has been determined in complex with several ligands (Bledsoe *et al.*, 2005).

These techniques were still not sufficient to provide soluble protein for the recalcitrant nuclear receptors GR and MR. Expression studies with GR demonstrated that mutating the phenylalanine at position 602 to a serine residue (F602S), as in PR, led to increased expression of this receptor (Bledsoe *et al.*, 2002; Fig. 2).

Although expression of soluble GR LBD increased with the F602S mutation, further investigations revealed that the ligand type had a dramatic effect on the expression levels (Fig. 3).

A multi-faceted approach of expression as a GST fusion, the presence of high-affinity ligands during cell growth, isolation under denaturing conditions, the F602S mutation and optimization of ligand type led to the growth of GR crystals and subsequent structure determinations (Bledsoe *et al.*, 2002; Fig. 4).

In similar expression studies with MR, the equivalent residue, Cys808, was also mutated to serine (C808S), producing an even more dramatic increase in expression of soluble GST MR LBD in *Escherichia coli* (Bledsoe *et al.*, 2005; Figs. 5 and 6).

Since the expression of these nuclear receptors is normally ligand-dependent, what strategy can one use if one is unable to express protein with an important ligand? In such an instance with PR, the protein was expressed in the presence of a low-affinity ligand ($\sim$50 nM). During the cell lysis, protein purification, dialysis and final concentration, a higher affinity ligand of interest ($\sim$5–10 nM) was included in molar excess. This was the only method by which crystals of the ligand of interest were grown (Fig. 7).

3. Use of ligands during protein purification

If you have optimized your system for protein expression, but the protein is not well behaved, the use of ligands during the protein purification may be a useful avenue to pursue. When ligands are included during the cell lysis, during part of or the entire purification process or are added during refolding, improvements in stability, solubility and aggregation have been observed. In some instances, the ligands have displaced other proteins, *e.g.* HSP90.

In one example, a recombinant enzyme (kinase 1) from a baculovirus was expressed in insect cells. The cell lysate was subjected to immobilized metal-affinity chromatography (IMAC) and size-exclusion chromatography (SEC). Fig. 8(*a*) shows a typical SEC chromatogram for this enzyme. All the protein elutes at 700 ml (the void volume for this column). The enzyme preparation is highly contaminated with other proteins and likely nucleic acids (given the high absorbance at 260 nm). Although this enzyme is active, it is unsuitable for structural studies. In an effort to prepare enzyme for X-ray diffraction studies, an inhibitor specific for the recombinant enzyme was included in the lysis and chromatography buffers. Fig. 8(*b*) is the SEC (size-exclusion chromatography) profile of the enzyme plus inhibitor. The proteins eluting at the 700 ml void are the same as seen in Fig. 8(*a*), but eluting at about 950 ml (the position expected for monomeric enzyme) is enzyme that is homogenous. The enzyme purified in the presence of inhibitor was successfully crystallized, enabling the three-dimensional structure to be determined.

Addition of the ligand during the cell-lysis step and throughout the entire protein-purification process was the key to obtaining pure monomeric kinase 1 (Fig. 8*b*). If no ligand was included, the resulting peak was a mixture of protein, DNA and lipids (Fig. 8*a*). Crystals grown from the pure monomeric protein routinely diffracted to 2–2.3 Å (Fig. 9).

Pure estrogen receptor α was obtained when the cells were lysed in the presence of urea and subsequently purified by estradiol affinity chromatography. The key to obtaining well behaved protein was refolding this protein in the presence of a ligand. This gave sufficient quantities of soluble protein without special growth conditions, *i.e.* inclusion of the ligand during protein expression (Brzozowski *et al.*, 1997; Tanenbaum *et al.*, 1998).

Work is still in progress on kinase 2, which copurifies with HSP90 on all chromatography media tested to date. We were not able to disrupt the kinase 2–HSP90 complex by the addition of a variety of salts, detergents or ATP. However, once a high-affinity ligand was included during the early steps of the purification, we obtained soluble kinase 2 without the HSP90. Although we do not yet have crystals of this protein, we now have monomeric protein to use for crystallization trials.

4. Cocrystallization

One of the common methods of obtaining crystals of a protein–ligand complex is cocrystallization, where the ligand is added to the protein to form a complex that is subsequently used in crystallization trials. This is often the method of choice when the compounds are quite insoluble or the protein aggregates easily. Cocrystallization is affected by temperature, protein concentration, ligand concentration, the use of additives to improve ligand binding, ligand exchange prior to cocrystallization and cross-seeding. A special type of cocrystallization, real time *in situ* competition crystallization (RTISCC), may also be employed.

4.1. Temperature

4.1.1. Temperature changes when complexing the protein and ligand. Often we complex our protein with the ligand of interest and incubate at 277 K for 30 min to 1 h before setting up the crystallization screens. If the ligand is rather insoluble, changing the temperature of this incubation may facilitate complex formation. Cocrystals of kinase 3 were obtained only when the protein–ligand mixture was incubated at room temperature for 30–60 min (Fig. 10). The samples were then transferred to ice immediately prior to setting up the crystallization screens.

4.1.2. Heat treatment of the protein–ligand complex/use of additives. We often have a protein that does not crystallize or gives poorly diffracting crystals. One quick experiment requiring minimal equipment that can be performed to see if

we can improve the quality of this protein is heat treatment. This can reduce or eliminate protein that is not folded properly, giving a more homogeneous protein sample. A time-course study can easily be performed to determine the most effective temperature and time for the heat treatment. Activity assays or dynamic light scattering (DLS) can be employed to monitor the protein.

Initial crystals of the viral protein–ligand complex in Fig. 11 showed no diffraction. However, when this protein complex was heated to 310 K for 5–10 min, followed by incubation on ice and centrifugation with a 0.2 µm filter, the resulting crys-

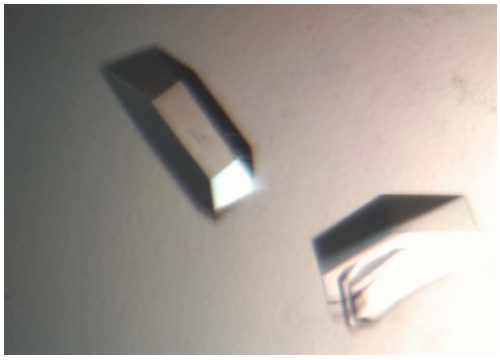

Figure 7
Progesterone receptor LBD. Cocrystals of the ligand of interest were obtained when the protein was initially expressed with a lower affinity ligand. The ligand of interest was then added in molar excess and included throughout protein purification.

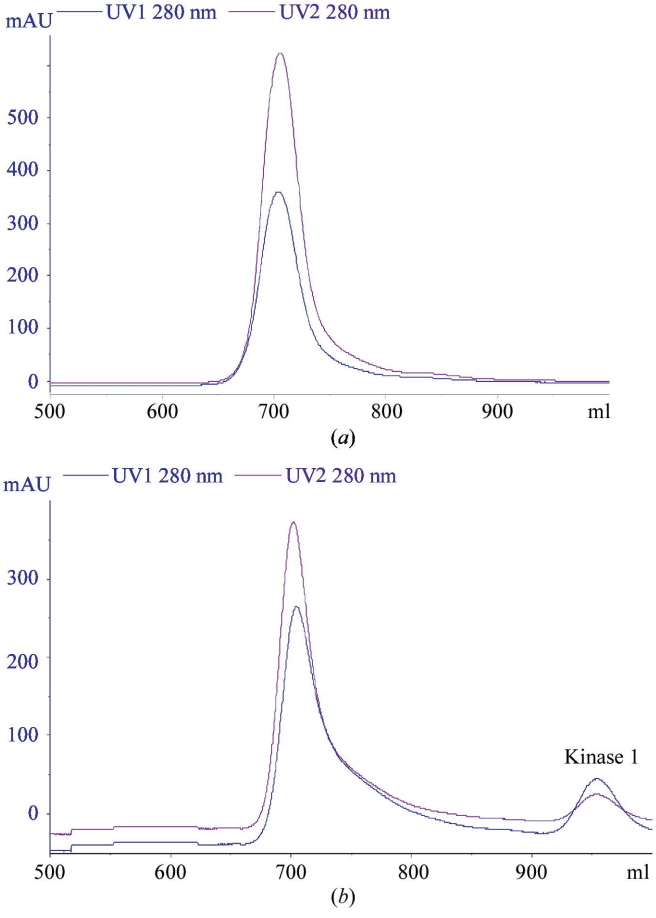

Figure 8
Ligand included throughout the protein purification of kinase 1 yielded pure monomeric protein (*b*). Purification without the ligand resulted in a mixture of protein, lipids and DNA (*a*).

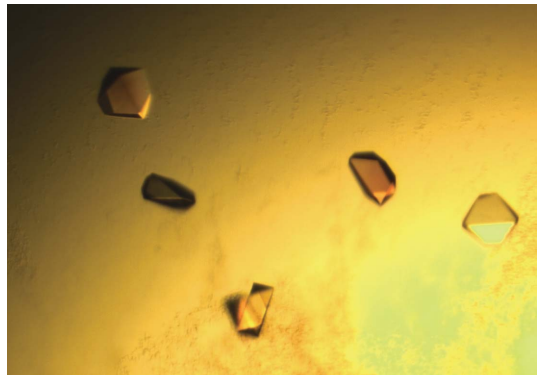

Figure 9
Crystals of the kinase 1 ligand complex routinely diffract to 2–2.3 Å.

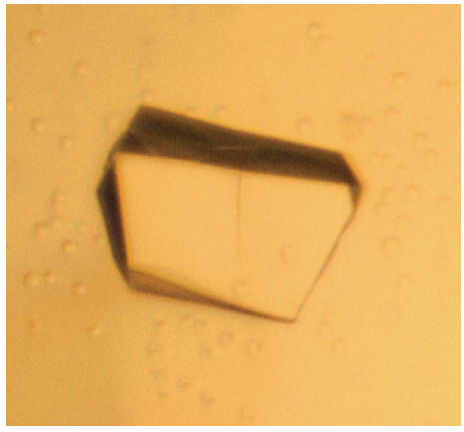

Figure 10
Incubation of kinase 3 with ligands at room temperature was critical in obtaining cocrystals of these complexes.

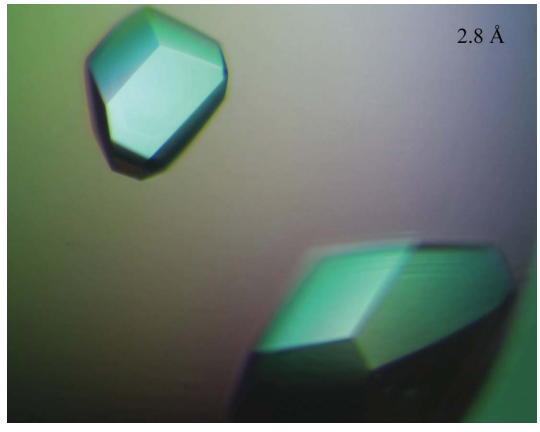

Figure 11
Crystals of a heat-treated viral protein–ligand complex diffract to 2.8 Å. The addition of 0.1% β-octylglucoside to the protein solution was required to grow crystals of some of these complexes.

tals diffracted to 2.8 Å (Wang & Nolte, 2006). The addition of 0.1% β-octylglucoside to the protein solution was key to obtaining crystals of some of these viral protein–ligand complexes.

4.2. Protein concentration/ligand concentration

Sometimes it is possible to concentrate our protein and then add the ligand to form the complex. However, if the ligand is insoluble, it may cause the protein to precipitate when it is at higher concentrations. It may be necessary to add dilute ligand to diluted protein to achieve good ligand binding with these very insoluble compounds. Kinase 4 had to be diluted to 1 mg ml^{-1} and then complexed with dilute ligand at a 1:3 protein:ligand ratio to achieve a stable complex that yielded well diffracting crystals (Fig. 12). The majority of these complex cocrystals were grown by cross-seeding using apo crystals.

Examples have been presented showing the effects of temperature, protein concentration, the use of additives and ligand concentration on the formation of protein–ligand complexes with insoluble compounds. However, there are additional ways to tackle the problem of insoluble ligands: homogenize the powdered with a small pestle, mix the ligand with tiny beads and vortex or sonicate to homogenize the powder and soak the crystal in cryoprotectant first before adding the ligand.

4.3. Ligand exchange prior to cocrystallization

What if the protein already has a natural ligand in the ligand-binding pocket (Fig. 13)? How do we obtain cocrystals of a protein–inhibitor complex?

Generation of cocrystal structures with new ligands has proven to be challenging for some of the nuclear receptors, primarily owing to the inability of synthetic ligands to displace the native phospholipids. After many trials, a protocol was developed that enhanced the displacement of native phospholipids in nuclear receptor 1 by our synthetic ligands and accelerated the determination of several ligand-complex structures.

The synthetic ligand is dissolved in dimethyl sulfoxide (DMSO) and mixed with phospholipid. This is lyophilized, dissolved in the protein buffer (liposome suspension) and complexed with the protein for 1–2 weeks at 277 K. Displacement of the native phospholipid by the synthetic compound is followed by mass spectrometry. The new complex is then purified by gel-filtration chromatography. The liposome-treated protein resulted in a reproducible system routinely giving crystals that diffracted to 2 Å (Fig. 14) for nuclear receptor 1.

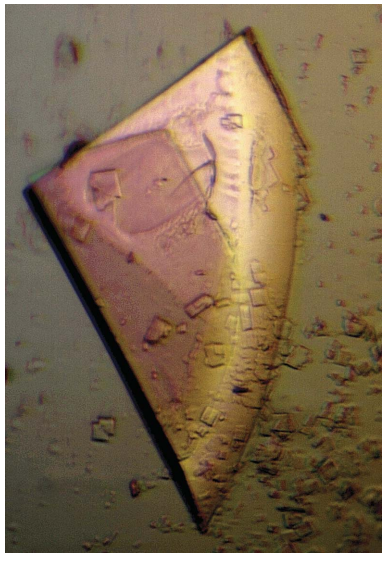

Figure 12
A protein concentration of 1 mg ml^{-1} during complex formation and cross-seeding were critical to obtaining cocrystals of the kinase 4 complexes.

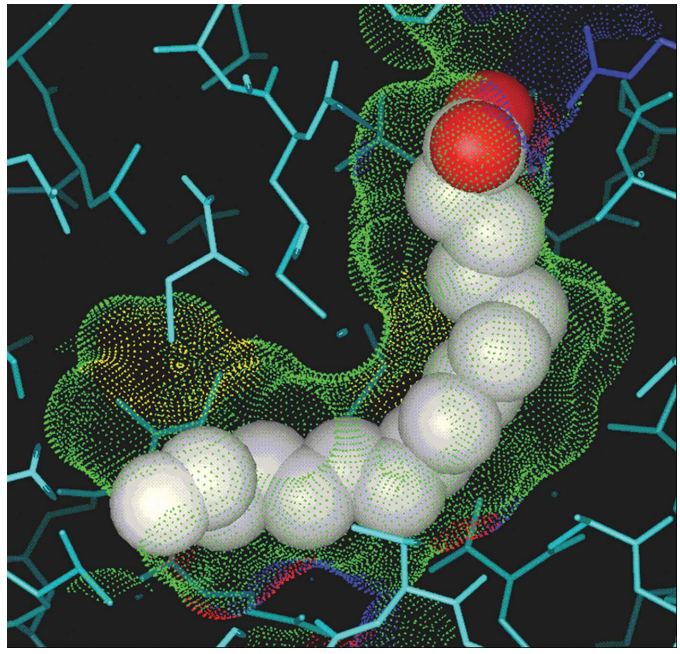

Figure 13
Nuclear receptor 1 with bound natural ligand.

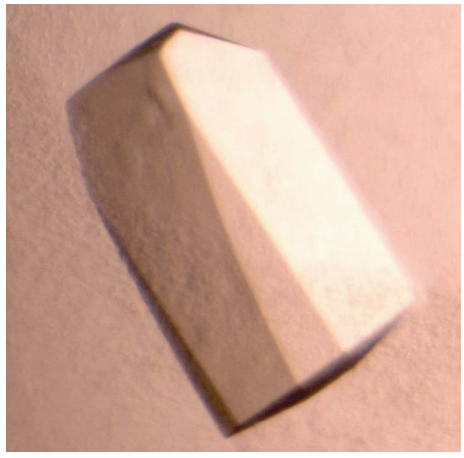

Figure 14
Crystals of the liposome-treated nuclear receptor 1 diffracted to 2 Å.

4.4. Cross-seeding

Apo crystals of kinase 5 were easily reproduced, but growing crystals of the ligand complexes proved difficult. Apo crystals were used for cross-seeding into Hampton Research Crystal Screen to find initial crystallization conditions for the ligand complexes. Crystal Screen reagent 28 gave the best results and was optimized. All of the subsequent cocrystals were grown by cross-seeding with either apo crystals or crystals of other ligand complexes into this reagent (Fig. 15).

4.5. Real time *in situ* competition crystallization (RTISCC)

Some proteins require the presence of a ligand during expression to obtain sufficient quantities of stable protein for crystallization trials. In some cases, the ligand used during protein expression is not the ligand of choice for structural studies. In RTISCC, the ligand of interest is added to the crystallization drop to compete out the first ligand used during expression (Fig. 16).

4.6. Use of a limited additive screen

It is not uncommon to have finite quantities of ligands of interest for cocrystallization studies, thereby limiting the experiments that can be performed. To address this problem, Lisa Shewchuk developed a limited additive screen that could be used in such instances. It is widely used in our laboratory for optimizing crystals and improving ligand solubility. Our initial additive screens use these reagents at a 5% concentration where the additive is added directly to the precipitating reagent. In other cases, these additives have been mixed with the ligand to improve its solubility.

5. Soaking ligands into existing crystals

Soaking crystals with ligands is often the method of choice to obtain crystals of protein–ligand complexes owing to the ease of the method. However, there are several factors to consider. The crystals may be fragile and soaking in a stabilization buffer or cross-linking may be required. The soaking time and inhibitor concentration need to be optimized, as many protein crystals are sensitive to the solvents used to dissolve the ligands. Additives may be required to achieve effective ligand binding during the soak time and/or during the subsequent cryoprotectant exchange. Finally, you may have cocrystals of one ligand complex and need to exchange the original ligand with a different ligand (replacement soaking; Skarzynski & Thorpe, 2006). The FAST fragment-based screening developed by Structural Genomix Pharmaceuticals is an example of a high-throughput soaking-type system that has been quite successful (Burley, 2004).

Although soaking ligands into crystals may be the method of choice for a particular protein, it is preferable to validate the soaking system with cocrystallization experiments when possible. The full range of conformational changes may not be seen in instances where the ligand has been soaked into the crystal. However, in the case of cyclin A–cdk2 crystals, the cyclin A restricts movement of the cdk2 and soaking in this system is a valid approach.

5.1. Stabilization of crystals/use of an additive during the soak

Crystals are often put into 'stabilization' buffers before they are immersed in the ligand solution. These buffers may contain increased concentrations of the precipitant(s) and a stepwise/gradual increase in reagent concentration or the introduction of a cryoprotectant may be required so that the crystals are not damaged.

Another option for stabilizing crystals is cross-linking with glutaraldehyde. This has been our method of choice with the cyclinA–cdk2 crystals that we routinely use as a surrogate kinase for projects where there is no protein available (Hassell *et al.*, 1998). These crystals are large and fairly sensitive to inhibitor soaks and cryoprotectant exchange. The method of Lusty (1999) using 25% glutaraldehyde in a microbridge for varied periods of time has worked quite well with this system. We routinely cross-link these crystals with 5 µl of 25% glutaraldhyde in a microbridge at 277 K for 30 min, but have found that these parameters vary greatly depending on the protein (5 min–18 h). We improved our success rate of soaks approximately twofold to threefold when this cross-linking

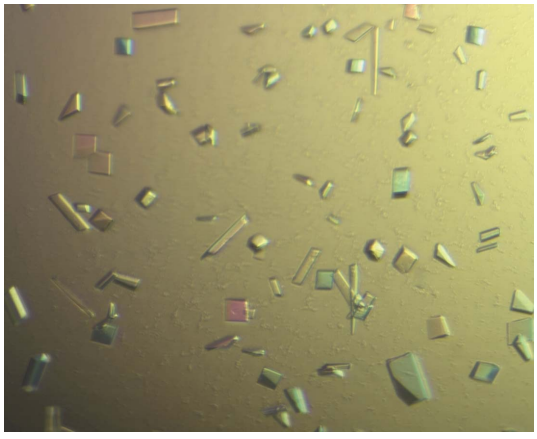

Figure 15
Crystals of the kinase 5 ligand complexes were obtained by cross-seeding.

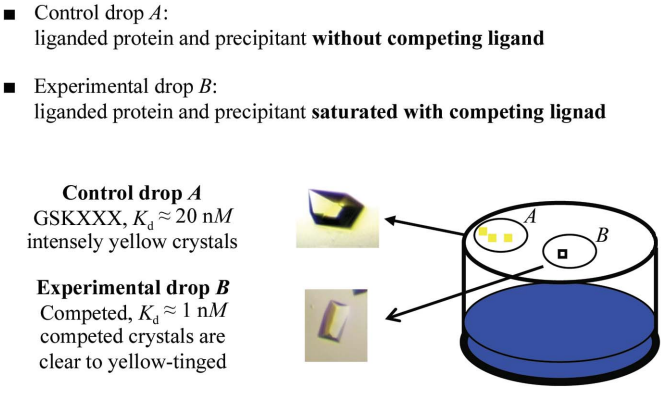

Figure 16
Real-time *in situ* competition crystallization (RTISCC). The ligand of interest competes out the original ligand used during protein expression.

procedure was employed. We also found that the use of PEG 400 increased our success rate with large ligands. The inhibitor is mixed with 50% PEG 400 in a 1:1 ratio. 1 µl of this mixture is then added to the cross-linked cyclinA–cdk2 crystals (Fig. 17).

Although PEG 400 has been useful for soaking large ligands into the cyclinA–cdk2 crystals, there are a variety of reagents that have proved useful in other systems, including Jeffamines, sugars, methylpentanediol (MPD) and a variety of PEGS. We often use some of the reagents in the limited additive screen in Table 1 to improve ligand solubility. This can be accomplished in several ways. The additive may be added directly to the precipitating reagent in the well and thoroughly mixed. Subsequently, the ligand is added to a drop of this additive/precipitating reagent mixture in a 1:1 ratio and then added to the protein drop. Alternatively, the ligand may be mixed with the additive and then added to the protein drop. The exact ratios of additive:ligand:precipitating reagent need to be optimized, as this can vary greatly depending on the type of ligand.

In protease 1, cocrystallizations yielded very few protein–ligand complexes, so a soaking strategy was devised. Since the apo crystals were rather fragile and the ligands were quite insoluble, an additive was needed that stabilized the crystal and improved the ligand solubility. Xylitol was added to the precipitating solution to a final concentration of ∼2–5%. 1 µl of this mixture was added to the protein drop to stabilize the crystals (∼15–60 min). Next, the inhibitor was added to the additive plus precipitating reagent mix (∼2–5 µl ligand plus 500 µl precipitating reagent). 1 µl of this ligand mixture was then added to the crystals (Fig. 18). This procedure was the only method that led to solution of structures of the protease–ligand complexes.

5.2. Soaking time/ligand concentration

The previous example showed crystals of cyclinA–cdk2 that were sensitive to handling where cross-linking and the use of an additive were critical for successful ligand soaks. However, in cdk2, a second approach of diluting the inhibitor and using longer incubation times worked well in obtaining inhibitor complexes. 1–3 µl of a 50 m*M* stock inhibitor solution was added to 500 µl well reservoir. 1 µl of this diluted ligand was then added to a 4–5 µl drop containing the cdk2 crystals (Fig. 19). Incubation times ranged from several days to 2–3 months.

Table 1
Limited additive screen (Lisa Shewchuk) used to improve crystal quality and ligand solubility.

1	0.10 *M* $MgCl_2$
2	0.15 *M* $CaCl_2$
3	1.0 *M* NaCl
4	25% Jeffamine T403
5	25% Jeffamine M600
6	20% Jeffamine M89
7	25% ethylene glycol
8	25% 1,6-hexanediol
9	20% glucose
10	1.0 *M* guanidine hydrochloride
11	35% dioxane
12	1.0 *M* imidazole pH 6.5
13	25% *tert*-butanol
14	25% MPD
15	0.10 *M* $MnCl_2$
16	10 m*M* $ZnCl_2$
17	0.1% TFA
18	0.14 *M* β-mercaptoethanol
19	25% 1,2-propanediol
20	25% PEG 10K
21	25% PEG 400
22	0.10 *M* triethylamine (TEA)
23	0.10 *M* spermidine
24	0.25 *M* arginine

5.3. Ligand exchange in crystals

There are instances where it is easy to grow crystals that contain a natural ligand or cocrystals with one inhibitor, but not with a new ligand or template. In this case, the new ligand of interest may be soaked into the existing cocrystals, substi-

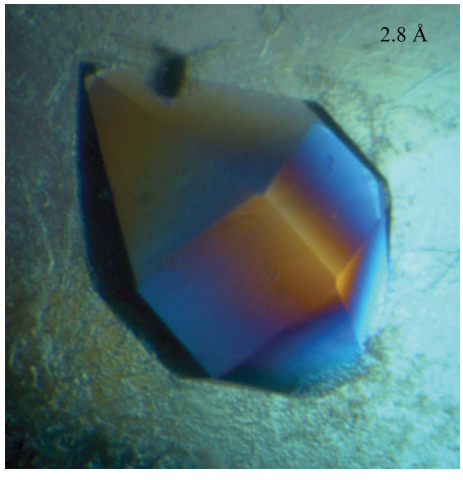

Figure 17
Cross-linking with glutaraldehyde and the use of PEG 400 with large ligands improved the success rate for soaks in the cyclinA–cdk2 system.

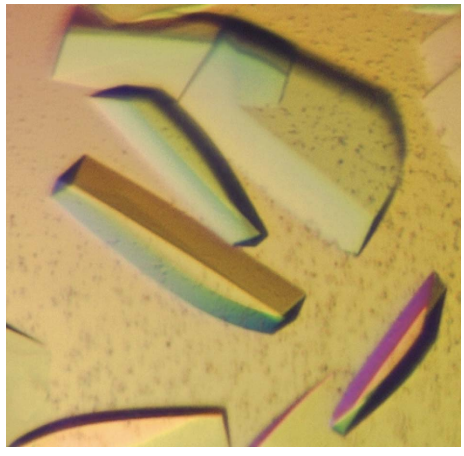

Figure 18
Protease 1. The use of xylitol during the soaking stabilized the crystal and improved the solubility of the ligands. If the ligand was not mixed in the xylitol plus precipitating mixture, the efficiency of obtaining protein–ligand complexes greatly decreased.

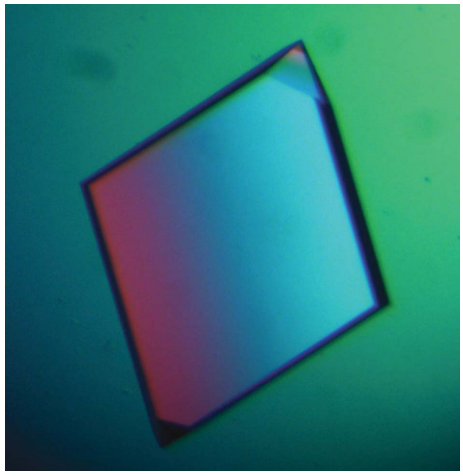

Figure 19
Inhibitor soaks of cdk2 gave crystals that diffracted to ∼2 Å. Dilute inhibitor concentrations coupled with long incubation times gave the best complexes.

tuting it for the original compound. When performing this 'replacement soaking' (Skarzynski & Thorpe, 2006), one must consider the binding constant of the new ligand. It may be difficult to introduce a new lower affinity ligand into the system and substitute it for a much higher affinity ligand that is already bound to the protein. The success of the ligand replacement can be seen when the electron-density map is calculated.

6. Conclusions

There are a wide variety of techniques available to the investigator for obtaining protein–ligand complexes. These include adding ligands during protein expression to obtain soluble protein, addition of the ligand during protein purification, cocrystallization of the ligand with the purified protein and soaking ligands into crystals. It is reasonable to start by soaking the ligands into crystals as this is the easiest method or to try cocrystallizing the ligand with the purified protein. If these are not successful, further optimization of the protein expression and purification may be necessary.

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