CRYOPROTECTION AND LIGAND SOLUBILIZATION: EVALUATION OF MULTICOMPONENT MIXTURES

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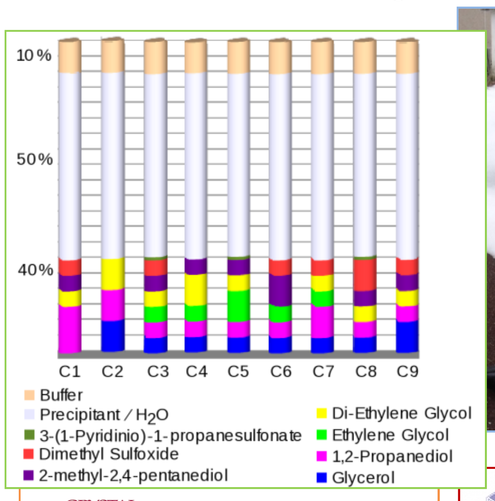
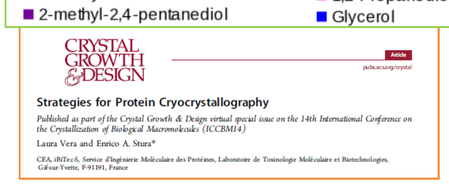
Crystallographic structure determination of protein-ligand complexes is an important step in the progress from a low affinity lead compound to an active molecule. The combination of low affinity and low solubility is a hurdle for structural studies of ligand-protein complexes. Complete solubilization of hydrophobic ligands is required for their use in co-crystallization or crystal soaking experiments to obtain interpretable electron density maps for the ligand. High-throughput screening is used to select among a library of millions of compounds those that bind to a target protein, inhibit a particular enzymatic reaction or block a cellular transport mechanism. Typically the chemical compounds are dissolved in dimethyl sulfoxide (DMSO)[1] to produce an aqueous solution. This solution is diluted during the screening to ensure that the concentration of DMSO does not exceed 10%; as higher concentrations can be damaging to the target protein.[2]




CRYSTAL GROWTH & DESIGN

Strategies for Protein Cryocrystallography

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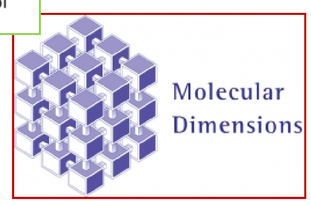

Figure 1

In a previous study (Figure 1), by mixing cryo-preserving compounds that act as precipitants with compounds that have the opposite effect we developed a set of multicomponent mixtures that could be combined with a precipitant and a buffer so as to be able to prepare crystals for X-ray data collection without hassle.[3]
We extended the set of multicomponent solutions for crystal cryoprotection to included additional components, namely dioxane and butanediol and have analysed the use of dioxane for ligand solubilization, alone and in conjunction with other cryoprotectant components and its compatibility for protein crystallization and crystal soaking.

The new cryosolutions were developed using Transthyretin as reference protein (Figure 2). About 200 samples were tested at synchrotron facilities, namely at the ESRF (beamlines ID23-1 and ID23-2) in Grenoble and on beamline Proxima-2 at the Soleil storage ring in Saclay

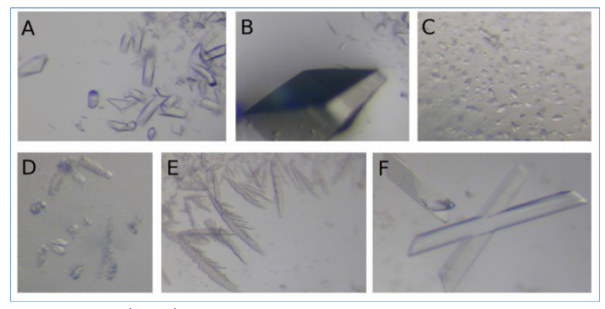

Figure 2

Crystals of wild-type human transthyretin (TTR) obtained under various crystallization conditions from the Stura footprint (Molecular Dimensions, Ltd) in the absence of any ligand. (A) Crystals obtained from 33% PEG-600, 0.2 M imidazole malate, pH 5.5. (B) Crystals from 2 M ammonium sulphate, 0.15 M sodium citrate, pH 5.5. (C) Shower of crystals from 1.5 M sodium citrate 10 mM sodium borate, pH 8.5. Larger crystals can be obtained by lowering the precipitant concentration. (D) Crystal grow in small attached blocks from 27% PEG 10,000, 0.1 M ammonium acetate, pH 4.5 (E) Feather-like crystals grow from 60% monomethyl PEG 550, 0.1 M HEPES, pH 8.2. (F) Large prismatic crystals are obtained from 36% monomethyl PEG 5,000, 0.1 M sodium acetate, pH 5.5.

PROBLEM

Among the analyzed compounds some result poorly soluble or insoluble in drop if solubilized with only DMSO (Figure 3).

Ligand insolubility is also a common problem in chemistry. There is no general rule on how to dissolve chemical molecules.

Figure 3. Photomicrograph of crystals of wild-type human transthyretin (TTR) grown in the presence of ligand. Crystals of the TTR-16α-bromo-estradiol complex. The steroid is well solubilized in the crystallization as the drop is set up, but it crystallizes as the drop shrinks to the final volume during vapour diffusion equilibration and crystallization.

SOLUTION

The strategy of using a mixture of solvents to solubilize ligands is well known. Two or more solvents together enhance the solubility of insoluble compounds.[4] The approach used to create cryoprotectant solutions by mixing together compounds that inhibit ice formation can be extended to solutions that are also effective in solubilizing ligands. For inhibitors poorly soluble or insoluble in DMSO, the mixed solutions with DMSO/dioxane/ethylene glycol mixes of different ratios should improve ligand solubilization. DMSO, ethylene glycol and dioxane belong to different selectivity classes: III, IV and VI, respectively (Figure 4)[5] and combination of these compounds should covers a relatively wide range of selectivity values to render water soluble a large variety of organic compounds.

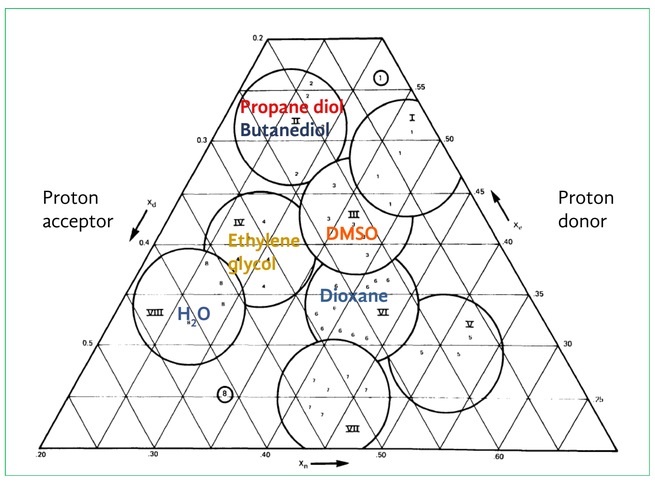

Figure 4

Classification of the Solvent Properties of Common Liquids

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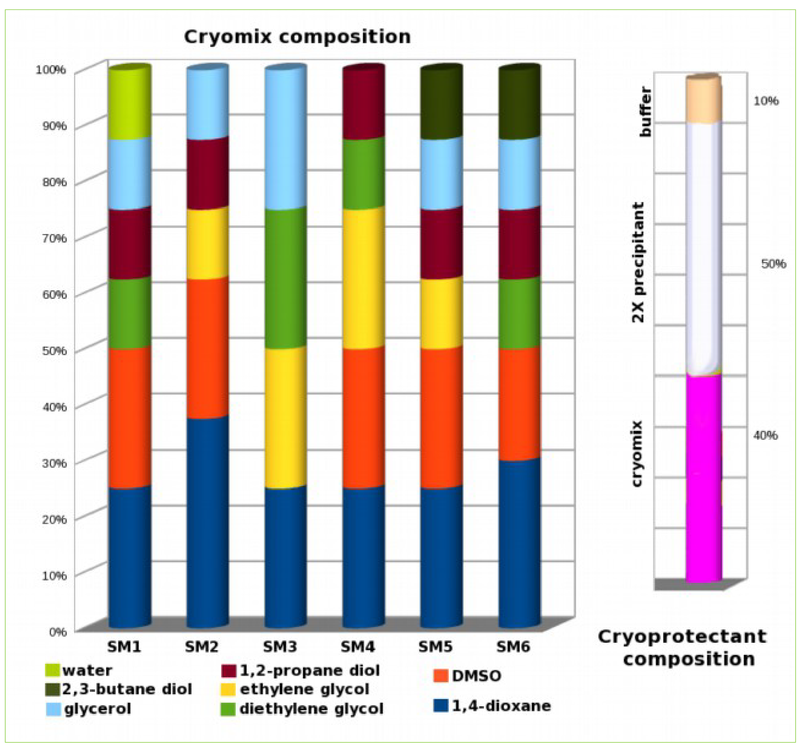

Figure 5

Composition of solubilizing cryoprotectant solutions

The composition of the solubilizing cryoprotectant solutions has been chosen on the same basis as those developed for cryoprotection of crystals.[3] Although ethanol and methanol are excellent solvents for ligands and can help reduce ice nucleation, it is impractical to use the in crystallization experiments or for cryoprotection of crystals because of their volatility. However, compounds like ethylene glycol and propylene glycol are good cryoprotectant compounds and used in water co-solvent mixture for pharmaceutical compounds.[4] Dioxane, a non volatile solvent that has been used as a crystallization additive, not present in the design of CryoProtX™ (Molecular Dimensions U.K. Ltd.) has been added to the new mixtures. Another addition is 2,3 butanediol, which is compatible with enzymatic activity.[6] The solubilizing mixes have been designed so they can be prepared easily and for consistency with the cryomixes formulated before. [3] This means that when 40 µL from the solubilizing cryoprotectant mixes are mixed with 10µL from the buffer and 50 µL of the precipitant at a concentration twice that used for the crystallization precipitant crystals can be soaked in the resulting solution without dissolving or cracking in the solution even if they remain in the solution 20 minutes or overnight (Figure 5)

Co-crystallization experiment

The **SM1-6** mixes were tested in co-crystallization experiments with TTR in the presence of curcumin and 16α-bromo-estradiol [7]. In these two cases, these hydrophobic compounds were solubilized in a DMSO/dioxane mix and co-crystallized with a precipitant consisting of high/low molecular weight PEG which helps to keep the inhibitor soluble in drop during crystal growth (Figure 6).

Precaution!!! An appropriate amount of cryomix was added to the reservoir to avoid disequilibrium between the drop and the reservoir. This is an important correction that must be applied to the reservoir solution after the protein-ligand drop has been mixed with the reservoir solution, because the cryomixes contain glycerol and other hygroscopic compounds that affect vapour diffusion equilibration.[8]

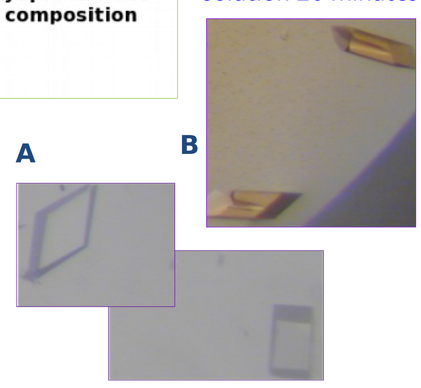

Figure 6 Photomicrographs of crystals of wild-type human transthyretin (TTR) grown in the presence of various ligands. (A) Crystals of the TTR-16α-bromo-estradiol complex. The crystals are grown with a lower concentration of PEG. PEG can participate in the solubilization of the steroid, but the small change in concentration has almost no influence on ligand crystallization. (B) Crystals of TTR grown in the presence of curcumin at pH 7.4 show an orange color denser than the background solution.

RESULTS

16α-bromo-estradiol is completely soluble in dioxane and not in DMSO, while curcumin is completely soluble in DMSO and not in dioxane. The combination of the two solvents has allowed the preparation of protein-ligand solutions suitable for crystallization (Figure 7). Dioxane acts as a precipitant in TTR crystallization, its effect is counterbalanced by glycerol and other diols that tend to increase protein solubility. The addition of 2,3 butanediol in the mixes contributes to ligand solubilization and to cryoprotection. This compound was considered an antifreeze agent more than 60 years ago [9].

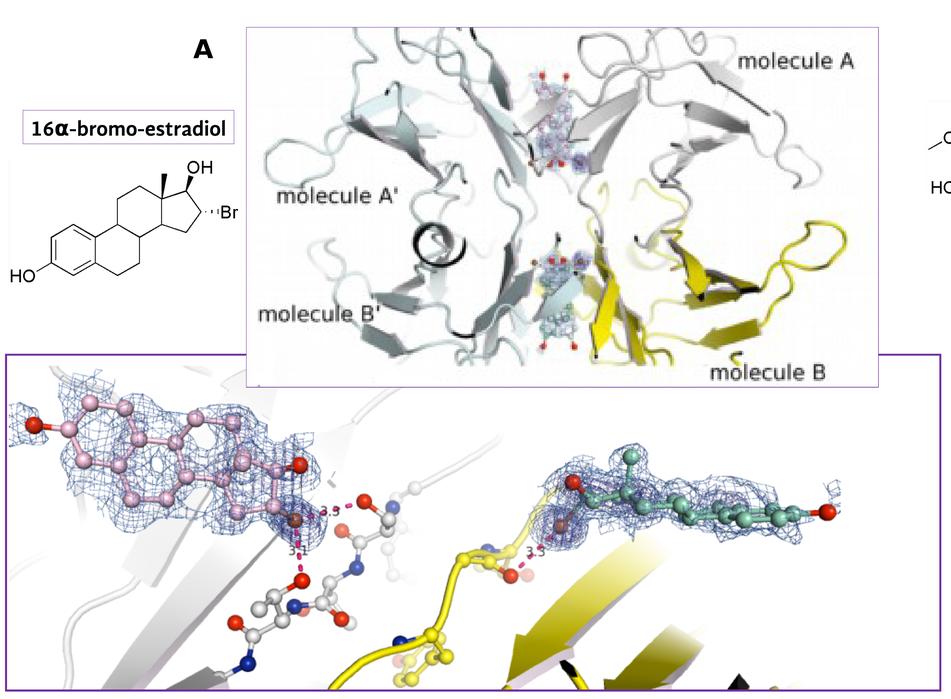

Figure 7 Electron density for the TTR-complexes with 16α-bromo-estradiol and curcumin. (A) Positioning of 16α-bromoestradiol within the TTR tetramer (top: PDB id: 4PM1). The bromine atom is bound in the halogen binding clearly identified by the higher electron density between Ser117 and Thr119. The electron density for the oxygen and carbon atoms of the steroid are smeared out due to the superposition of the two symmetric ligands with each at occupancy=0.5 for a fully occupied ligand binding site.
(B) Electron density for the two curcumin complexes (top: PDB Id: 4PME and bottom: 4PMF). Soaking with curcumin for 20 minutes during the cryopreservation step allows the exchange of ferulic acid and the replenishment of curcumin in the site.

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