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Reverse Screening

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Abstract

Major emphasis has been placed in recent years on kits for screening crystallization conditions of macromolecules. Such approaches have undoubtedly speeded up the initial screening and, to a certain extent, helped in reducing the amount of protein required for the initial survey. Factorial screening techniques, either full-factorial or sparse-matrix approaches, have proved successful in the crystallization of many proteins. However, in cases where the amount of protein is limited a systematic approach based on an *a priori* choice of precipitants may be preferable to an extensive search. The approach described here targets such situations. The approach consists of the determination of the solubility characteristics of the macromolecule under study as a function of precipitant and macromolecule concentrations to define a working range for these parameters. Conditions under which the protein is highly supersaturated, and hence more conducive to nucleation, are established so as to favor the formation of an initial stable nucleus which can be one of the dominant problems that hinders successful crystallization of proteins. Later, changes in solubility as a function of pH and as a result of the introduction of additives are evaluated. In addition, when ligands are available for the formation of macromolecular complexes, screening of different complexes is used as a means to increase the probability of obtaining crystals. Solubility information derived from one, or more, complexes that have been screened can be used for comparison and to aid in the crystallization of other complexes. Cross-seeding between complexes is an intrinsic part of the method and provides an efficient way of obtaining crystals when spontaneous nucleation is hard to achieve. In the example presented here, reverse screening has enabled the production of crystals of several peptide complexes with an anti-malaria antibody.

Introduction

Several physical conditions have to be established to achieve nucleation and subsequent crystal growth. Screening protocols that sample many possible crystallization conditions can yield crystals only when the physical requirements for nucleation and crystal growth are met. When extensive screening is not possible, it may be preferable to choose a particular precipitant. By analyzing the solubility of the macromolecule, the conditions needed for crystallization may be established. Subsequently, conditions may be varied within a range so that the crystallization trials will remain within the high-supersaturation region of the solubility curve considered more favorable for the formation of a stable nucleus and subsequent crystal enlargement. In choosing the first precipitant (or set of precipitants, see Stura, Nemerow & Wilson, 1992) we may use prior experience or specific information about the macromolecule that may come from a variety of sources. For example, analysis of the crystallization database (BMCD; Gilliland, 1988; new software release: Gilliland, Tung, Blakeslee & Ladner, 1994) reveals that ammonium sulfate and polyethylene glycol 4000 and 8000 are the most commonly used precipitants (Fig. 1*a*). This observation is highlighted in the cartoon summarizing the Bischenberg meeting in 1987 (Drenth, 1988). Hence, it may be expected that good success will be achieved when using a precipitant that has been successful for other macromolecules in the same class as the one for which crystallization trials are being set. In our experience this has certainly been true for antibodies and their complexes.

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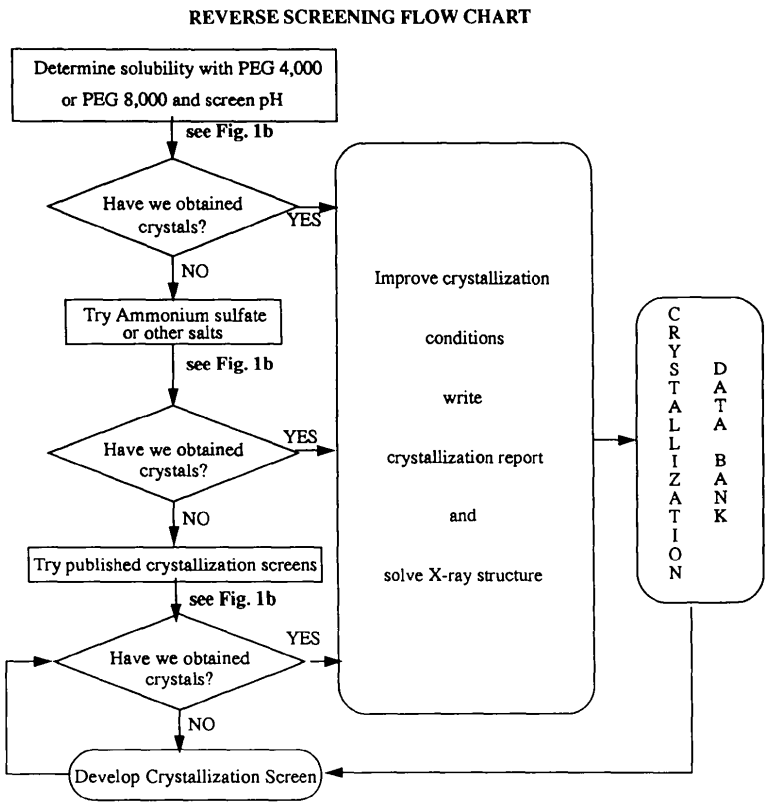
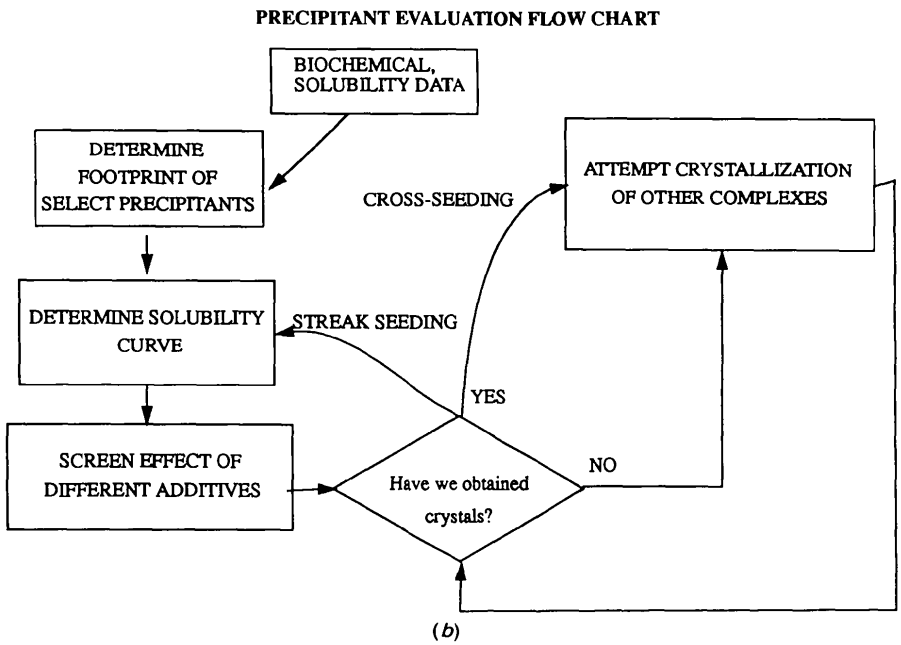
Reverse screening is based on solubility principles and experience. As experience with a particular precipitant system is acquired, the conditions encompassed by the system will become more successful and crystallization reports will tend to reflect this. Reverse screening, like most screening methods and protocols, does not comprehensively screen all possible crystallization conditions. In effect, investigators rarely define the full range of crystallization conditions, not by choice, but because of a limited supply of macromolecule.



(*a*)



(*b*)

Fig. 1. (*a*) Flow diagram to illustrate the reverse screening approach and its relationship with the development of a crystallization data bank. Precipitants that have been successful for other crystallizations are the most likely to be tested first, and if suitable to grow crystals, no other experiments are done. The results will then be reported in the literature and the precipitant will be more likely to be chosen by other investigators. Data will continue to be accumulated more rapidly for the more standard precipitants such as ammonium sulfate, PEG and for precipitants included in published screens. (*b*) Flow diagram to illustrate the precipitant evaluation procedure. A footprint type screen is set up for a limited number of precipitants depending on the supply of macromolecule, and the one that fulfills the desired criteria is chosen for further solubility determination and subsequent additive search. The same path can be taken with as many precipitants as desired or as the available sample will allow. The selection of the precipitant will be guided by various biochemical considerations or the crystallization data bank as illustrated in (*a*) above.

Reverse screening

The method is referred to as reverse screening because instead of screening for a precipitant, the first step consists of making the best guess of the possible crystallization conditions, and checking whether the macromolecule is likely to crystallize under such conditions. In total the method consists of four steps.

(i) *Choice of precipitant, pH and buffer system.* Often polyethylene glycol (PEG) or ammonium sulfate are used (Figs. 1*a* and 1*b*). PEG is preferred to ammonium sulfate or other salts because certain heavy atoms are insoluble under high salt conditions.

(ii) *Determination of solubility properties.* The results of experiments carried out with different precipitants and protein concentrations are mapped to yield a solubility curve for the macromolecule. This is checked as a function of pH and molecular weight of PEG.

(iii) *Screening of additives.* If the precipitant or buffer used has any specific effects on crystallization, then the precipitant, or buffer, is replaced with a related precipitant, or buffer, and the first added to the crystallization medium as an additive, or co-precipitant, in a carefully controlled manner. The goal is to have the precipitant play no role other than that of inducing supersaturation, and the buffer no role other than controlling the pH. Hence, the method promotes the use of 'precipitant-additive' combinations as a part of the linearization of the crystallization search and as a means to better control the crystallization environment. The screening of additives is carried out close to the precipitation point of the precipitant/protein system.

(iv) *Improvement of crystallization conditions.* Once crystals have been obtained, seeding can be an effective way to produce improvements while avoiding the production of highly supersaturated conditions to induce nucleation. Without seeding, nucleation can be a serious and ongoing problem in the crystallization. At times this may vary from one batch of protein to the next, and seeding can be used to maintain the ability to grow crystals from different protein preparations. Streak seeding (Stura & Wilson, 1990, 1991) is also used to determine the range of the supersaturated zone.

In reverse screening, solubility information is used to select conditions under which nucleation and crystal growth are more likely to occur (for detailed discussion see Feher & Kam, 1985). Even when all the parameters appear to be consistent with the possibility of nucleation, crystals may not be obtained. Selected conditions in screens currently being developed may become noteworthy for their ability to induce nucleation in difficult cases.

Footprint solubility screen

We give the term footprint to a screen which maps out the solubility characteristics of a macromolecule. Footprint experiments carried out in our laboratory consist typically of four different precipitant concentrations at two or more protein concentrations to map out a precipitant/protein concentration plot as shown graphically in Fig. 2(*a*). Vapor diffusion is the preferred method because it has the ability to concentrate (relatively slowly) the protein and the precipitant twofold, or by a different amount depending on the relative volumes of the drops mixed. When analyzing the results of each footprint, the morphology of various precipitates and the separation of the protein into phases are taken into account and correlated with biochemical analysis (see Lorber & Giegé, 1992). The various precipitates may arise from heterogeneous components in the protein solution, each of which may have a separate precipitation point. The use of a standardized footprint type screen (Stura, Nemerow & Wilson, 1992) has certain advantages in comparing solubility changes between macromolecules and their complexes, and between different preparations and purification schemes. Footprint-type screens are designed from previous experience or using the BMCD crystallization database (Gilliland, 1988; Gilliland *et al.*, 1994). Footprint solubility results (Fig 2*a*) are used in the design of a subsequent finer search and to refine precipitation conditions. Footprint screens are also used to compare the solubility of different protein preparations, and the effect of ligands and additives.

Precipitation and solubility curves

For a crystal to grow, macromolecules must be transferred from solution onto the crystalline lattice. Because a large crystal ($0.5 \times 0.5 \times 0.5$ mm) contains about 0.1 mg of protein, a 2.5 µl protein drop at a concentration of 30 mg ml^{-1}, or more, would be required to grow a crystal of this size. The same total amount of protein could generate a shower of around 100 crystals ($0.1 \times 0.1 \times 0.1$ mm). For most protein crystals the protein concentration in the crystal is around 600 mg ml^{-1} (assuming about 50% solvent). The precipitant must act to concentrate the protein, 20× initially and up to 2000× towards the end, assuming that growth stops when the protein concentration drops to 0.3 mg ml^{-1}. Such calculations make some very specific assumptions about the shape of the solubility plot. In this example, it has been assumed that with a protein concentration of 30 mg ml^{-1}, at a particular precipitant concentration, it is possible to achieve sufficient supersaturation for nucleation, and that crystal growth will continue at the same precipitant concentration until the protein concentration drops to 0.3 mg ml^{-1}.

Solubility plots are a valuable tool for designing an effective screening strategy, for obtaining initial crystals, establishing conditions for seeding and maintaining sufficient supersaturation for continued crystal growth. As the degree of supersaturation decreases, the growth

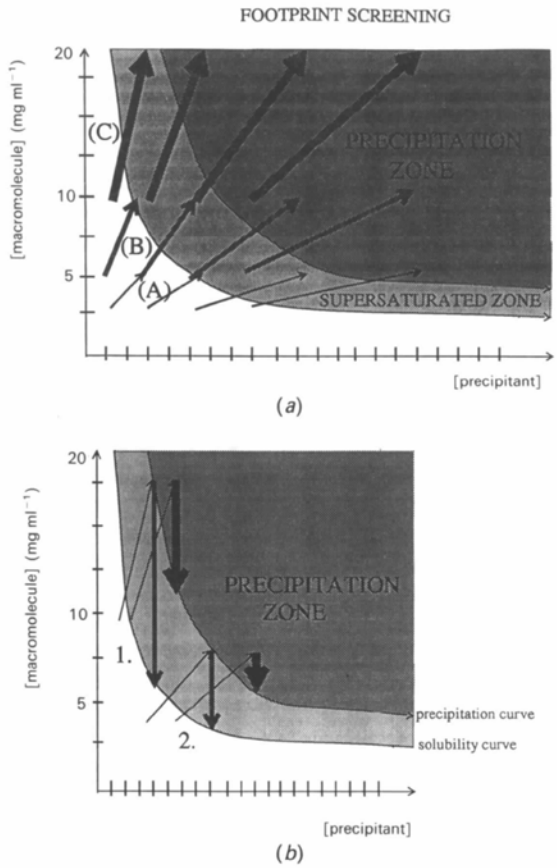


Fig. 2. (*a*) Diagram to illustrate the precipitant evaluation procedure. The thickness of the arrows is a visual aid to separate experiments *A*, *B* and *C*, performed with different initial protein concentration, the thicker the arrow the higher the protein concentration. The arrows map macromolecule and precipitant concentrations at the start and at the end of equilibration process by vapor diffusion. The diagram illustrates the manner in which the precipitant–macromolecule space is scanned, footprinted, by the arrows. By observing the drops from the beginning to the end of the experiment we can deduce the path taken during equilibration. In practice, observations are made after the drops have been mixed with the precipitant, sometimes 2 to 3 times during equilibration and at the end. Observation during equilibration is avoided when it involves a temperature change as observing the experiment may alter the results. (*b*) Diagram to illustrate the effect of the two different combinations of precipitant and macromolecule concentrations. In experiment 1, the precipitant and protein become concentrated by vapor diffusion and on equilibration a substantial amount of precipitation is obtained as a result of a small change in precipitant concentration. This fulfills the requirements and further investigations will be set up around such conditions. If the amount of precipitated macromolecule is small, as in experiment 2, the experiment can be repeated at higher concentrations of macromolecule or a more effective precipitant chosen. The thick arrows indicate experiments performed at higher precipitant concentration that leads to precipitation while the thin arrows indicate experiments performed at a lower precipitant concentration that leads to nucleation and crystal growth. The length of the thick line is proportional to the amount of protein which will appear as a precipitate. Once equilibration is achieved, crystal growth occurs at constant precipitant concentration; hence, we must select precipitant and macromolecule concentrations to maximize the amount of protein that can be transferred from the solution to the drop. The transfer of protein from the solution to the crystal is indicated by the thin lines. The vertical projection of the line is proportional to the amount of protein transferred to the crystal.

process will loose its driving force and eventually stop. The amount of protein available to the crystallization can be evaluated from the solubility plot by considering that once equilibration is achieved, crystal growth continues at constant precipitant concentration. Crystallization conditions are, at times, relatively broad, and, since the rate of growth is proportional to the surface area of the crystal, large crystals will grow faster, and as the solubility boundary is approached they will continue to grow even at the expense of the smaller ones. For example, in multinucleated drops, a large crystal may be observed in a clearing in the middle of a shower of small crystals. Macroseeding is a convenient way to divert all the protein available to the growth of a single crystal. For solubility plots to be of practical value, they should be determined with minimal use of time and protein, possibly while screening for crystallization conditions.

Determination of precipitation and solubility limits

The limits of the supersaturated zone are defined by microscopic observations. The top limit of the supersaturated zone is determined by the precipitation of the macromolecule while the lower limit occurs at the protein and precipitant concentrations at which a crystal will barely dissolve. An initial screen, limited to a small set of conditions, is used to map out the limits of the supersaturated zone (Fig. 2*a*). This will also enhance the chances of achieving nucleation since the supersaturated zone will be scanned during equilibration. When screening with limited protein samples, experiments are set out row by row, or point by point, over several days, slowly accumulating information and excluding conditions which are outside the supersaturation range. For a protein which is believed to be very soluble, a high precipitant concentration is checked first. The opposite is done for proteins of low solubility. Conditions from sparse-matrix or factorial-type screens (Jancarik & Kim, 1991; Carter, 1990; McPherson, 1992) are a good starting point for a footprint screen. Such conditions are set up at multiple precipitant and protein concentrations to obtain solubility information.

While it is relatively easy to determine the precipitation curve delimiting the precipitation zone, it is difficult to calculate the solubility curve in the absence of visible crystals. In the absence of crystals or seeds, we choose a precipitant on the basis of *significant precipitation as a result of a small change in precipitant concentration*, and then adjust the conditions to favor nucleation and crystal growth. This is shown schematically in Fig. 2(*b*).

Once crystals or microcrystals are obtained, we use streak seeding (Stura & Wilson, 1990, 1991, 1992) to define the lower solubility limit and to define the width of the supersaturated zone. A streak will become visible in a protein drop only if the conditions are suitable for crystal growth, and, even at low supersaturation, a faint

line may be discernible across the protein-precipitant drop. The rate at which crystals grow and their final size can be used to choose one crystallization condition over another. Even a rough determination of the crystallization environment in terms of the precipitation and solubility curve, as shown in the example in Fig. 3, can give the confidence to set up experiments where crystals are grown over long periods of time. This approach has helped in reducing the number of precipitants that must be screened, and has provided a good starting point for co-precipitant and additive screening.

Since solubility is dependent on temperature, we attempt to provide a constant temperature environment for crystallization experiments. We have had good success with sitting-drop vapor diffusion (Stura *et al.*, 1989; Stura & Wilson, 1991, 1992) in a constant-temperature incubator (Precision Scientific, Chicago) and we now use solid glass inserts (Glass Tech. Supplies, Anaheim, California) to increase the heat capacity of the system when greater thermal stability is needed.

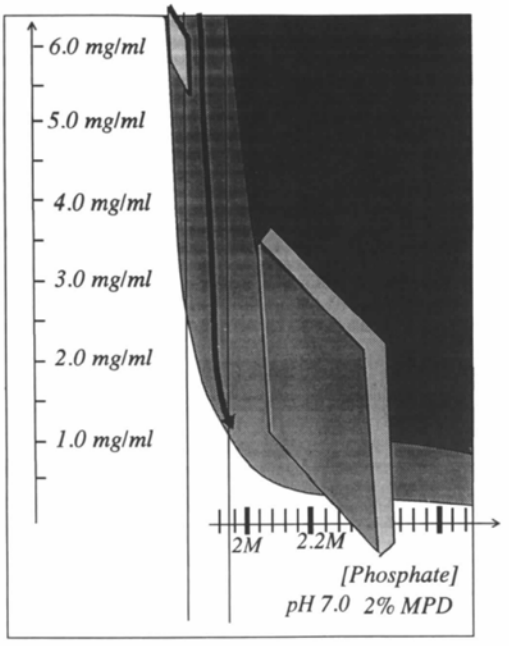


Fig. 3. Diagram to exemplify how the crystallization of MHC-I H-2K^b-SEV-9 complex was evaluated. A 2.2 µl drop of H-2K^b-SEV-9 complex at 6 mg ml^{-1} was mixed with 2.0 µl of reservoir solution (1.98 *M* NaH_2PO_4/K_2HPO_4, pH 7.0, 2.0% 2-methyl-2,4-pentane diol, crystallization grade from Fluka) and allowed to equilibrate. An initial seed (estimated to be 0.1 × 0.1 × 0.01 mm) was introduced into the solution. The final crystal grew to an estimated size of 0.3 × 0.3 × 0.2 mm, and total protein of about 0.011 mg was transferred to the crystal. From this we can make some very simple calculations: Mass of protein in crystal = 0.011 mg; estimated residual protein in solution = 0.0022 mg in 2 µl, hence we can estimate the residual protein concentration to be about 1.1 mg ml^{-1}. Although calculations like these are simply an estimate, they can give indications of how well protein is being transferred from the solution to the crystals, and possibly give indications on how the conditions may be improved.

Additive screening

Additives have been used extensively in crystallization. They play very different roles such as counter ions to modify the dielectric constant of the solution, or as surfactants to minimize aggregation (McPherson *et al.*, 1986; their role in membrane protein crystallization is beyond the scope of this discussion). Novel additives, such as electrostatic linkers (Cudney, Patel, Weisgraber, Newhouse & McPherson, 1994) are new developments in the field. Whatever function is assigned to them, it is important to evaluate their effectiveness. While some may bind specifically to the protein, such as phosphate and sulfate ions, and divalent metals at their respective binding sites, others may have a catalytic effect on the crystallization. Some additives may be incorporated specifically into the lattice, while others help mainly by altering the physical or chemical properties of the solution and are used to modify nucleation and crystal growth. In some cases additives may induce large changes in crystallization conditions and have profound effects on the crystallization. Those that substantially alter the solubility properties of the macromolecule are treated as a completely independent crystallization experiment. Here we will only deal with those that have a secondary effect such as altering the crystallization dynamics. We have found that such additives provide a means to fine tune crystallization experiments.

Examples are as follows: (i) in the crystallization of the various steroid complexes of Fab′ DB3 under virtually identical conditions (Stura, Feinstein & Wilson, 1987; Stura, Arevalo *et al.*, 1987), ethanol is used to help increase the solubility in aqueous solution of progesterone and other steroids. [Ethanol can be modeled in the electron density as discussed in Arevalo, Stura, Taussig & Wilson (1993).] Ethanol acts as a modulator, and increases the concentration of precipitant (ammonium sulfate) needed for crystallization. The increase is statistically significant and is within the 5–10% precipitant concentration range which is typically used to bracket the crystallization experiment.

(ii) In the crystallization of mouse MHC-I H-2K^b protein (Stura, Matsumara *et al.*, 1992), the additive, 2-methyl 2,4-pentanediol (MPD), is used to inhibit nucleation and growth of crystals. In this case, MPD affects differentially the nucleation rate and the crystal growth of three (or more) different crystal forms. Nucleation of an undesirable crystal form is suppressed, and crystals of a form that diffracts to high resolution are grown with the use of macroseeding. Different concentrations of MPD favor different crystal forms; 0.3% favors triclinic crystals, while at 1.5% or more, orthorhombic crystals are obtained.

(iii) Additives have been used to promote nucleation, and special attention has been given to the role of PEG–salt mixtures which form coacervate droplets. In such systems, as clearly illustrated photographically in

Ray & Bracker (1986), nucleation can be catalyzed at the phase boundary (see also Ray, 1992). In the PEG-salt system, evaluation of the effect of additives is carried out by first determining the solubility limits for the protein in the salt solution before introducing PEG (0.01% PEG 20 000 may be sufficient; Stura *et al.*, 1987) preferably as part of a comparative study of the effect of organic additives (such as those in Box 3; McPherson, 1992) on protein precipitation. An additive can act as a co-precipitant as in the case of thiocyanate in combination with ammonium sulfate (Diller, Shaw, Stura, Vacquier & Stout, 1994). The effect of additives can be analyzed more quantitatively using a full-factorial screen (Carter, 1990).

Reverse screening using different complexes

Many issues have to be addressed for the crystallization of complexes, including stability and the induction of conformational changes as a result of complexation and how this may affect the heterogeneity of the system. In general, each complex is treated as a separate crystallization trial and the results are compared (see Stura & Wilson, 1992; D'Arcy, 1994, for a discussion of these issues).

Some mutant proteins and complexes may crystallize under very similar conditions (Brennan, Wozniak, Farber & Matthews, 1988) with only minor changes in precipitant concentrations and pH needed to compensate for changes in solubility and charge. In the crystallization of the anti-malaria antibody 4B7, the intact antibody as well as an Fab-fragment complex have been crystallized with many different linear and cyclic peptides (see below and Stura, Kang *et al.*, 1994; Stura, Satterthwait *et al.*, 1994), and although the crystallization conditions remained similar from one peptide complex to the next, the same was not generally true for the chances of nucleation which were found to be very different. Hence, in order to increase the likelihood of nucleation, it may be advantageous to attempt crystallization of many different complexes. We found that solubility information obtained from crystallization trials with one complex was applicable to the crystallization of other complexes. The different Fab-peptide complexes also provided a means to study the effect of single amino-acid changes on crystallization parameters more efficiently than mutating proteins, even with the current molecular biology techniques. We highlight the use of reverse screening and some of the conclusions of this study in the example below.

Example: transmission-blocking anti-malaria antibody 4B7

Biological system

We have used an antibody, which binds to a conformational determinant in a 25 kDa antigen (Pfs25) present on the surface of the zygote and the ookinete stages of the malaria parasite, to test some of the concepts of reverse screening. This antibody, MAb 4B7, recognizes and blocks the action of Pfs25 of the malaria parasite, *Plasmodium falciparum*, as it develops in the midgut of the mosquito (Kaslow *et al.*, 1988). The epitope has been defined as lying within the ILDTSNPVKTGV sequence contained in the third EGF-like domain of the Pfs25 molecule. Cyclic peptides containing this sequence were made by replacing the main-chain amide-carbonyl hydrogen bonds (NH···O=C*R*NH) with a covalent hydrogen-bond mimic (N—N=CHCH$_2$CH$_2$) (Chiang *et al.*, 1991) to improve binding to the antibody. The epitope was modeled on the basis of a predicted β-hairpin in the third EGF-like domain of Pfs25 (Kaslow *et al.*, 1988) from the solution structure of the EGF-like domains derived from NMR studies (Cooke *et al.*, 1987; Kline *et al.*, 1990). Cyclic peptide C2 {sequence: JILDTSNPVKTGVGZG; the peptide is cyclized with a hydrazone link between residues **J**, [N=CH—(CH$_2$)$_3$CO], and **Z**, (NCH$_2$CO)} was designed to mimic the prototypical EGF *B* loop which locates two cystines directly opposite one another at the base of the loop, placing the SNPV sequence located at the apex in a type VI reverse turn (Wilmot & Thornton, 1990). Cyclic peptide C1 (JILDTSNPVKTGZG) places TSNP at the apex in a type VIII turn (Wilmot & Thornton, 1990) as predicted by *GORBTURN* (Wilmot & Thornton, 1988), a program that identifies tetrapeptide sequences with propensities for specific turn types. Both cyclic peptides show significantly higher affinities for 4B7 than the corresponding linear peptides: C1 binds better than C2. Other cyclic peptides have also been included in the study to understand the effect of peptide conformation on antibody binding. These studies provide a means to study crystallization and are important for the development of a vaccine to reduce the transmission of the malaria parasite from the vertebrate host to the mosquito vector. Transmission blocking is important to prevent the proliferation of escape mutants and drug resistant strains. These studies will contribute structural information to the global program for the control of malaria.

Solubility characteristics of IgG 4B7 and Fab 4B7

A footprint screen [consisting of PEG solutions buffered with 0.2 *M* imidazole malate: 14-42% PEG 600 at pH 5.5; 10-25% PEG 4000 at pH 7.0; 7.5-22.5% PEG 10 000 at pH 8.5; and salt solutions: 0.75-2.0 *M* ammonium sulfate, in 0.15 *M* sodium citrate, pH 5.5, mixed 0.8-2.0 *M* Na_2HPO_4 and KH_2PO_4, pH 7.0; and 0.75-1.5 *M* sodium citrate, in 0.01 *M* sodium borate at pH 8.5; see Stura, Nemerow & Wilson (1992) for further details] was the first step in the solubility analysis. The screen was carried out at 10 and 20 mg ml^{-1}

protein concentration. The best precipitants, evaluated according to the amount of precipitate obtained, were citrate and polyethylene glycol. The latter was chosen because polycrystals were obtained with polyethylene glycol for the IgG 4B7-peptide complex L2 (Ac-ILDTSNPVKTGVGGG-NH_2: corresponding to cyclic peptide C2) and for a complex of the Fab with the cyclic peptide C1. 16% PEG (M_r from 2000 to 10 000) was the precipitation limit for most cyclic and linear peptide complexes for both the Fab and IgG. The morphology of the precipitate varies depending on the molecular weight of the PEG. The morphology changes from granular at low molecular weight to a continuous precipitated phase with the higher molecular weight PEGs (8000 and 10 000). The lower limit of solubility was determined by streak seeding with seeds from the polycrystals and was found to be around 10% for most complexes of both the IgG and the Fab. In the second stage, the molecular weight of the PEG was chosen to optimize crystal quality. PEG 4000 was used for the IgG and PEG 8000 for the Fab crystallizations. An additive search was carried out within this range for both the Fab and the IgG. NaCl was found to change the morphology of the precipitate when used in conjunction with polyethylene glycol. Diffraction-quality crystals (to about 5 Å resolution) of the IgG 4B7 were later obtained with several linear peptides, including L2(**H**) Ac-ILDTSNPVKTG**H**GGG-NH_2 (10–11% PEG 4000, 0.2 *M* imidazole malate, 250 m*M* NaCl, 120 m*M* mixed Na_2HPO_4 and KH_2PO_4, pH 5.5–6.5), seeding was used to favor the crystal form most likely to yield X-ray quality crystals. It is interesting to note that many of features of the crystallization of the IgG (Stura, Satterthwait *et al.*, 1994) and the Fab (Stura, Kang *et al.*, 1994) are similar apart from their dependence on additives.

The best data set currently available was collected from cocrystals of the Fab with peptide L2(**H**) crystallized from 12–15% PEG 8000, 0.2 *M* imidazole malate, pH 5.2 to 6.3, with and without 100 m*M* NaCl (Stura, Kang *et al.*, 1994).

In conclusion, for several of the crystal forms observed, the pH optimum for the crystallizations appeared to overlap for the IgG and Fab peptide complexes. Further crystallization studies with other Fab, $F(ab')_2$ and IgG are in progress to determine whether such results are applicable to other immunoglobulins. Such studies are of interest as they may provide a model for the crystallization of fusion proteins. We would like to determine whether a reverse-screening-type search can be applied to the crystallization of the two linked proteins. The crystallization of the different classes of immunoglobulins IgG2a, IgG2b and IgG1 which have different length tethers in the hinge region between the Fc and two Fab moieties may mimic to some extent the crystallization of a protein linked to its fusion partner.

Comparison of the crystallization of complexes with peptides of modified sequence

Cyclic peptides C2 with the exact sequence in Pfs25, C2(**H**) (JILDTSNPVKTG**H**GZG) and C2(**T**) (JILDTSNPVKTG**T**GZG) with histidine and threonine replacements for valine were synthesized specifically for the crystallization studies (residues in bold are not in the sequence of Pfs25). Similar replacements were made in linear peptides. The results of these studies have shown that peptide C2(**H**)-Fab 4B7 complex crystals grow at a 30% lower concentration of PEG than the C2(**T**)-Fab complex. A similar change in solubility was found from studies in the crystallization of an anti-HIV-1 V3 loop Fab with cyclic peptides (Stura *et al.*, in preparation). A decrease in solubility (10% PEG 4000 against 20% needed to grow crystals) was recorded for the replacement of a histidine for a serine. It is encouraging to note that in both cases the histidine-containing peptide complexes showed a decrease in solubility. However, no definite conclusions can be reached at present from such limited examples. The target of the work is to establish a replacement series which can be used to alter protein solubilities to favor crystallization of proteins that are otherwise difficult to crystallize and to understand the extent to which, if any, single amino-acid changes affect the protein solubility, nucleation and crystal growth, and the resolution to which crystals diffract.

Concluding remarks

The crystallization conditions for the Fab 4B7 complexes are by no means unique. Anti-peptide Fab's against influenza virus hemagglutinin (HA) in complex with HA peptides (Stura & Wilson, 1991) share similar crystallization conditions to the Fab 4B7-peptide complexes, and so does the peptide complex of anti-HIV-1 Fab 50.1 (Stura, Stanfield *et al.*, 1992) and others currently under study. The overall range for these crystallizations is from 10 to 24% polyethylene glycol of molecular weights from 2000 to 10 000, and the pH range scans from 5.5 to 8.5. This information is important for the application of reverse screening to the crystallization of Fab's and their complexes.

Among the various complexes, the sC3 (JILDTSNPVKGZG) complex of Fab 4B7 nucleates readily both under the same conditions as all the other complexes and also gives morphologically identical crystals from 0.8 *M* sodium citrate, with 10 m*M* sodium borate buffer, pH 8.5. Crystallization under both these sets of conditions has been observed for other Fab's, such as the anti-tissue factor Fab 5G9 (Ruf *et al.*, 1992; the crystals obtained from the two different precipitants have identical space group and similar

lattice parameters) and the humanized anti-IL-2 receptor (p55) antibody HAT (Stura, Fieser & Wilson, 1993).

The use of the reverse screening can speed up investigations when the number of possible complexes which can be screened becomes too large for a comprehensive screen or with limited supply of macromolecule. In such cases the search is focused on a reduced set of conditions which are more likely to yield crystals. Crystallization potential is tested by cross-seeding if spontaneous nucleation does not occur within a period of time no longer that twice than observed for a self-nucleating complex. Situations where several purified fractions are combined with a set of ligands present the same logistic problem.

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