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The liquid protein phase in crystallization: a case study—intact immunoglobulins

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Abstract

A common observation by protein chemists has been the appearance, for many proteins in aqueous solutions, of oil like droplets, or in more extreme cases the formation of a second oil like phase. These may accompany the formation of precipitate in “salting out” or “salting in” procedures, but more commonly appear in place of any precipitate. Such phase separations also occur, with even greater frequency, in the presence of polymeric precipitants such as polyethyleneglycol (PEG). In general the appearance of a second liquid phase has been taken as indicative of protein aggregation, though an aggregate state distinctly different from that characteristic of amorphous precipitate. While the latter is thought to be composed of linear and branched assemblies, polymers of a sort, the oil phase suggests a more compact, three-dimensional, but fluid state. An important property of an alternate, fluid phase is that it can mediate transitions between other states, for example, between protein molecules free in solution and protein molecules immobilized in amorphous precipitate or crystals. The “liquid protein” phase can be readily observed in many crystallization experiments either prior to the appearance of visible crystals, or directly participating in the crystal growth process. In some cases the relationship between the liquid phase and developing crystals is intimate. Crystals grow directly from the liquid phase, or appear only after the visible formation of the liquid phase. We describe here our experience with a class of macromolecules, immunoglobulins, and particularly IDEC-151, an IgG specific for CD4 on human lymphocytes. This protein has been crystallized from a Jeffamine- LiSO_4 mother liquor and, its crystallization illustrates many of the features associated with the liquid protein, or protein rich phase. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Liquid protein droplets, or larger domains of the liquid protein phase, sometimes coexist in crystallization mother liquors with protein crystals. Fig. 1 illustrates just a few examples. In some cases the relationship between crystals and the

liquid phase is intimate. Crystals appear to grow directly from the liquid phase, or develop only after the formation of a visible liquid phase, which is subsequently consumed as the crystals grow.

In general, the phenomena have been taken as indicative of protein aggregation, though aggregation distinctly different from that yielding amorphous precipitate. While the latter is thought to be linear and branched assemblies, polymers of a sort, the liquid phase is characterized by high protein density, which approaches that of the solid state,

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Fig. 1. Examples of macromolecular crystals associated with, or growing from droplets or oils of a liquid protein phase. In (a) bovine α lactalbumin. Note here the roughening of the surfaces of the droplets and the single cluster of needle crystals in the background at top. (b) cytochrome c from levidura, a microbial parasite, forms leaflike crystals within a vast aqueous-liquid protein matrix. Droplets of the protein rich phase are dark red. (c) Masses of dendritic porcine insulin crystals among droplets of the liquid protein phase. (d) Needle crystals of a human Fab have droplets in direct contact with their surfaces as well as free in the surrounding mother liquor. (e) Crystals of a leaf lectin from the Japanese Pagoda Tree develop from the surface of an oil smear on the glass vessel. (f) Trypsin crystals grow as a mass from an oil phase of the PEG containing mother liquor. (g) Cubic crystals of satellite panicum mosaic virus contain liquid droplet inclusions. (h) Needle crystals of the β subunit of bovine leuteinizing hormone grow in contact with the surfaces of droplets of an alternate phase in the mother liquor.

Fig. 2. Examples of prominent multilayer stacks of growth layers on the surfaces of crystals of (a) and (f) STMV, (b) canavalin, (c) and (d) beef catalase and (e) lysozyme.

by promiscuous, non-specific intermolecular interactions, by molecular mobility within clusters of molecules, and a frequently transient existence.

Biomolecules, such as proteins, nucleic acids, and viruses, are lyophilic (liquid loving) colloids. In concentrated salt solutions, due to screening by ions, long-range electrostatic repulsion is diminished and the macromolecule's behavior is dominated by short-range attractions. As a consequence, phase diagrams with "hidden" gas-liquid phase boundaries, for example, have been delineated for γ -crystallins [1], and for lysozyme [2,3]. This may be pertinent to crystallization, because theoretically, and experimentally it has been shown that the nucleation rate of a crystalline phase occurs more readily in the vicinity of a gas-liquid critical point.

A phenomenon that we have frequently recorded using atomic force microscopy (AFM) was the appearance of large multilayer stacks of

growth steps, and small microcrystals on the surfaces of growing STMV, canavalin, lysozyme, catalase and thaumatin crystals [4–9]. Examples of these are seen in Fig. 2. Multilayer stacks are not crystalline in solution, but appear to arise from volume elements of liquid protein phase that sediment upon and adhere to growing crystal surfaces. Once in contact with a substrate crystal, the liquid phase transforms into crystalline, multilayer stacks of growth steps as directed by the underlying lattice. The multilayer stacks form by ordering of the liquid phase from bottom to top, layer after layer [10,11].

An important question however, continues to be the role of the liquid phase in the initial formation of crystal nuclei in solution in the absence of a preexisting solid, crystalline phase. Nucleation of two-dimensional islands and multilayer stacks on crystalline surfaces does, after all, occur by similar processes and according to the

same principles, as does three-dimensional nucleation in solution.

The liquid protein phase has entered into practical crystallization in another way. Ray first described protein rich droplets [12–14], which he called “coacervate droplets,” that were formed when low concentrations of PEG were mixed into a concentrated protein (phosphoglucomutase) – salt (ammonium sulfate) solution. These droplets, consisting of a PEG-water matrix also contained protein concentrations, as measured by refractometry, of about 400 mg/ml, or about the same protein density as a crystal. He further observed that crystals of the protein appeared to nucleate at the surface (not within) the droplets, and grow *into the protein poor phase* (not inward to the interior of the drop). These “coacervated droplets” of Rays’ are likely similar in character to the liquid protein phase discussed above.

The crystallization of intact immunoglobulins [15–17] provides a good example where the appearance of a liquid protein phase is common. Some examples are seen in Fig. 3. IgG, the major immunoglobulin of serum, appears to share characteristics with membrane and lipophilic proteins, though it is otherwise completely different in its solubility properties. While soluble in saline solutions to high concentrations, it becomes insoluble at low ionic strength. When it emerges from the solution it generally does not do so as a conventional amorphous precipitate, but as “oil droplets”, gums, gels, or a viscous liquid phase. This propensity to aggregate, almost certainly mediated through interactions of Fc segments on different molecules, may be a reflection of its physiological response to antigen binding. Upon antigen triggering, IgG activates complement by forming specific aggregates through Fc interactions [18,19].

We report here an experience with one particular antibody, and preliminary observations with several others, that may suggest approaches to manipulating, or exploiting the liquid protein phase to promote the nucleation of immunoglobulin crystals. The results we report are certainly reminiscent of those from the laboratory of Ray some 15 years ago using an entirely different system. Both sets of observations indicate that

protein rich phases, or liquid protein phases may be produced in solutions of high salt concentration, but modified in character by the inclusion of small amounts of polymers such as PEG, and that these droplets can indeed promote crystal nucleation.

2. Experimental procedure

The monoclonal antibody IDEC-151 (clinical title: clenoliximab) is produced by IDEC Pharmaceutical Company (La Jolla, CA) and has been approved for clinical use in patients with rheumatoid arthritis. The antigen is CD4 on T lymphocytes, and its properties have been reviewed elsewhere [20,21]. IDEC-151 is of isotope IgG4/ λ and is a primatized human immunoglobulin. The variable regions of both the heavy and light chains are derived from monkeys, while the remainders of both the light and heavy chains are of human origin. One particular variant of this antibody is a double mutant in which serine 241 has been replaced by proline, and leucine 248 is substituted by glutamic acid. The purpose of the mutations was to reduce the number of half molecules produced because of interchain disulfide formation in the hinge between C239 and C242.

The antibody was supplied by IDEC Pharmaceutical Company and formulated in phosphate buffered saline at 5 mg/ml. The antibody, prior to crystallization trials was dialyzed for 24 h at room temperature against several changes of distilled water, concentrated in Centricon tubes at 2000 g to a final concentration of 9 mg/ml, centrifuged for 30 min at 10000 g for clarification, and filtered through a 0.22 μ m syringe filter (Millipore Corp., Bedford, Mass.). In later experiments, described below, no dialysis was employed before concentration, clarification and filtering.

Initially, three sets of trials were carried out to crystallize IDEC-151, and these were Crystal Screen, Crystal Screen II, and Low Ionic Strength Screen (Hampton Research, Laguna Nigel, CA). All trials were carried out using sitting drops [22] in Cryschem plates [23]. After a period of 2 weeks, crystals like those seen in Fig. 4 were obtained in sample 16 of Crystal Screen. No crystals in any

Fig. 3. Intact monoclonal antibody crystals growing from, or in association with alternate liquid phases within crystallization samples. The proteins are (a) MK2-23, a murine antibody against human melanoma cells, (b) a human–murine chimeric antibody against human carcinoembryonic antigen, (c)–(e) crystals of a human IgG4 immunoglobulin, and (f) a murine antibody specific for CD20 on human lymphocytes.

other sample were ever observed. Sample 16 is 1.5 M LiSO_4 in 0.1 M HEPES titrated to pH 7.5 with HCl. The crystals were obtained at 18°C but were more stable at 4°C. Attempts were immediately made to optimize the crystallization conditions by composing fine interval matrices to determine optimal (1) LiSO_4 concentration, (2) pH, (3) temperature, and (4) whether LiSO_4 was obligatory by testing other salts as the precipitant by pairing Li with other anions and SO_4 with other cations. No crystals were produced in any

optimization sample. Attempts to reproduce the crystals using an equivalent solution 16, made with identical components to those used by Hampton Research, even after detailed consultation with the company, failed. The crystals, however, could be reproduced if we once again used the original tube 16 from Crystal Screen.

We suspected that tube 16 of Crystal Screen must be contaminated by some component of which we were ignorant. After some reflection, we further concluded that the most likely source of

Fig. 4. In (a), one of the first crystals grown of the human–simian antibody IDEC-151 against CD4 on human lymphocytes. The hexagonal crystal, containing myriad imperfections, is seen amidst a background of alternate phase droplets that form in the concentrated LiSO_4 solution which contains, here, PEG 4000. In (b) are monoclinic crystals of the same antibody, again accompanied by droplets of an alternative phase.

the contamination would be the contents of tube 15, which preceded it in the kit, and in the deployment of samples. Remnants of solution 15 could be carried over into tube 16 if pipette tips were not conscientiously changed during the disposition of samples. Tube 15 of Crystal Screen contains 30% PEG 3350, thus a small carry over of solution 15 could indeed contaminate solution 16 significantly. To test this hypothesis, 1 μl of 10% PEG 3350 in water was added to a number of existing sitting drops deployed in the failed optimization attempts. In some of these intentionally contaminated samples crystals began to appear within 6 h time. Others formed crystals in the following days.

While reproducibility in crystallizing IDEC-151 resulted from the inclusion of small amounts of

Table 1
Initial polymer screen

4 μl	2 μl	4 μl
1.75 M LiSO_4	2.5% 1.25% 0.625%	antibody
1.	PEG 20,000	
2.	PEG 8,000	
3.	PEG 3,350	
4.	PEG 1,000	
5.	PEG 400	
6.	Jeffamine ED 2,000	
7.	Jeffamine ED 4,000	
8.	MPD	

PEG, we were not certain that PEG was the best nucleation promoter, nor of its optimal concentration. To evaluate this question the screen in Table 1 was applied. From this screen, it was quite clear that the best nucleation promoter was not PEG of any size, but Jeffamine ED-2000, and that the optimal concentration in the initial drop was about 0.5%. Lower percentages yielded poorer results while higher concentrations of the Jeffamine resulted in considerable phase separation in the presence of 1.7 M LiSO_4 (this best salt concentration was further determined in the presence of Jeffamine ED2000 in subsequent optimization experiments). Attempts to optimize pH and temperature showed pH to have little influence over two pH units, but 19°C was shown to be the better temperature as far as ultimate crystal size and quality was concerned.

Because we were still uncertain whether Jeffamine ED-2000 was the best nucleation promoter, we expanded the test set as in Table 2. No member of this test set performed as well as Jeffamine ED-2000, though some also induced crystallization. Jeffamine and PEG again emerged as the best promoters. After optimization of all of the available parameters our final drop composition was 4 μl of the 9 mg/ml antibody, 4 μl of 1.5 M LiSO_4 in 0.1 M HEPES, and 2 μl of 2.5% Jeffamine ED-2000, and the crystallization temperature was 19°C.

Two distinct crystal forms of IDEC-151 have been grown and they are illustrated in Fig. 5. Both forms appear under similar conditions and

Table 2
Secondary polymer screen

1.	Mixture of all PEG sizes
2.	PEG 2,000 monostearate
3.	PEG 600 monostearate
4.	Jeffamine ED-600
5.	Jeffamine ED-90
6.	PEG 550 MME
7.	Jeffamine ED-4000
8.	Jeffamine ED-2000
9.	Polyvinylpyrrolidone K15
10.	Polyacrylic Acid 5100
11.	Ultra low Visc. Carboxymethylcellulose
12.	Medium Visc. Carboxymethylcellulose
13.	High Visc. Carboxymethylcellulose
14.	Polyvinyl alcohol 15,000
15.	Polypropylene glycol P400
16.	Polyacrylic Acid 2100

Fig. 5. In (a) a crystal, greater than 1 mm in length, of the hexagonal form of antibody IDEC-151. In (b) the monoclinic form of the same antibody, again greater than 1 mm in the longest dimension.

frequently coexist in the same sample. The hexagonal form is usually earliest to appear, but seems less stable over time. After several weeks the hexagonal crystals tend to show dissolution, the monoclinic crystals, however, show no signs of age even after several months.

Though they will grow at 19°C, the hexagonal form seems favored by lower temperature (2–10°C) and lower LiSO₄ concentrations of 1.5–1.6 M. The monoclinic crystals, on the other hand, grow most reliably at 19°C with reservoir concentrations of 1.65–1.75 M LiSO₄. Neither crystal form appears if PEG or Jeffamine is omitted from the mother liquor.

Crystals of IDEC-151 in many cases, appear before droplets of a second liquid phase are visible by light microscopy. In other instances, crystals appear simultaneous with, or following formation of droplets. Inevitably, however, samples become filled with both crystals (generally only a few) and hundreds of small droplets. As in other examples cited previously, the number of droplets far exceeds, by at least two orders of magnitude, the number of crystals, ultimately formed. In the case of IDEC-151, crystals are not observed to nucleate directly on surfaces of droplets. This in spite of the need for a sufficient concentration of a polymer to produce phase separation. Crystals are also never seen to grow *into* the droplets, but develop entirely from protein on their exteriors.

Because of the success of these conditions with IDEC-151, we sought to determine whether these same, or similar conditions might be successful with other antibodies and Fab fragments. Without going into unnecessary details, attempts were made to apply these conditions to 18 other immunoglobulins or their fragments. From these trials, three other antibodies yielded crystals, only one of which [15] had we previously succeeded in crystallizing. In addition, four different Fab fragments from various antibodies crystallized that had previously not done so. These are listed in Table 3.

3. Results and discussion

The observation that polymers could act to promote crystal nucleation under high salt

Table 3
Anitbodies and fab fragments crystallized

IgG4 – prinitized
IgG1 + antigen (phenobarbital)
MK2–23 Ab
MK2–23 Fab
GH1081 Fab
GH 518.2.30 Fab
GH 464.2.34 Fab

conditions is not novel. Ray [12,14] reported nearly 15 years ago that crystals of phosphoglucosyltransferase from rabbit muscle could only be grown from concentrated ammonium sulfate solutions if 2% PEG was also included. In that case, crystals nucleated at the surfaces of droplets of the PEG rich phase suspended in the salt solution and then proceeded to grow into the salt solution. In this, the most thoroughly studied case, Ray concluded that protein became highly concentrated within the PEG containing droplets to give a protein rich phase, and that these local, supersaturated regions produced nuclei. Further studies suggested (Ray, *perso. discuss.*) that some PEG remaining in solution along with the salt had the effect of increasing the solubility of the protein in the salt phase, which somehow further enhanced crystal growth. Other investigators have also noted the influence of PEG on crystal growth in salt solutions, usually in amounts of about 2%. Some screening matrices, such as Crystal Screen from Hampton Research, for example, have some trial solutions composed of high salt concentrations along with 2% PEG.

It is not clear that what we observe with IDEC-151 is simply another case like that above. First, at least here, the crystallization of the antibodies appears to be specific to LiSO_4 , although more experiments are required to confirm this. Second, we find a pronounced improvement in the promotion of nucleation using Jeffamine ED-2000, and furthermore, within both the PEG and Jeffamine size range, we find a distinct preference for polymers in the range of 2000–4000. Finally, and perhaps most importantly, the optimal concentration of the polymers appears, at least here, to be about an order of magnitude less than previously

used, i.e. around 0.1–0.3%. One conclusion to be drawn from these experiments is that LiSO_4 in the range of 1–2 M appears to provide a good set of conditions for the crystallization of some proteins, particularly antibodies and their fragments, but particularly when a polymeric nucleant is included. That recommended from these results is Jeffamine ED-2000.

Regarding the influence or importance of the liquid protein phase on crystal nucleation, we would like to call attention to the following observations and suggest some tentative conclusions. These tend to call into question, we believe, some ideas currently under consideration.

- (1) Broide et al. [1] demonstrated that a liquid phase of γ globulin, produced by lowering temperature at high protein concentration, was stable. It gave rise, over a long period of time, to neither crystals nor precipitate.
- (2) In the examples of Fig. 1, as well as many other examples we have observed but not shown here, there is either a large volume of the liquid protein phase, or many droplets of that phase, but there are only a very limited number of crystals. That is, most of the liquid phase is not associated with crystals, and the number of crystals is never proportional to the volume of the liquid protein phase.
- (3) Kuznetsov et al. (accompanying paper, these proceedings) show by AFM, that for the protein thaumatin, extremely high protein concentrations enhance growth rate, but do not increase the occurrence of two-dimensional nucleation on surfaces. Other investigations (not published) show this to be true for satellite tobacco mosaic virus crystallization as well.
- (4) Quasi-elastic light studies [24–26] and neutron diffraction studies [27] show for several systems the presence of vast numbers of large clusters and aggregates, likely manifestations of the liquid protein phase separation. The number of these large aggregates, which are ultimately incorporated into crystals, far exceed the number of crystals ultimately formed. That is, most of the large aggregates do not give rise to nuclei.

All of these observations seem somewhat inconsistent with the idea that the liquid protein phase mediates or promotes nucleation; growth yes, but no evidence for nucleation. There is, however, an additional observation which is:

- (5) Crystals are seen to nucleate on the surfaces of the liquid phase droplets, oils, or PEG protein rich droplets, and *they always grow outward* into the exterior (low concentration) protein containing medium, *not inward* into the (high concentration) protein rich phase.

The important point here is that crystals nucleate on the surface of the liquid protein phase, at the interface between the liquid protein and exterior medium. This suggests that perhaps the most important feature of the liquid protein phase is not its high internal protein concentration or its molecular fluidity. Its salient quality, as far as nucleation is concerned, may lie in the structure and physical properties of the interface it forms with other materials or fluids. Indeed, there are numerous examples in the literature of both conventional and macromolecular crystals, where surfaces or interfaces of various sorts provide crucial sites for nucleus formation. The phase boundaries discussed here may be yet another example.

It is easy to see with the unaided eye that 1% Jeffamine 2000 or PEG 4000 alone produces an emulsion of, presumably, polymer rich droplets suspended in a 50% saturated solution of ammonium sulfate or other salts. Ray showed that protein concentrates in these droplets. It may well be, however, that it is the surface of this “quasi” liquid protein phase that is responsible for the nucleation.

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