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# Large Single Crystals of the *Neurospora crassa* Plasma Membrane $H^+$-ATPase: an Approach to the Crystallization of Integral Membrane Proteins

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## Abstract

Large single crystals of the dodecylmaltoside (DDM) complex of a polytopic integral membrane transport protein, the *Neurospora* plasma membrane $H^+$-ATPase, have been obtained using an approach that attempts to take into account the possibly radically different physicochemical properties of the protein surfaces and the detergent micellar collar. The overall goal of the crystallization strategy employed was to identify conditions in which the protein surfaces of the DDM–ATPase complex are moderately insoluble and in which the DDM micellar collar is also near its solubility limit. The first step was to screen a variety of commonly used protein precipitants for those that were able to induce the aggregation of pure DDM micelles. The concentration at which any precipitant induced DDM micellar aggregation was hoped to be close to the concentration at which it might induce insolubility of the detergent micellar collar of the DDM–ATPase complex. Of the nine precipitants tried, seven, all polyethylene glycols (PEGs), were able to induce DDM micelle insolubility. The seven PEGs were then tested for their effect on the solubility of the DDM–ATPase complex at a concentration slightly below that necessary to induce DDM micellar aggregation. Three of the PEGs caused extensive precipitation of the ATPase at this concentration and were, therefore, shelved. The other four PEGs did not induce precipitation at the concentration employed and were subsequently used at this concentration for crystallization trials in which the protein concentration was varied. Encouragingly, crystalline plates of the ATPase were obtained for each of the four PEGs tried, indicating that the overall approach may be valid. Unfortunately, the crystals obtained were visibly flawed, suggesting that the correct balance of protein surface and DDM micelle insolubility had not yet been reached. The ionic strength of the crystallization trials was then raised, which was known from other experiments to render the protein surfaces of the ATPase less soluble while having no effect on the DDM micellar aggregation point. For one of the PEGs, PEG 4000, this brought on a new, well formed hexagonal crystal habit. Subsequent optimization of the initial conditions has yielded large single hexagonal crystals of the $H^+$-ATPase roughly $0.4 \times 0.4 \times 0.15$ mm in size, holding promise for exploration of the structure of the ATPase by X-ray diffraction analysis.

## Introduction

Many of the great remaining secrets in biology at the molecular level of dimensions are kept by integral membrane proteins. While our understanding of many cellular processes involving cytoplasmic proteins has grown in phenomenal fashion over the last few decades, the events that transpire when integral membrane proteins perform their function is still only vaguely understood. Thus, in spite of intense experimental attention over many years, we still know very little regarding the molecular mechanisms catalyzed by membrane transporters, numerous hormone receptors, voltage and ligand-gated ion channels, and a variety of other integral membrane proteins also. The primary reason for the rapid expansion of knowledge regarding soluble proteins is the availability of detailed information about the molecular structures of these proteins. Our lack of understanding of membrane proteins is due to the dearth of such information regarding their structures. Thus, it is generally acknowledged that what is desperately needed for progress toward understanding membrane protein-mediated events is high-resolution structural information about the membrane-bound catalysts.

Considering the size of most integral membrane proteins of interest, it is unlikely that emerging two-dimensional nuclear magnetic resonance techniques are going to provide the necessary structural information in the foreseeable future (Bax, 1989). The only other approaches available in order to obtain this information are electron microscopic and X-ray diffraction analysis of two- and three-dimensional crystals. The recent impressive solution of the general structure of bacteriorhodopsin by electron microscopic techniques (Henderson *et al.*,

1990) offers promise for obtaining structural information from two-dimensional and tubular crystals of other integral membrane proteins, and progress in this regard has been made with several of these (Kühlbrandt, 1992; Toyoshima, Sasabe & Stokes, 1993; Wang, Kühlbrandt, Sarabia & Reithmeier, 1993; Unwin, 1993). However, it is unclear whether or not an adequate level of resolution will be attainable with this approach generally, at least in the near future. Thus, the most powerful approach to obtaining high-resolution structural information for integral membrane proteins remains the technique of X-ray diffraction analysis of three-dimensional crystals, and the paradigm of this approach is the spectacular solution of the structure of the bacterial photosynthetic reaction center (Deisenhofer, Epp, Miki, Huber & Michel, 1985). Unfortunately, although this approach held great promise for membrane proteins early on (Michel, 1983), enthusiasm appears to have waned owing to the subsequent general failure to obtain many other crystals of integral membrane proteins suitable for X-ray diffraction analysis.

With these thoughts in mind, I have been working for several years now on developing procedures for obtaining useful three-dimensional crystals of the integral membrane protein of primary interest in my laboratory, the proton-translocating ATPase from the plasma membrane of *Neurospora crassa*. Although most of the early work along these lines yielded little, recent developments suggest that a rational solution to the problem for the $H^+$-ATPase may be at hand. In this article I describe the experiments that led to the development of a method for growing large single crystals of the $H^+$-ATPase along with the rationale that guided these experiments. It is hoped that what has been learned about crystallizing the $H^+$-ATPase may encourage others to try a similar approach to crystallizing their integral membrane protein of interest. Any success in this regard could contribute to the future development of reliable general guidelines to be followed for crystallizing integral membrane proteins, which would be a most welcome advance in many areas of contemporary biology.

## The problem

The driving force for the crystallization of asymmetric objects such as protein molecules is the minimization of free energy. When a protein solution becomes supersaturated, a fraction of the protein molecules in the solution proportional to the degree of supersaturation is driven toward a solid state, and the free energy of that solid state is at a minimum (maximum probability) when the protein molecules are arranged in a symmetrical and repetitive manner, *i.e.* in a three-dimensional crystalline array. This is in contrast to the normally higher free energy of the solid state when it is present in the form of a disordered amorphous precipitate. The multiple forces that give rise to crystalline ordering are complex and not precisely understood for protein molecules, and for this reason, protein crystallization is largely an empirical process (McPherson, 1982). Nevertheless, the underlying strategy behind virtually all protein crystallizations is the same. By a great variety of different means, the protein in solution is simply brought to a state of limited supersaturation, usually slowly so that precipitation does not occur, and then with sufficient time and some good fortune, the protein molecules will come out of solution in a crystalline form as they seek their solid-state free-energy minimum.

This process has been studied in great depth and placed on a quantitative basis by Feher & Kam (1985). The extremely important and fundamental conclusion that has arisen from this work is that the crystal size and quality are a critical function of the supersaturation ratio, *i.e.* the ratio of the protein concentration in a crystallization trial to the concentration of the protein at its solubility limit. These findings are graphically illustrated in the phase diagram of Fig. 1(*a*), adapted from Feher & Kam (1985). The solubility line delineates protein and precipitant concentrations at which a typical soluble protein remains in solution and above which it comes insoluble. The shaded region above the solubility line indicates protein and precipitant concentrations at which the solution is supersaturated and conducive to crystal growth. The boxes indicate that the crystal size is greatest in the center of the crystallization zone and smaller near the periphery. Above the crystallization zone is the undesirable region of amorphous precipitation. This figure shows clearly, and almost counterintuitively, that large single protein crystals can be obtained at several different protein concentrations and precipitant concentrations, as long as the supersaturation ratio is maintained at a certain critical value. Thus, the attainment of the correct supersaturation ratio would appear to be the primary goal for successful crystallization of most soluble proteins.

In the case of integral membrane proteins, which almost always must be present as a detergent complex, the situation is significantly more complicated. Fig. 1(*b*) attempts to summarize the situation. The diagram at the top depicts a typical detergent complex of an integral membrane protein. The term protein surfaces indicates the region of the molecule normally present in the aqueous media on either side of the membrane. The term detergent micellar collar describes a torus of detergent molecules surrounding and solubilizing the highly hydrophobic region of the

molecule normally embedded in the lipid bilayer. Such complexes are thus chimeras comprising regions with potentially very different physicochemical characteristics. In a manner strictly analogous to that described above for the crystallization of soluble proteins, it is a simple matter to bring solutions of such detergent complexes of integral membrane proteins to a state of supersaturation using protein precipitants, but rarely do crystals ever form when this is tried. Consideration of the phase diagram in Fig. 1(*b*) suggests a reasonable explanation for this common disappointing occurrence. There is no reason to believe that the protein surfaces of integral membrane protein–detergent complexes are any different than the protein surfaces of soluble proteins. Thus, it can be expected that there exists a solubility line, a crystallization zone and an amorphous precipitation region for these surfaces analogous to those depicted in Fig. 1(*a*) for soluble proteins. This solubility line and potential crystallization zone are indicated in Fig. 1(*b*) by the line and shaded area as in Fig. 1(*a*). The amorphous precipitation region is not labeled. By varying the protein and precipitant concentrations, it is likely that the potential protein crystallization zone for the protein surfaces could be found empirically for most membrane protein–detergent complexes. But even if the correct protein and precipitant concentrations necessary to achieve the correct supersaturation ratio to induce crystallization of the protein surfaces of the protein–detergent complex could be found, it is not very likely that the same conditions would induce a suitable level of insolubility of the detergent micellar collar, owing to its usually different physicochemical properties.

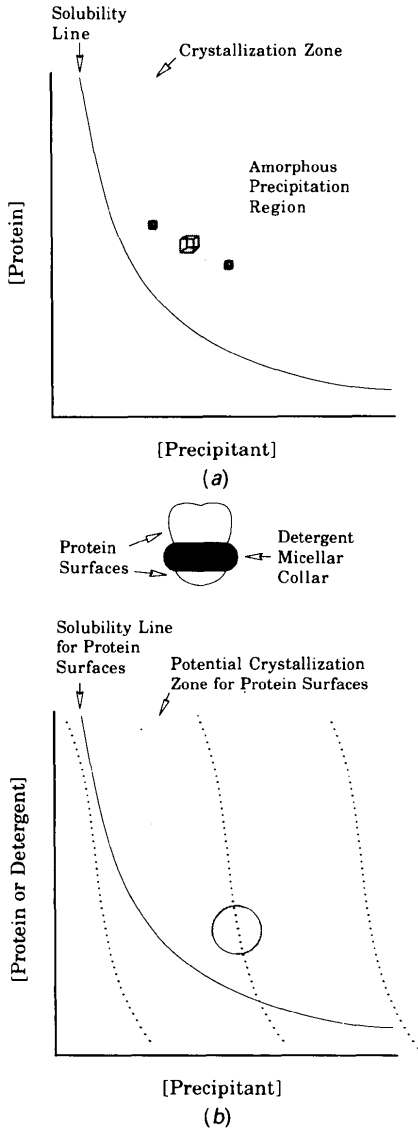



Fig. 1. Hypothetical phase diagrams for the crystallization of soluble proteins (*a*) and integral membrane protein–detergent complexes (*b*). See text for details.

To define the dilemma more explicitly, the dotted lines in Fig. 1(*b*) indicate three hypothetical solubility modes for the detergent micellar collar of a membrane protein–detergent complex as a function of the precipitant concentration. The lines indicate detergent and precipitant concentrations above which the detergent micelles aggregate to form a separate, detergent-rich phase analogous to a proteinaceous solid phase. The shapes of the lines are extremely approximate and can be expected to vary with individual detergent/precipitant systems. Depending on the detergent and precipitant in question, any of the micellar solubility modes depicted in the figure is equally possible, as are infinitely many others. But importantly, for a system with the potential protein surface crystallization zone depicted in the figure, it is likely that neither the detergent micellar solubility mode on the left nor that on the right would be very conducive to crystal formation. That is, for the micellar solubility mode on the left, the detergent micellar collar would be hopelessly insoluble at the protein and precipitant concentrations suitable for crystallization of the protein surfaces. On the other hand, for the micellar solubility mode on the right, the protein surfaces would be in the amorphous precipitation region near the detergent micellar collar solubility line. In order to attain conditions in which both the detergent micellar collar and the protein surfaces are at an acceptable level of insolubility, a detergent/precipitant system with solubility characteristics near that indicated by the dotted line in the center would be required. In this case, there should be precipitant, protein surface and detergent micellar collar concentrations at which crystals of the complex will form. This desired zone in the phase-diagram space is

indicated approximately by the circle in Fig. 1(*b*). Thus, it is proposed that the key to crystallizing membrane protein–detergent complexes is the attainment of conditions in which the protein surfaces are moderately supersaturated and the detergent micellar collar is also at or near its solubility limit so that it is not overly or underly willing to coalesce into a solid state. The fact that the conditions for most successful integral membrane protein crystallizations are near the micellar aggregation point of the detergent used (Garavito & Picot, 1990) supports at least part of this contention.

### An experimental solution

The considerations above suggest that simple attainment of supersaturation using protein precipitants and detergents chosen more or less at random is not likely to be a very successful approach to the crystallization of most integral membrane protein–detergent complexes. Numerous early attempts to crystallize the $H^+$-ATPase using a variety of protein precipitants supported this general notion. With some confidence that the foregoing hypothesis might be valid, it was decided, therefore, to approach the problem more systematically in an attempt to find conditions in which the supersaturation ratios of the protein surfaces of the ATPase and the detergent micellar collar were properly matched. *A priori*, it is obvious that it is impossible to study the solubility properties of the protein surfaces of most polytopic membrane protein–detergent complexes in the absence of their detergent micellar collar, because the two surfaces are inextricably interconnected. However, it is possible to study entities analogous to the detergent micellar collar in the absence of the protein using pure detergent solutions. Although the information obtained from such studies is useful only to the extent that pure detergent micelles reflect the properties of the detergent micellar collar of the protein–detergent complex, the successful outcome of the experiments described herein suggests that it is reasonably safe to operate under this assumption. Earlier stability experiments had indicated that the ATPase is suitably stable, active and soluble when prepared as its dodecylmaltoside (DDM) complex, so the experiments carried out so far have involved only this detergent. These studies, therefore, began with an extensive but relatively easy series of experiments designed to determine the concentrations of several different protein precipitants necessary to induce the aggregation of DDM micelles at several DDM concentrations at 277 K in a weakly buffered, low ionic strength, 30% glycerol solution in which the ATPase has been shown to be quite stable (Hennessey & Scarborough, 1988). The micellar aggregation point or cloud point is readily determined visually by the appearance of turbidity. The precipitants chosen included $(NH_4)_2SO_4$, and polyethylene glycols (PEGs) with average molecular weights of 200 (Aldrich), 400 (Sigma), 600 (Sigma), 600 (Aldrich), 1000 (Sigma), 1000 (Aldrich), 2000 (Fluka) and 4000 (Fluka). The commonly used organic solvent type protein precipitant, 2-methyl-2,4-pentanediol, could not be used as it was shown in preliminary experiments to be deleterious to the ATPase activity. Two of the precipitants tried, $(NH_4)_2SO_4$ and PEG 200, did not induce DDM micellar aggregation over a broad concentration range and were thus shelved. The other seven PEGs did induce DDM micellar aggregation over a range of PEG concentrations. The effective PEGs and the concentration (*w*/*v*) at which they induced DDM micellar aggregation at 5 mg ml$^{-1}$ DDM under the specific conditions of these experiments were as follows: PEG 400, 26%; PEG 600 (Sigma), 19%; PEG 600 (Aldrich), 19%; PEG 1000 (Sigma), 15%; PEG 1000 (Aldrich), 15%; PEG 2000, 15%; PEG 4000, 13%. Interestingly, the micellar aggregation point was relatively insenstitive to the DDM concentration at the concentrations used (2–5 mg ml$^{-1}$) for each of the seven effective PEGs. A more striking dependence of precipitant-induced micellar aggregation on the detergent concentration has been noted by Garavito & Picot (1990) for the detergent $\beta$-octylglucoside.

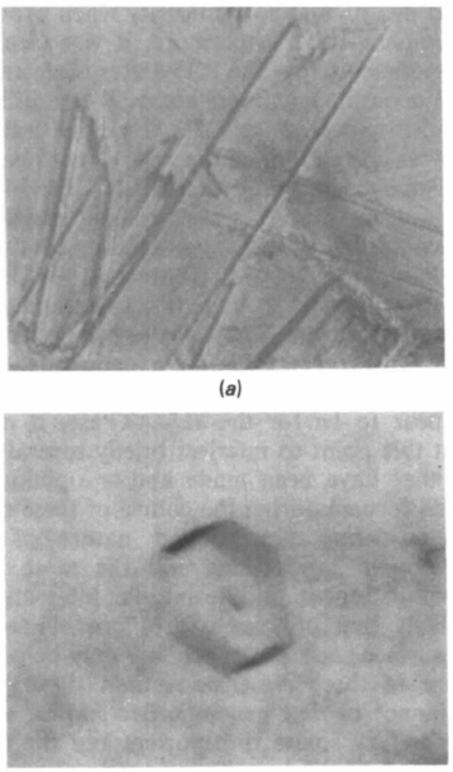
The next step of the process was to determine the approximate solubility of the DDM–ATPase complexes at concentrations of the effective DDM precipitants slightly (2%) below the concentrations at which they induce visible micellar aggregation. The idea was that this may reflect, at least approximately, the solubility of the protein surfaces of the complex because the DDM micellar collar should not be insoluble at these levels of precipitants. These experiments were carried out using a microscale protein solubility assay designed expressly for this study. Aliquots of DDM–ATPase complex solutions (11–22 mg of protein ml$^{-1}$) were mixed with equal volumes of a double-strength solution of each of the seven effective precipitants containing enough DDM to yield a final concentration of 5 mg ml$^{-1}$ in clear plastic microfuge tubes in a final volume of 10 $\mu$l, and the mixtures incubated overnight at 277 K, centrifuged for 15 min at top speed in a microfuge, and the protein concentration of the supernatant fluids determined by a modified Lowry assay which eliminates interference by detergents and precipitants (Bensadoun & Weinstein, 1976). The results indicated that both PEG 1000s elicit extensive amorphous precipitation of the DDM–ATPase complexes at the concentration used leaving a soluble protein concentration of less than 0.3 mg ml$^{-1}$. Thus, referring to the phase diagram of Fig. 1(*b*), for

these two precipitants it seemed unlikely that conditions could be found in which the potential crystallization zone of the protein surfaces and the DDM micellar solubility line could possibly coincide. That is, the potential protein surface crystallization zone below and to the left of the amorphous precipitation region would lie too far to the left of the detergent micellar solubility line as would be the case for the hypothetical detergent micellar solubility line on the right. The PEG 1000s were also shelved, therefore. PEG 600 (Aldrich) also induced amorphous precipitation but left a soluble protein concentation around 2 mg $ml^{-1}$. This PEG may be useful for future experiments but has not been explored further since good crystals rarely grow from solutions with such a low protein concentration (McPherson, 1982).

The other four precipitants, PEG 400, PEG 600 (Sigma), PEG 2000 and PEG 4000 did not induce amorphous precipitation at a concentration 2% below the concentration at which they induce visible DDM micellar aggregation. Therefore, it was considered possible that conditions might be found for these precipitants in which the micellar solubility line and the potential protein surface crystallization zone did coincide, *i.e.* the circle region in the phase diagram of Fig. 1(*b*). Therefore, the next step was to prepare crystallization trials in microdialysis cells (Cambridge Repetition Engineers Ltd, Cambridge, England) with several different concentrations of the DDM–ATPase complexes up to 22 mg of protein per ml and dialysis against solutions of the above four precipitants at concentrations 2% below the concentration at which they induce DDM micellar aggregation. By doing this, the DDM micellar collar was held near its solubility limit as the protein concentration increased, hopefully approaching the potential crystallization zone for the protein surfaces and the circle region of Fig. 1(*b*). Encouragingly, for each of the four PEGs used, crystalline plates were obtained at the higher protein concentrations, lending credence to the overall idea being pursued. Fig. 2(*a*) shows the crystals obtained with PEG 4000; the crystals obtained with the other PEGs are very similar. These crystals can grow to lengths of at least 1 mm. Importantly, the crystals were seen only inside the microdialysis wells and never outside, corroborating their identity as crystals of the DDM–ATPase complexes.

While the crystalline plates obtained are reasonably large in two dimensions, they are quite thin in the third dimension. Even worse, as can readily be seen in Fig. 2(*a*), the crystals are ragged at the edges and of an overall unsatisfactory appearance. These characteristics suggested, if the overall idea was correct, that the correct balance between the insolubility of the protein surfaces and the DDM micellar collar had not yet been achieved. The fact that the formation of these crystals is relatively insensitive to the protein concentration used, suggested that it was the protein surface insolubility that was in need of adjustment. Additional increases in the protein concentration, which might have helped, were impractical, but earlier experiences with the ATPase, not to be reviewed here, had suggested that its protein surfaces might be rendered substantially less soluble by relatively minor increases in the ionic strength of the solution. This was confirmed in microscale protein solubility experiments carried out as described above. Importantly, the DDM micellar aggregation determinations carried out earlier had indicated that $(NH_4)_2SO_4$ has no measurable effect on the DDM micellar solubility. Therefore, crystallization trials were set up similar to those described above but with the addition of 100 m*M* $(NH_4)_2SO_4$, to decrease the solubility of the protein surfaces while holding the DDM micellar collar solubility constant. For PEG 4000, this adjustment brought on the hexagonal crystal habit displayed in Fig. 2(*b*). Numerous subsequent experiments to optimize the size and quality of these crystals have so far yielded well formed hexagonal crystals approximately 0.4 × 0.4 × 0.15 mm in size.

The evidence that the hexagonal crystals are indeed ATPase crystals is convincing. First, the crys-



(*a*)

(*b*)

Fig. 2. (*a*), (*b*) Two crystal habits of the $H^+$-ATPase. See text for details.

tals are never seen outside of the sample well of the microdialysis cells. Second, analyses of washed crystals by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and staining reveals that the ATPase is the only protein present in the crystals and that it is present in about the amount expected if the crystals are roughly half protein and half solvent. Third, and most importantly, preliminary X-ray diffraction analyses indicate that the unit cell of the crystals has a hexagonal space group with its smallest dimension greater than 165 Å, far too large for a small-molecule crystal. All of the evidence thus indicates that the crystals obtained comprise $H^+$-ATPase molecules. Thus, at least in the case of the $H^+$-ATPase, the bimodal solubility problem posed above appears to be realistic, and the experimental approach used to solve this problem has proved to be reasonably effective.

### Additional comments

The next question of obvious interest regards the X-ray diffraction quality of the hexagonal $H^+$-ATPase crystals that have been obtained. Initial efforts to ascertain the diffraction quality of the ATPase crystals at room temperature yielded reflections out to only about 15 Å. However, during these experiments it appeared that the crystals, which are grown at 277 K, may lose integrity when brought to room temperature. Furthermore, it was clear from these experiments that the ATPase crystals are quite fragile. Encouragingly, more recent X-ray diffraction experiments carried out at 277 K yielded reflections to nearly 8 Å, even though the crystals used had been visibly damaged during mounting (D. Madden, W. Kühlbrandt & G. A. Scarborough, unpublished experiments). Thus, there is good reason to hope that the ATPase crystals will eventually be found to diffract well beyond 8 Å with conventional X-ray diffraction equipment. They may also be good candidates for synchrotron radiation experiments.

If the ideas proposed in this paper are valid, as they appear to be for the $H^+$-ATPase, it may be useful at this point to interject briefly several observations that have been made and/or opinions that have been formed during the course of these studies. First, considering the pointed nature of many protein–protein interactions and the more diffuse nature of detergent micelle–micelle interactions, it seems likely that proteins with a predominance of protein surface such as the ATPase may be easier to crystallize in three dimensions than those with a predominance of detergent micellar surface such as most secondary solute transporters and the numerous members of the bacteriorhodopsin/$\beta$-adrenergic receptor family. This point has been made earlier by others (Kühlbrandt, 1988; Garavito & Picot, 1990). Possibly the attachment of more water-soluble proteins to the non-membrane surfaces of proteins with limited extramembranous mass by gene fusion or immunological techniques could render them more crystallizable by the approach described in this paper. Second, the studies described herein were limited to considerations of the detergent DDM because of its special compatibility with the ATPase. For other membrane proteins with less rigorous detergent requirements, the availability of multiple safe detergents would add additional dimensions of variable space for exploration. It has also been noted in these studies that excess DDM micelles may poison the ATPase crystallization process. It thus may not be good to work with much more detergent than is necessary to saturate the protein of interest. Binding studies to determine the stoichiometry of detergent binding would, therefore, appear to be advisable. Third, as the amount of PEG 4000 is increased in 0.5% increments in the region of ATPase crystallization, the number of crystals increases dramatically and the size decreases accordingly. Therefore, it appears that ATPase crystal nucleation and growth are reciprocally related as is often the case for soluble proteins. The difference for the ATPase is that there is a more drastic transition over a very small range of PEG 4000 concentrations than is usually seen for soluble proteins crystallized with PEGs (McPherson, 1982). This may be related to the rather sharp dependence of the DDM micellar aggregation point on the PEG concentration noted in the micellar aggregation point determinations described above, which actually looks something like that depicted by the dotted lines in Fig. 1(*b*). Fourth, if during preliminary detergent micellar aggregation point determinations, precipitants are found that do not induce aggregation, such precipitants may be useful in later experiments in combination with another precipitant to modulate the solubility of the protein surfaces without affecting the detergent micellar solubility. The idea of using mixtures of precipitants to selectively modulate the solubilities of the protein surfaces and the detergent micellar collars seems attractive, but complicated. Fifth, although the ATPase was crystallized following the general plan described above, the final adjustment into the quality crystallization zone was made by taking advantage of ATPase solubility properties learned during the development of the ATPase solubilization and purification procedure. Thus, while this approach may be a useful general guideline for crystallizing a new membrane protein, the details are bound to vary extensively. For this reason, the more that is known about the protein of interest at the outset, the better the chances for crystallization success. And finally, it must be emphasized if it is not already obvious that the assumptions made herein

about the solubilities of the protein surfaces and detergent micellar collars of integral membrane protein–detergent complexes are so simplifying that they are extremely approximate, at best. They did, however, directly lead to the development of a method for crystallizing the ATPase and, therefore, would seem to be reliable enough to be useful until a more formal treatment of the problem is developed.

## Concluding remarks

In conclusion, a hypothesis regarding a general approach to the crystallization of integral membrane proteins has been proposed. The central idea is that it should be possible to crystallize the detergent complexes of integral membrane proteins if conditions can be found in which the protein surfaces of the complexes are moderately insoluble and in which the detergent micellar collar is also near its solubility limit. With this idea as a general guideline, a reasonably systematic procedure for finding such conditions for the DDM complex of the *Neurospora crassa* plasma membrane $H^+$-ATPase was devised, and it yielded large, single crystals of this transport enzyme. Considering the widely experienced difficulties in obtaining crystals of integral membrane proteins, this success with the $H^+$-ATPase suggests that the formulation of the problem described herein and its experimental solution may prove to be useful for the crystallization of other integral membrane proteins also.

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