Improved Protein Crystal Detection in Detergent and Lipidic Meso-Phases

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Abstract: Texas Red® dyes were used to partially label proteins for crystallization in both detergent and lipidic meso-phases. Fluorescence detection of Texas Red® can then be used to differentiate the protein crystals from salt crystals and other phase separations in the crystallization drop. Whereas ultraviolet light absorption and fluorescence of protein crystals in lipidic meso phase crystallization trials using glass sandwich plates was difficult to discern, the fluorescence of the partially labeled protein can be used to distinguish protein crystals. With as little as 0.05% Texas Red labeling of the protein, protein crystals showed up very clearly in both detergent and the lipid meso-phase crystallization setups.

Keywords: Protein, crystallization, crystal detection, fluorescent labeling, lipidic meso-phases.

INTRODUCTION

Membrane proteins reside in highly insulating phospholipid bilayers and mediate vital cell physiology such as solute transport, charge separation, conversion of energy, as well as signal transduction. Little is known about the structural basis of such proteins since the number of membrane protein structures is significantly lower than that of soluble protein structures [1-4]. Although most membrane proteins structures have been determined *via* X-ray crystallography, the major bottleneck is obtaining well-diffracting membrane protein crystals [5, 6]. Membrane proteins have been crystallized in bicelles [7, 8], detergent [5], vesicle [9], lipidic meso [6, 10-12], and lipidic sponge phases [13]. Typically the most challenging problem in evaluating membrane protein crystallization setups is being able to distinguish protein crystals from other phases (i.e. salts, etc.) in the crystallization milieu. This problem is complicated by the addition of non-protein components required in membrane protein crystallization such as lipids, detergents, and other additives. Such challenges can be also present for soluble proteins that are in buffers that contain detergents and lipids.

Typically protein crystals can be differentiated from other phases by using protein specific dyes such as IZIT (Hampton Research: Aliso Viejo, CA., USA) [14], cross polarization [15], ultraviolet light absorption [16] and protein UV fluorescence [17]. Protein UV fluorescence or intrinsic UV fluorescence refers primarily to the ultraviolet fluorescent properties of tryptophan which is present in most proteins. However, these methods are difficult to perform in the presence of detergents and lipids that are found in membrane protein crystallization experiments. The protein staining dyes such as IZIT do not distinguish protein crystals from the inhomogeneous phase separations of detergent and lipid phases during crystallization experiments. Likewise, the presence of different detergent and lipid phases induced during protein crystallization can make resolution of protein and non-protein crystal detection using cross polarization extremely problematic. The use of detergents such as TritonX100 and Emulgen 911 have aromatic functional groups that can interfere with UV absorption detection [18]. Some proteins have weak intrinsic UV fluorescence. The glass plates that are used in the sandwich drop lipidic meso-phase crystal screens [19] can reduce the effectiveness of protein UV absorption and fluorescence because of the optical properties of the glass.

Soluble proteins can be labeled covalently and noncovalently with fluorescent dyes and used in conventional protein crystallization trials [20-22]. We adapt this method to label proteins in the detergent and lipid phases in order to robustly differentiate protein crystals from salt crystals, detergent crystals, and detergent/lipid phase transitions. The combination of intrinsic UV fluorescence alongside with detection of the fluorescent dye labeled proteins resulted in better resolution of protein crystals from other phases in different protein crystallization experiments that contain detergent, lipids, and precipitants.

MATERIALS AND METHODS

Hen egg white lysozyme purchased from Sigma Chemicals (L-6876, St. Louis, MO., USA.) 11-beta-hydroxysteroid dehydrogenase type 1 (11-β-HSD-1) was prepared for crystallization as noted previously [23, 24]. Texas Red® C_2 Maleimide, and Texas Red®-X succinimidyl ester dye were purchased from Invitrogen Corporation (Carlsbad, CA., USA). Monoolein was purchased from NuChek prep, Inc. (M-239; Elysian, MN., USA.) Pall Microsep 10,000 MW Omega filters (Pall Corporation; Ann Arbor, MI, USA) were used for protein concentration. Bio-Rad 10DG desalting columns (Bio-Rad Laboratories; Hercules, CA., USA) were used to separate unbound dye from protein. Absorbance readings were taken using a NanoDrop® ND-1000 spectrophotometer (NanoDropTechnologies; Wilmington, DE., USA) with a 0.10 cm cell path length. All protein buffers and crystallization solutions were

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purchased from Hampton Research (Aliso Viejo, CA., USA).

Protein Labeling and Preparation

Lysozyme was dissolved in buffer A (2.5 mM HEPES [4(2-hydroxyethyl)-1-piperazineethanesulfonic acid] pH 7.5) to make a 5 mg/mL solution. Texas Red®-X succinimidyl ester dye was dissolved in dimethylsulfoxide to make a 0.01 M solution. An equimolar ratio of dye (8.75 µL of the 0.01 M Texas Red® X Succinimidyl ester stock) was added to 1.0 mL of the 5mg/mL lysozyme solution and allowed to incubate at 4 °C. The reaction was covered with aluminum foil to protect the reaction from the light. After 18 hours, the reaction was quenched with 20 µL neat ethanolamine.

11-β-HSD-1 at 14mg/mL was diluted from 100 µL to 1 mL with buffer B (20mM *Tris*[*tris*(hydroxymethyl)amino-methane] pH 8.0, 5 % w/v glycerol, 2 mM TCEP (*Tris*(2-carboxyethyl)phosphine), 0.5% w/v Triton X100). The diluted protein was then mixed with 2 µL 0.01M Texas Red® C2-maleimide in dimethylsulfoxide at 4 °C. The reaction was covered in aluminum foil to protect it from the light for 1 h at room temperature. The reaction was quenched with 1 µL neat ethanolamine.

After labeling, the samples were loaded onto desalting columns at 4 °C and equilibrated respectively with buffer A for lysozyme and buffer B for 11-β-HSD-1. The void volume containing dye-labeled protein was collected and concentrated at 6480g at 4 °C. Final concentration of labeled 11-β-HSD-1 was 3.5 mg/mL and labeled lysozyme was 22.7 mg/mL.

Crystallization

Crystallization of 11-β-HSD-1 was conducted using 83.3% (v/v) unlabeled protein at 14.0 mg/mL, and 16.7% (v/v) labeled protein at 3.5 mg/mL for a protein sample with a final concentration of 12.25 mg/mL. This solution of protein required a high amount of detergent (100 fold higher than the critical micellar concentration of TritonX100 in water) for purification. Crystals of 11-β-HSD-1 were grown at 17 °C in hanging drop vapor diffusion method [23]. Crystallization drops were 2 µL protein + 2 µL well solution. Well solution (1 mL) was 14% (w/v) PEG3350, 0.1M *Bis-Tris* pH 5.6. Crystals grew overnight.

Lysozyme crystals were grown in the lipidic meso-phase using the sandwich drop method [19]. The lysozyme sample consisted of 1%(v/v) labeled lysozyme at 22.7mg/mL, and 99%(v/v) unlabeled lysozyme at 50mg/mL, for a final concentration of 49.7mg/mL. The doped lysozyme sample was added to molten monoolein for a final ratio of 35% (w/v) lysozyme solution to 65% (w/v) monoolein. Labeled lysozyme in the lipidic meso-phase was loaded into a lipidic cubic phase dispensing robot (patent US 688727B2). The sandwich drop plates were made by adding 200nL of the lysozyme in the lipidic meso-phase to each well on a 96 well glass plate with wells defined by a double-sided sticky middle layer (adhesive 96 well template tape). Then 600nL of lysozyme crystallization solution (0.5M sodium chloride, 0.1M sodium acetate pH 4.8) was added over each cubic phase drop. A layer of glass was placed on top with the sticky middle layer sealing each well completing the sandwich plate.

The plates were initially viewed in visible light to look for possible crystals on a UV detecting microscope (patent US 20060215156 A1). The crystals were then examined for protein UV fluorescence [17]. Finally the crystals were examined for Texas Red® fluorescence using 540nm excitation with a 0546/12 excitation filter (Newport Corp./ Corion; Franklin, MA, USA) and 610 nm emission with a HQ610/75M emission filter (Chroma Technology; Rockingham, VT., USA).

RESULTS AND DISCUSSION

The levels of fluorescent dye labeling can vary for proteins depending on access of reactive amine and thiol containing amino acid residues in different buffer and solution conditions. Hence quantification of the fluorescent dye bound to the protein was required and was determined using optical absorption measurements at 280 nm and 594 nm. Proteins typically have an absorbance peak at 280nm that can be used to calculate protein concentration. Free Texas Red® dye has absorbance maxima at 269nm and 594nm. The absorbance peak of Texas Red® at 269nm caused an additive effect with the absorbance of proteins at 280nm. To deconvolute this effect, the quotient of Texas Red absorbance (Abs 280 nm / Abs 594nm = 0.30) was determined for Texas Red® X-succinimidyl ester reacted with spermine. This conjugate kept Texas Red® dye in solution but did not interfere with the absorbance readings. The concentration of Texas Red® in solution can be calculated using its extinction coefficient at 594nm, 1.12×10^5 $M^{-1}cm^{-1}$. By knowing the absorbance of Texas Red® at 594nm and the absorbance ratio (0.30), the contribution of Texas Red® at 280nm was determined. By subtracting the effect of Texas Red absorbance at 280nm from the total absorbance at 280nm, the protein concentration was calculated. Labeling efficiency was equal to the concentration of Texas Red® divided by the concentration of protein.

These calculations were based on the ability to remove unbound Texas Red® dye from Texas Red® bound to protein. The labeling efficiency of lysozyme with Texas Red® succinimidyl ester dye was calculated to be 11.3%. The concentrated labeled lysozyme was doped into unlabeled lysozyme to make the sample used for crystallization. A range of doping from 0.01% to 0.1% of lysozyme labeled with Texas Red® succinimidyl ester dye was crystallized and tested for sensitivity of detection. Fluorescence measurements with 300 ms exposures could be obtained with as low as 0.05% Texas Red® labeled lysozyme.

The percentage of labeled 11-β-HSD-1 was calculated to be 10%, before doping into unlabeled protein for crystallization trials, but was difficult to determine accurately. The presence of Triton X100 in the protein buffer resulted in some of the unbound dye remaining in the sample after running it through a desalting column. Therefore the concentration of Texas Red® in the sample was not an accurate reflection of Texas Red® bound to 11-β-HSD-1. Nonetheless, the calculations can provide an upper boundary of the potential protein-dye labeling efficiency.

Fig. (1). Crystals of 11-β-HSD-1 doped with Texas Red ® C_2-maleimide viewed under conditions of (a) visible light, (b) protein UV fluorescence, and (c) Texas Red ® fluorescence.

Fig. (**1**) illustrated the case where the protein crystals exhibited little or no protein UV fluorescence. In the case of 11-β-HSD-1, the lack of strong protein UV fluorescence could be due to absorbance of the excitation light by the Triton X100 or weak intrinsic fluorescence of the protein. With the weak protein UV fluorescence, these crystals could be overlooked. However, the Texas Red® fluorescence from the doped 11-β-HSD-1 crystals was clearly visible in the presence of the other phases.

The fluorescent labeling technique was also useful in distinguishing protein crystals in the lipidic meso-phase where the glass from the sandwich drop plates diminished the intrinsic UV fluorescence. Using the sandwich drop method, crystals can be seen in visible light without obstruction from the irregularities caused by the application of the protein containing lipidic meso-phase onto a given surface [25]. As illustrated in Fig. (**2**), the lysozyme crystals were easily seen in visible light. Under protein UV fluorescence conditions, the glass from the plates reduced the protein UV fluorescence. The Texas Red® fluorescence was brighter and was less affected by the glass plates. Like the labeled 11-β-HSD-1 crystals in Fig. (**1**), the labeled lysozyme crystals in Fig. (**2**) were easily distinguishable as protein crystals by using Texas Red® fluorescence.

The fluorescent labeling technique was also useful in distinguishing protein crystals from salt crystals, and detergent-lipid phase transitions in the lipidic meso-phase. Fig. (**3**) illustrated crystals, precipitates, and other phase

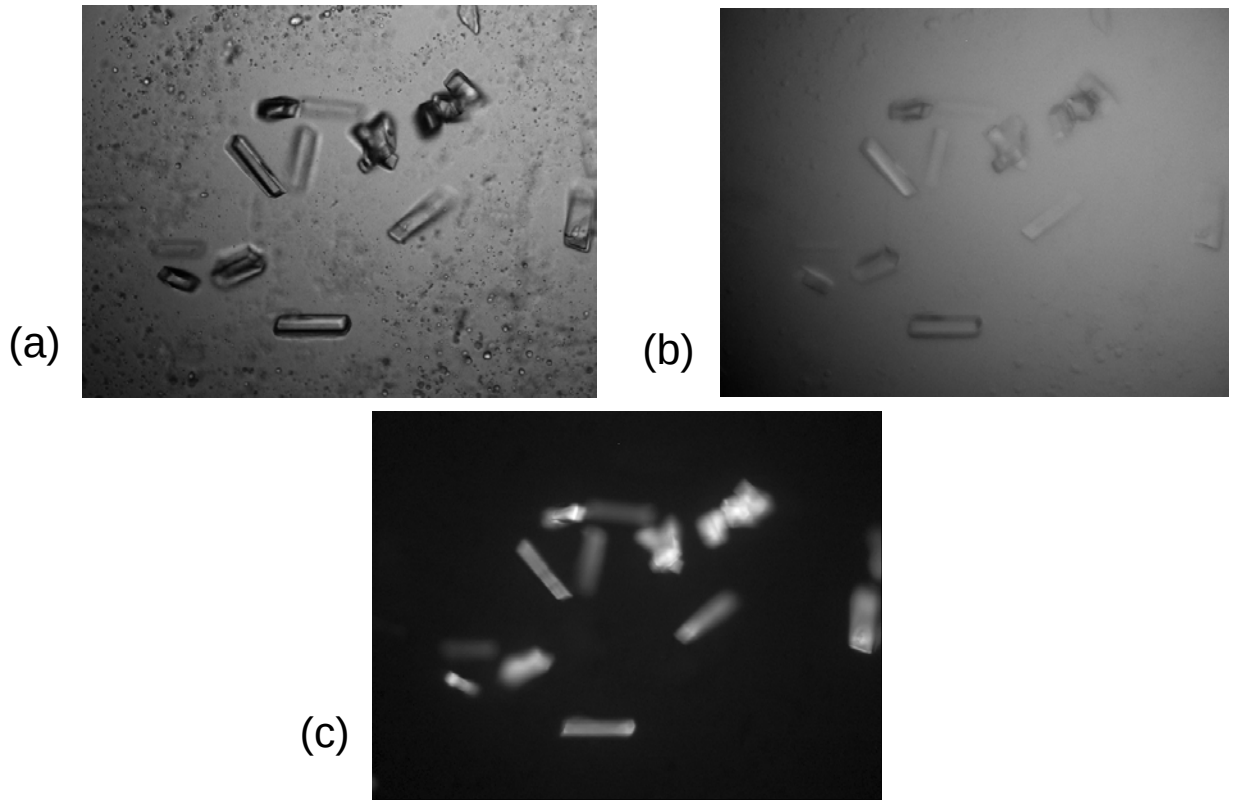

Fig. (2). Lysozyme labeled with Texas Red ®-X succinimidyl ester in the lipidic meso-phase glass plate-sandwich drop format viewed under conditions of (a) visible light, (b) protein UV fluorescence, and (c) Texas Red® fluorescence.

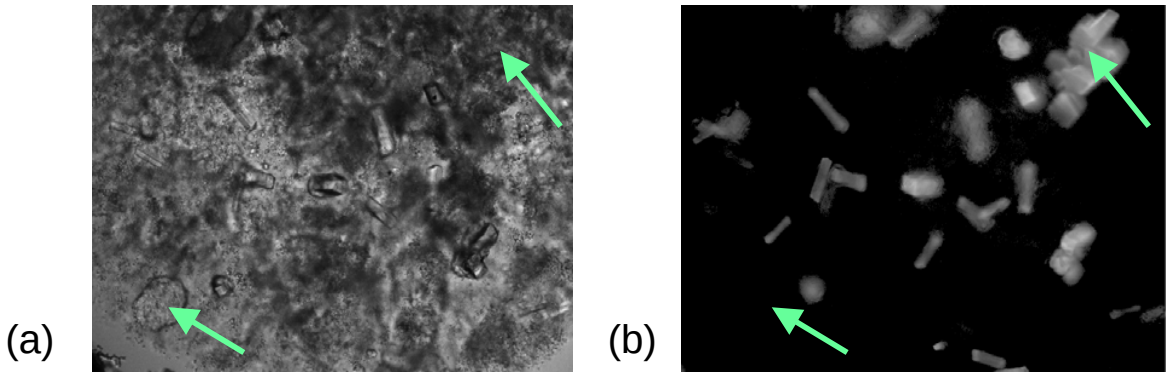

Fig. (3). Lysozyme labeled with Texas Red ®-X succinimidyl ester in lipidic meso-phase glass plate-sandwich drop format viewed under (a) visible light and (b) viewed at Texas Red ® fluorescence conditions. Monoolein phase change in lower left hand corner did not fluoresce under Texas Red® fluorescence conditions. Crystals in upper right hand corner were not visible through protein precipitate, but were visible in Texas Red® fluorescence conditions.

changes in the crystallization drop. It was unclear in visible light whether any of these objects contained protein. Protein UV fluorescence could be hampered by the glass used in the sandwich drop format, as shown in Fig. (**2**). When viewed using Texas Red® fluorescence, the labeled crystals were easily distinguished from non-protein crystals and phases. The objects in the lower left hand corner, which did not show any fluorescence under these conditions, were ruled out as protein crystals. The Texas Red® labeled lysozyme fluorescence also helped us distinguish protein crystals through protein precipitate, as seen in the upper right hand corner. These crystals were not noticeable in visible light through all of the precipitate. However with Texas Red® fluorescence, the labeled lysozyme crystals stood out clearly.

Fluorescent labeling of proteins is an easy and robust method to enhance protein crystal detection and is applicable to both soluble and membrane proteins. This method is especially useful with lipidic meso-phase glass sandwich drop plates, which diminish the use of protein UV fluorescence detection. This also provides a potential avenue for automated protein crystal detection in both vapor diffusion and lipid meso-phase crystallization formats. This method relies on the use of low level doping of unlabeled protein. Although high resolution diffraction quality lysozyme crystals could be obtained with <10% labeling with Texas Red, it can be possible that the presence of the doped protein could prevent the formation of x-ray diffracting crystals. Thus to minimize any of these effects due to Texas Red labeling, the amount of Texas Red labeled protein added to the unlabeled protein can be as low as 0.05%. Alternative fluorescent dyes could be used especially if the protein of interest has spectral properties that could interfere with dye detection. Fluorescent labeling also holds potential use in other crystallization formats that have yet to be explored.

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