Published in final edited form as:

Cryst Growth Des. 2008 ; 8(1): 347–350. doi:10.1021/cg0703745.

Selection of Protein Crystal Forms Facilitated by Polymer-Induced Heteronucleation

Adam L. Grzesiak and Adam J. Matzger

Department of Chemistry and the Macromolecular Science and Engineering Program, The University of Michigan, Ann Arbor, Michigan 48109-1055

Abstract

Crystallization of biological macromolecules as high quality single crystals is critical for determining their structure and facilitates the rational design of drugs. Because macromolecules often crystallize in multiple phases that have unique diffraction properties, the selective production of phases is desirable. Furthermore, determining multiple structures allows for a greater understanding of the relationship between crystal packing and conformation. With the aim of exploiting the polymer-induced heteronucleation approach to selectively nucleate multiple macromolecule crystal forms, hen egg white lysozyme (HEWL) was chosen as a model. Selective phase production was achieved under conditions that, in the absence of added heteronuclei, result in crystallization of a single crystal form. Moreover, nucleation rate, which in turn affects the size and quality of HEWL crystals, was controlled by various polymer surfaces. Thus, the polymer-induced heteronucleation approach provides an additional diversity element which can be easily implemented to complement standard crystal growth techniques for the selective production of high quality protein crystals.

Introduction

The determination of protein, nucleic acid, and virus structure by X-ray crystallography, perhaps more than any other method, has led to significant understanding of the structure and function of biological macromolecules.^{1,2} Furthermore, the rational design of drugs targeting specific sites within a protein requires structural information.² However, several substantial hurdles still exist in obtaining a data set of quality sufficient for structure determination, the most important of which is the formation of the crystal itself. Commercial implementation of a variety of empirical screens^{3,4} has led to a substantial increase in the number of quality crystals obtained. These screening kits offer a multitude of crystallization conditions by varying salts, buffers, and precipitants in a format that is compatible with small quantities of sample. Screening methods, however, do not always lead to suitable crystals, but salt/buffer/precipitant conditions may be identified yielding microcrystals that, under slightly different conditions, may grow to larger single crystals suitable for structure determination.²

It is often observed that multiple crystal forms can arise from subtle changes in crystallization conditions.² In the case of macromolecules, this can mean the difference between obtaining a high quality structure or only a unit cell determination. For example, one form may not be amenable for structure determination because it crystallizes in a low-symmetry space group or with more than one molecule in the asymmetric unit thus requiring significantly more data to

Adam J. Matzger Department of Chemistry 930 N. University University of Michigan Ann Arbor, MI 48109-1055 Phone: (734)-615-6627 FAX: (734)-615-8553 E-mail: matzger@umich.edu.

Supporting Information Available. Tables of crystallization outcomes from the tetragonal-, triclinic-, monoclinic-, and orthorhombic-inducing conditions in the presence of libraries A-C. This material is available free of charge via the Internet at <http://pubs.acs.org>.

accurately describe the structure. An additional motivation for controlling form production in proteins arises from the observation that each phase has a characteristic morphology and a given phase may be better suited for diffraction studies because it grows with more favorable dimensions. Moreover, determining the structures of multiple phases allows one to observe changes in molecular conformation that cannot be assessed with a single structure. For these reasons developing methodology that can yield multiple crystal forms of macromolecules is highly desirable. Approaching this problem at the level of nucleation is attractive because it is at this stage that crystal form is typically determined.

Because the barrier to homogeneous nucleation is relatively large, most laboratory crystallizations proceed by heterogeneous nucleation, the process by which the surface of a foreign substance lowers the energy barrier for aggregation of molecules and induces nucleation.⁵ Controlling the process of heterogeneous nucleation in a reproducible manner for the selective production of multiple crystal forms can be accomplished by creating libraries of surfaces bearing a variety of functionalities and compositions; this is readily achieved using polymers.^{6,7} We have shown that this technique of polymer-induced heteronucleation is successful in the selection and discovery of crystal phases of organic molecules,⁶⁻⁸ inorganic complexes⁹ and extended networks.¹⁰ However, protein crystallization is typically performed on a scale associated with homogenous nucleation and much care is taken to avoid contaminants in solution. The challenge, then, is to affect nucleation behavior of proteins to control crystal form based on heteronucleation from the surface in contact with the protein solution. Heterogeneous nucleation of proteins has been explored with minerals,^{11,12} human hair,¹³ polymers,¹⁴⁻¹⁶ porous glass and silicon,^{17,18} silicon-based devices,¹⁹ and chemically treated glass surfaces²⁰ to facilitate nucleation and even orient crystal growth through a heterogeneous pathway, but high throughput screening based on altering the heteronucleus surface chemistry is unexplored. Therefore, polymer-induced heteronucleation is an ideal approach to investigate the nucleation behavior of proteins in a parallel fashion.

Hen egg white lysozyme (HEWL), the most widely studied crystalline protein, has five structurally characterized phases.²¹⁻²⁵ However, these five forms should not be considered polymorphs, and indeed the term polymorph should not generally be applied to proteins, because each has a unique composition arising from variable water content and buffer components. HEWL is employed here as a model system because it presents an opportunity to selectively crystallize its multiple phases from a given solution and to readily monitor the nucleation behavior of each phase. HEWL was crystallized by different methods (see Experimental Section) reported in the literature to afford pure samples of tetragonal,^{26,27} orthorhombic,²⁸ monoclinic,^{26,27} and triclinic²⁹ crystal forms. Each of these was identified by morphology (Figure 1) and unit cell determination.³⁰ These crystallization procedures differ in protein concentration, salt, and/or temperature and each condition was employed in the presence of diverse libraries of insoluble polymers (see Experimental Section) to examine the effect polymer heteronuclei have on the nucleation of multiple crystal forms of HEWL and their rates of nucleation.

Experimental Section

Polymer Synthesis

Cross-linked polymer libraries were prepared by a previously described method.^{7,9} Monomer solutions in ethanol (1:1) were combined pairwise in six ratios (86:14, 71:29, 57:43, 43:57, 29:71, 14:86) in 96 well polypropylene plates. Thus, 90 pairwise monomer mixtures and 6 pure monomer solutions were prepared each with a volume of 100 μ L. To these 96 solutions was added 50 μ L of a 1:1 (v:v) solution of the cross-linker divinylbenzene (DVB) in ethanol containing 2 mol% azobisisobutyronitrile (AIBN) with respect to DVB as initiator. The plates were subsequently flushed with nitrogen gas and irradiated for three hours with an array of

four 15 watt UVA lamps to yield random cross-linked polymers. Following irradiation, the polymers were annealed at 85 °C in a vacuum oven (<1 Torr) for four hours to remove residual solvent, monomer, and short oligomers. Composition of library A: 4-acetoxystyrene (AOS), *n*-butyl methacrylate (*n*-BuMA), *t*-butyl methacrylate (*t*-BuMA), benzyl methacrylate (BzMA), styrene (STY), and 2,2,3,3-tetrafluoropropyl methacrylate (TFPMA) with DVB; library B: 2-vinylpyridine (2VP), 4-vinylpyridine (4VP), 2-dimethylaminoethyl methacrylate (DMAEMA), *N,N*-dimethylmethacrylamide (DMMAA), methacrylonitrile (MAN), and 2-methyl-2-nitropropylmethacrylate (MNPMA) with DVB; library C: acrylic acid (AA), 2-ethoxyethyl methacrylate (EEMA), ethylene glycol methacrylate phosphate (EGMAP), 2-hydroxyethyl methacrylate (HEMA), methacrylic acid (MAA), methyl methacrylate (MMA) with DVB.

Lysozyme Crystallization

Tetragonal HEWL was crystallized from 20 mg/mL protein solutions containing 0.1 M acetate buffer at pH 4.5 and 50 mg/mL NaCl.^{26,27} Crystallization occurred over the period of hours to days at room temperature. Orthorhombic HEWL was crystallized at 40 °C from a 200 mg/mL protein solution dissolved in 50 mM acetate buffer at pH 4.5 with 25 mg/mL NaCl.²⁸ Monoclinic HEWL was crystallized from 30 mg/mL protein solutions containing 0.05 M acetate buffer at pH 4.5 and 20 mg/mL NaNO₃.^{26,27} Crystallization of monoclinic HEWL occurred at room temperature after a couple of days. Triclinic HEWL was prepared by cooling solutions of 10 mg/mL protein, 0.05 M acetate buffer at pH 4.5 and 20 mg/mL NaNO₃ to 4 °C for 6 hours, by which time protein precipitates as microcrystalline aggregates, and then the solution is warmed to room temperature and large triclinic single crystals form within a day.²⁹ All solutions were filtered through a 0.2 µm syringe filter and were dispensed as 5-10 µL drops onto each polymer and each well was sealed to prevent evaporation. All crystallizations were performed with six ratios of comonomer pairs and each of these was conducted in duplicate to gauge reproducibility (see Supporting Information).

Raman Spectroscopy

Raman spectra were obtained using a Renishaw inVia Raman spectrometer equipped with a Leica microscope, RenCam CCD detector, 785 nm diode laser or 633 nm HeNe laser, and 1200 lines/mm grating. Spectra were collected and analyzed using the WiRE 2.0 software package. Calibration was performed using a silicon standard. Spectra were typically collected using an Olympus SLMPlan 20x objective (numerical aperture = 0.35) or a Leica N Plan 100x objective (numerical aperture = 0.90) and 50 µm slit in extended scan mode with a range of 100-3500 cm⁻¹.

X-ray Diffraction

Unit cells were determined by single crystal X-ray diffraction recorded at room temperature on a Rigaku R-Axis Spider diffractometer equipped with a CuK α source ($\lambda = 1.5406 \text{ \AA}$) operated at 2000 W power (50 kV, 40 mA) and an image plate detector with samples sealed in glass capillaries. Powder X-ray diffraction (PXRD) was performed using the same instrument with samples packed in sealed glass capillaries. Typical scans were 30 minutes with χ and ω fixed while ϕ was spun at a rate of 5°/sec to remove the effects of preferred orientation.

Results and Discussion

Crystallization of HEWL from the tetragonal-inducing condition^{26,27} results in exclusively tetragonal crystals of HEWL in the absence of added polymers in 1-2 days. However, when HEWL is allowed to crystallize from the identical solution in the presence of polymer libraries A-C a dramatic effect on the nucleation behavior is observed. For example, polymers derived from the aromatic monomers AOS, 4VP, 2VP or STY induce nucleation of tetragonal crystals

at least 10 to 25 times faster than without added polymers.³¹ Moreover, nucleation enhancement of up to 100 times was observed between the fastest (within 2 hours) and slowest (>10 days) nucleating wells. The size and quality of tetragonal crystals was also found to vary due to the polymer heteronucleus (Figure 2). In some cases, a few large tetragonal HEWL single crystals were observed in a single crystallization. However, depending on the functionality of the polymer, thousands of very small tetragonal HEWL crystals could be produced. For example, polymers derived from monomers with relatively non-polar and/or aromatic functionalities, such as AOS, STY, *n*-BuMA, *t*-BuMA, BzMA or MMA, primarily nucleate a few large crystals, whereas polymers derived from basic monomers including DMAEMA had an increased propensity for nucleating thousands of minute tetragonal crystallites.

The orthorhombic-inducing condition, which consists of crystallizing HEWL at 40 °C from a 20% protein solution dissolved in 50 mM acetate buffer at pH 4.5 with 2.5% NaCl, results exclusively in the production of orthorhombic crystals in the absence of added polymers. However, in the presence of polymers, selective crystallization of both the orthorhombic and tetragonal phases can be achieved. The majority of crystallizations result in orthorhombic form production, but those with a relatively polar and/or acidic functionality in library C selectively induce crystallization of thousands of tetragonal crystals.

HEWL was also crystallized utilizing a monoclinic phase-inducing condition, consisting of 3% HEWL dissolved in 50 mM sodium acetate buffer at pH 4.5 with 2% NaNO₃,^{26,27} in the presence of polymers. This condition results in the exclusive production of monoclinic HEWL in volumes greater than 100 µL, but when the drop size is decreased to 5-10 µL a mixture, often concomitant, of three crystal phases is observed in the absence of polymers on glass (Figure 3a). However, when polymers are added to the solution selective crystallization of each of these three crystal phases can be achieved. The monoclinic form can be selectively nucleated from polymers with nitrogen-containing functionality, including those derived from 2VP, 4VP, DMAEMA and DMMAA (Figure 3b). Another of the observed phases was nucleated with exceptionally high selectivity from both polar and acidic functionalized polymers composed of AA, MAA, and HEMA. The crystals are readily identified by their distinctive morphology as tetragonal (Figure 3c), but this form has never previously been observed from a condition utilizing NaNO₃ as the salt and has been referred to as the chloride form in the literature.^{26, 27,32} Therefore, the unit cell of crystals grown directly in the presence of polymers was determined and the known tetragonal lattice of $a = b = 79.07(18)$, $c = 38.33(11)$ Å, $\alpha = \beta = \gamma = 90^\circ$ was observed. Raman spectra of these crystals contain a strong peak near 1050 cm⁻¹, characteristic of nitrate ions. The nitrate-containing tetragonal crystals slowly transformed to monoclinic or triclinic phases of HEWL, proving that polymers can facilitate the selective nucleation of metastable protein crystals as high quality single crystals. The observation of tetragonal crystals under these conditions challenges the conventional wisdom that multiple crystal forms are best accessed by altering the crystallization solution components, i.e. salt, buffer, pH or concentration.

The third crystal phase observed employing the monoclinic-inducing condition in the presence of polymers has a needle morphology (Figure 3d), which has been previously observed with both chloride³³ and nitrate³⁴ salt conditions. It was not certain that this was a new crystal form of HEWL and further characterization was warranted. Therefore, Raman spectroscopy and PXRD of the needle-shaped crystals was performed. The Raman spectrum confirmed that they were chemically similar to HEWL (Figure 4). PXRD of this form showed characteristic diffraction at $2\theta = 2.84, 3.68, 4.93, 5.61, 7.31, 8.20, 9.44, \text{ and } 9.89^\circ$, consistent with previous results,³³ and this diffraction is clearly distinct from other known crystal forms of HEWL (Figure 5) and does not match simulated PXRD data from known crystal structures.³⁵ This form was selectively crystallized from acidic polymers, such as those derived from EGMAP,

AA or MAA, and also from non-polar polymers, including those derived from AOS, *t*-BuMA or *n*-BuMA, as the heteronucleant. Needle-shaped crystals were also observed from the tetragonal and triclinic-inducing conditions, often from acid-functionalized polymers, indicating that this form may be crystallized under a number of conditions and from heteronuclei with diverse surface chemistry.

HEWL can also crystallize as a triclinic form, which is accessible by combining a 2% protein solution dissolved in 50 mM sodium acetate buffer at pH 4.5 with an equal volume of a 4% NaNO₃ solution dissolved in the same buffer. The solution is cooled to 6 °C for 6 hours leading to a white microcrystalline precipitate that upon warming to room temperature redissolves and triclinic crystals nucleate and grow over a period of a couple days.²⁹ In the presence of insoluble polymer heteronuclei, the transformation rate from microcrystalline precipitate to triclinic HEWL is affected based on the functionality of the polymer. For example, when the aromatic, nitrogen-containing 4VP is copolymerized with 2VP, DMAEMA or DMMAA and DVB, the transformation from microcrystals to triclinic HEWL is accelerated. In these cases, triclinic HEWL was the exclusive form observed after 4 hours at room temperature, while microcrystals were still observed after 21 hours in the absence of added polymers. In general, high quality single crystals of triclinic HEWL resulted after crystallization was complete. However, tetragonal HEWL crystals were also selectively obtained in the presence of polymers derived from the polar monomer HEMA copolymerized with AA or MAA. This again confirms the utility of the polymer-induced heteronucleation approach for modifying nucleation behavior and selectively nucleating crystal phases by simply changing the identity of the polymer.

Conclusions

Polymer heteronuclei have the ability to accelerate the rate of nucleation by lowering the barrier to assembly through favorable interactions between the protein and polymer. This can have dramatic effects on the size, quality, and even crystal phase produced from a given set of conditions and has the potential to control whether a single crystal of sufficient quality for structure determination can be obtained. The polymer-induced heteronucleation approach may be utilized with any crystallization technique, including batch and vapor diffusion experiments, as an additional diversity element to facilitate the growth of high quality single crystals. Having a greater range of crystal forms to investigate structurally, especially when these are all produced from nominally identical solutions, has the potential to shed light on the role of crystal packing in modifying observed protein structure and offers a glimpse of conformational flexibility not obtainable from studies on a single phase.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENT

This work was supported by the National Institutes of Health (GM072737) and the Alfred P. Sloan Foundation.

REFERENCES

- (1). McPherson, A. Preparation and Analysis of Protein Crystals. John Wiley & Sons, Inc.; New York: 1982.
- (2). McPherson, A. Crystallization of Biological Macromolecules. Cold Spring Harbor Laboratory Press; New York: 1999.
- (3). Jancarik J, Kim SH. J. Appl. Crystallogr 1991;24:409–411.

- (4). Cudney B, Patel S, Weisgraber K, Newhouse Y. *Acta Crystallogr., Sect. D* 1994;50:414–4123. [PubMed: 15299395]
- (5). Mullin, JW. *Crystallization*. Vol. 4th. Butterworth-Heinemann; Oxford, England: 2001.
- (6). Lang MD, Grzesiak AL, Matzger AJ. *J. Am. Chem. Soc* 2002;124:14834–14835. [PubMed: 12475310]
- (7). Price CP, Grzesiak AL, Matzger AJ. *J. Am. Chem. Soc* 2005;127:5512–5517. [PubMed: 15826189]
- (8). Grzesiak AL, Matzger AJ. *J. Pharm. Sci.* 2007*in press*
- (9). Grzesiak AL, Matzger A. *J. Inorg. Chem* 2007;46:453–457.
- (10). Grzesiak AL, Uribe FJ, Ockwig NW, Yaghi OM, Matzger AJ. *Angew. Chem. Int. Edit* 2006;45:2553–2556.
- (11). McPherson A, Schlichta PJ. *J. Cryst. Growth* 1987;85:206–214.
- (12). McPherson A, Schlichta PJ. *Science* 1988;239:385–387. [PubMed: 17836869]
- (13). Leung CJ, Nall BT, Brayer GD. *J. Mol. Biol* 1989;206:783–785. [PubMed: 2544732]
- (14). Wood SP, Janes RW, Sweeney E, Palmer RA. *J. Cryst. Growth* 1992;122:204–207.
- (15). Malkin AJ, Cheung J, McPherson A. *J. Cryst. Growth* 1993;126:544–554.
- (16). Fermani S, Falini G, Minnucci M, Ripamonti A. *J. Cryst. Growth* 2001;224:327–334.
- (17). Chayen NE, Saridakis E, Sear RP. *Proc. Natl. Acad. Sci. U. S. A* 2006;103:597–601. [PubMed: 16407115]
- (18). Stolyarova S, Saridakis E, Chayen NE, Nemirovsky Y. *Biophys. J* 2006;91:3857–3863. [PubMed: 16920829]
- (19). Sanjoh A, Tsukihara T, Gorti S. *J. Cryst. Growth* 2001;232:618–628.
- (20). Tsekova D, Dimitrova S, Nanev CN. *J. Cryst. Growth* 1999;196:226–233.
- (21). Sauter C, Otalora F, Gavira JA, Vidal O, Giege R, Garcia-Ruiz JM. *Acta Crystallogr., Sect. D* 2001;57:1119–1126. [PubMed: 11468395]
- (22). Saijo S, Yamada Y, Sato T, Tanaka N, Matsui T, Sazaki G, Nakajima K, Matsuura Y. *Acta Crystallogr., Sect. D* 2005;61:207–217. [PubMed: 15735330]
- (23). Harata K, Akiba T. *Acta Crystallogr., Sect. D* 2006;62:375–382. [PubMed: 16552138]
- (24). Walsh MA, Schneider TR, Sieker LC, Dauter Z, Lamzin VS, Wilson KS. *Acta Crystallogr., Sect. D* 1998;54:522–546. [PubMed: 9761848]
- (25). Brinkmann C, Weiss MS, Weckert E. *Acta Crystallogr., Sect. D* 2006;62:349–355. [PubMed: 16552135]
- (26). Alderton G, Fevold HL. *J. Biol. Chem* 1946;164:1–5.
- (27). Steinrauf LK. *Acta Crystallogr* 1959;12:77–79.
- (28). Nagatoshi Y, Sazaki G, Suzuki Y, Miyashita S, Matsui T, Ujihara T, Fujiwara K, Usami N, Nakajima K. *J. Cryst. Growth* 2003;254:188–195.
- (29). Vidal O, Robert MC, Arnoux B, Capelle B. *J. Cryst. Growth* 1999;196:559–571.
- (30). The following room temperature unit cells were determined: tetragonal with $a = b = 79.27(10)$, $c = 38.04(7)$ Å; orthorhombic with $a = 30.54(4)$, $b = 56.57(8)$, $c = 73.95(11)$ Å; monoclinic with $a = 28.06(3)$, $b = 63.01(7)$, $c = 60.59(6)$ Å, $\beta = 90.73(4)^\circ$; and triclinic with $a = 27.28(1)$, $b = 31.97(3)$, $c = 34.31(3)$ Å, $\alpha = 88.7(2)$, $\beta = 71.5(1)$, $\gamma = 68.1(2)^\circ$.
- (31). The assumption that nucleation events and crystal appearance track each other is implicit here and seems reasonable considering the fact that growth occurs from nominally identical conditions.
- (32). Jones FT. *J. Am. Chem. Soc* 1946;68:854–857.
- (33). Bhamidi V, Skrzypczak-Jankun E, Schall CA. *J. Cryst. Growth* 2001;232:77–85.
- (34). Heijna MCR, Theelen MJ, van Enkevort WJP, Vlieg E. *J. Phys. Chem. B* 2007;111:1567–1573. [PubMed: 17263569]
- (35). Powder diffraction patterns were simulated using the following crystal structures from the Protein Data Bank: 1IEE, 1WTM, 2D4I, 4LZT, and 2FBB.

Figure 1.
Photomicrographs of a) tetragonal, b) orthorhombic, c) monoclinic, and d) triclinic HEWL grown by their respective literature methods.

Figure 2. Photomicrographs showing the change in crystal size of tetragonal HEWL grown from the tetragonal-inducing condition in the presence of a) AA/MMA/DVB terpolymer, b) 2VP/MAN/DVB terpolymer, and c) DMAEMA/MAN/DVB terpolymer.

Figure 3.

Photomicrographs showing a) the concomitant formation of monoclinic, tetragonal, and needle forms on glass, b) monoclinic crystals growing from EEMA/MMA/DVB terpolymer, c) tetragonal crystals growing from *t*-BuMA/TFPMA/DVB terpolymer, and d) needle crystals growing from HEMA/MMA/DVB terpolymer all crystallized using the monoclinic-inducing condition.

Figure 4.

Raman spectra of five crystal forms of HEWL. Tetragonal, orthorhombic, monoclinic, and triclinic HEWL crystals utilized for analysis were grown by their respective literature procedures and needle-shaped crystals were grown from the monoclinic-inducing condition in the presence of polymers. Note the peak near 1050 cm^{-1} for monoclinic, triclinic, and needles which indicates the presence of nitrate.

Figure 5.
PXRD patterns of five crystal forms of HEWL.