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Pentaerythritol propoxylate: a new crystallization agent and cryoprotectant induces crystal growth of 2-methylcitrate dehydratase

Andrew M. Gulick,[a] Alexander R. Horswill,[b] James B. Thoden,[a] Jorge C. Escalante-Semerena[b] and Ivan Rayment[a]*

[a]Department of Biochemistry, University of Wisconsin, 433 Babcock Drive, Madison, WI 53706, USA, and [b]Department of Bacteriology, University of Wisconsin, 1550 Linden Drive, Madison WI 53706, USA

Correspondence e-mail: ivan_rayment@biochem.wisc.edu

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In the search for macromolecular crystallization conditions, the precipitant is probably the most important variable, such that when problematic crystals are encountered there is always the question of whether an alternative precipitant might resolve the problem. During an effort to obtain high-quality crystals of several problematic proteins, two new agents, pentaerythritol propoxylate and pentaerythritol ethoxylate, yielded well ordered quality crystals where more traditional precipitants were unsuccessful. Pentaerythritol propoxylate and pentaerythritol ethoxylate contain a pentaerythritol backbone to which organic polymers are bound, forming a branched polymer. As such, they are larger than small organic precipitants such as low molecular-weight alcohols or 2-methyl-2,4-pentanediol, but behave differently to polyethylene glycols. These compounds have been used to crystallize an enzyme encoded by the *Salmonella enterica prpD* gene that catalyzes the dehydration of 2-methylcitrate to form 2-methyl-*cis*-aconitate. While the PrpD protein has crystallized readily under a number of conditions, the resultant crystals were unsuitable for a crystal structure determination. The new crystals obtained with 25–40% pentaerythritol propoxylate belong to the orthorhombic space group $C222_1$, with unit-cell parameters a = 73.2, b = 216.4, c = 214.3 Å, and diffract beyond 2.0 Å with synchrotron radiation. A further benefit of this precipitant for crystallization is its ability to function as a cryoprotectant, allowing the crystals to be transferred directly from the mother liquor to the nitrogen stream at 113 K.

1. Introduction

Protein crystallization is influenced by a number of factors including pH, precipitant, ionic strength, temperature and the conformational state of the protein, as influenced by the presence of ligands, substrate or substrate analogs. Of these factors, pH and precipitant are probably the most important variables. Since it is impossible to predict whether a new protein will crystallize or what the composition of the final crystallization solution will be, the first step in a crystallization project is normally to screen a wide range of precipitants and pH with a sparse matrix (Carter & Carter, 1979; Jancarik & Kim, 1991). These screens commonly include a variety of small organic molecules, salts (McPherson, 2001) and polyethylene glycols (McPherson, 1985) that vary in molecular weight and structure. Thus, success in this initial stage requires a broad repertoire of precipitants.

Once initial parameters have been identified, the next challenge is to refine the conditions to produce large well diffracting crystals. In those cases where crystals are obtained from solutions of polyethylene glycol, a variety of polymer molecular weights and chain-termination moieties are typically surveyed in the search for the optimal precipitant. Likewise, when crystals are derived from organic precipitants such as low molecular-weight alcohols, 2-methyl-2,4-pentanediol or polyethylene glycol, variations in the size and isomer are often observed to influence the quality of the crystals. In an effort to expand the range of precipitants available for the initial screening and subsequent refinement of crystallization conditions, a new class of polymers has been tested and found to yield crystals for several problematic proteins and macromolecular complexes. The compounds, pentaerythritol propoxylate and pentaerythritol ethoxylate, are branched polymers built on a pentaerythritol backbone as indicated in Table 1. They are liquids and are available in a variety of molecular weights, thus providing a spectrum of crystallization properties.

Of the proteins tested, one has been crystallized from pentaerythritol propoxylate in a form superior to those obtained from conventional precipitants. This protein, PrpD, is involved in the metabolism of propionate in *S. enterica*. In this bacterium, propionate is

metabolized to pyruvate *via* the 2-methylcitric acid cycle encoded by the *prpBCDE* operon (Horswill & Escalante-Semerena, 1999*b*). In this pathway, the propionate is first combined with oxaloacetate by sequential catalytic activity of PrpE, a propionyl-CoA synthetase (Horswill & Escalante-Semerena, 1999*a*), and PrpC, the 2-methylcitrate synthase (Horswill & Escalante-Semerena, 1999*b*). The 2-methylcitrate thus formed is converted to 2-methylisocitrate, which is subsequently cleaved to form succinate and pyruvate by PrpB (Horswill & Escalante-Semerena, 2001). The conversion of 2-methylcitrate to 2-methylisocitrate is analogous to the reaction catalyzed by aconitase in the citric acid cycle in which a single enzyme catalyzes the dehydration of citrate to *cis*-aconitate and the subsequent rehydration to form isocitrate. The hydration and rehydration of 2-methylcitrate, however, has recently been shown to be catalyzed by two separate enzymes: the dehydration is catalyzed by PrpD, while the rehydration can be catalyzed by either of two aconitase enzymes present in *S. enterica* (Horswill & Escalante-Semerena, 2001). PrpD is unrelated to aconitase: it does not contain an iron–sulfur cluster and is not metal-ion dependent.

Likewise, PrpD appears to be unrelated to the enolase superfamily which also catalyzes the vinylogous elimination of a water molecule *via* a common enolic intermediate (Gerlt & Babbitt, 1998), as PrpD does not appear to contain the necessary residues to allow placement in this family. To further understand the catalytic basis for this novel enzyme and the evolutionary relationships to these seemingly unrelated aconitase and enolase families, a project has been initiated to determine the atomic structure of this enzyme. We report here the use of a new crystallization agent that allowed the production of crystals that diffracted beyond 2.0 Å at the Structural Biology Center-CAT at the Advanced Photon Source.

2. Materials and methods

2.1. Reagents

Pentaerythritol propoxylate with average molecular weights 426 and 629 Da (PEP 426 and PEP 629, respectively) and pentaerythritol ethoxylate with average molecular weights 270 and 797 Da (PEE 270 and PEE 797, respectively) were purchased from Aldrich. The propoxylate and ethoxylate compounds are highly viscous liquids that are completely miscible with water and so it was possible to make buffered 50% stock solutions which were used for crystallization experiments. All other chemicals were of reagent grade.

Table 1
Properties of the pentaerythritol-based precipitants.

Precipitant	Formulae	⟨*n*⟩	Average MW (Da)
Pentaerythritol propoxylate (5/4 PO/OH)	$C\{CH_2[OCH_2CH(CH_3)]_nOH\}_4$	∼1.3	426
Pentaerythritol propoxylate (17/8 PO/OH)	$C\{CH_2[OCH_2CH(CH_3)]_nOH\}_4$	∼2.1	629
Pentaerythritol ethoxylate (3/4 EO/OH)	$C[CH_2(OCH_2CH_2)_nOH]_4$	∼0.75	270
Pentaerythritol ethoxylate (15/4 EO/OH)	$C[CH_2(OCH_2CH_2)_nOH]_4$	∼3.75	797

2.2. Protein expression

Recombinant PrpD protein tagged with a His_6 purification tag was produced as described previously (Horswill & Escalante-Semerena, 2001). The protein was dialyzed into 10 m*M* *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES) pH 7.5 at 277 K and concentrated to 11.7 mg ml^{-1}. The protein was frozen as small droplets by direct pipetting into liquid nitrogen. The frozen pellets were stored at 193 K and thawed as required for crystallization experiments.

2.3. Crystallization

A preliminary 48-well crystallization screen was prepared that included 12 entries for each of the pentaerythritol precipitants at a concentration of 40% and an equal distribution of conditions in the pH range 5.0–9.0. Across this regular array of pH and precipitant a variety of salts were distributed including NaCl, KCl, $LiSO_4$, $MgCl_2$ and ammonium sulfate, ranging in concentration from 100 to 500 m*M*. Although this was not an exceedingly diverse screen, it was sufficient to illustrate several important features and differences between these precipitants. It is noteworthy that with 40% PEP 629 it was difficult to prepare solutions that contained more than 100 m*M* of divalent cations or anions owing to phase separation, although at lower concentrations of PEP 629 such solutions could be prepared. Crystallization was examined with the hanging-drop method where equal volumes of protein and precipitant were mixed.

3. Results and discussion

3.1. Crystallization

Initially, pentaerythritol propoxylate and pentaerythritol ethoxylate were used to screen for crystals for a Tn5 transposase–DNA complex, N-acylamino racemase as well as PrpD protein, all of which crystallize poorly from PEG. Preliminary crystals were observed for both the transposase–DNA complex and PrpD, but not for the racemase. Of these, better crystals were obtained for PrpD with PEP 426 than with PEG, as described below.

Prior to investigating pentaerythritol derivatives as precipitants, PrpD protein was shown to crystallize readily under a number of different conditions identified in a conventional broad-screen sparse matrix. Hexagonal rods are observed using moderate to high salt concentrations (0.4–2.2 *M*), polyethylene glycols and small organics. The protein crystallizes with a variety of salts to form highly anisotropic crystals in space group *P*622 that diffract to 2.5 Å perpendicular to the sixfold rotation axis but only to ∼4 Å along the *c* axis. Additionally, a second isozyme of this enzyme from a different organism has been studied and also yielded multiple crystals that either diffracted very poorly or exhibited crystal twinning. The protein also crystallized in a second form using the hanging-drop method and a precipitant consisting of 12% PEG 2000 methyl ether, 50 m*M* $MgCl_2$, 100 m*M* NaCl, 10% MPD, 50 m*M* HEPES pH 8.0 at 277 K. This form belonged to the rhombohedral space group *R*3; however, the crystals were shown to be twinned. Addition of the substrate and product analogs citrate, *cis*-aconate and mesaconate often provided different crystal forms, but did not yield any crystals more suitable for structure determination.

The difficulty in finding suitable crystals led to a search for additional crystallization agents. A series of small organics was identified that contain four polymeric chains bound to a central quaternary C atom. In several proteins studied, crystallization was promoted in hanging drops using a range of concentration from 20 to 45% of the precipitant.

PrpD was subjected to a crystallization screen with 12 conditions using each of the new precipitants. The precipitant was

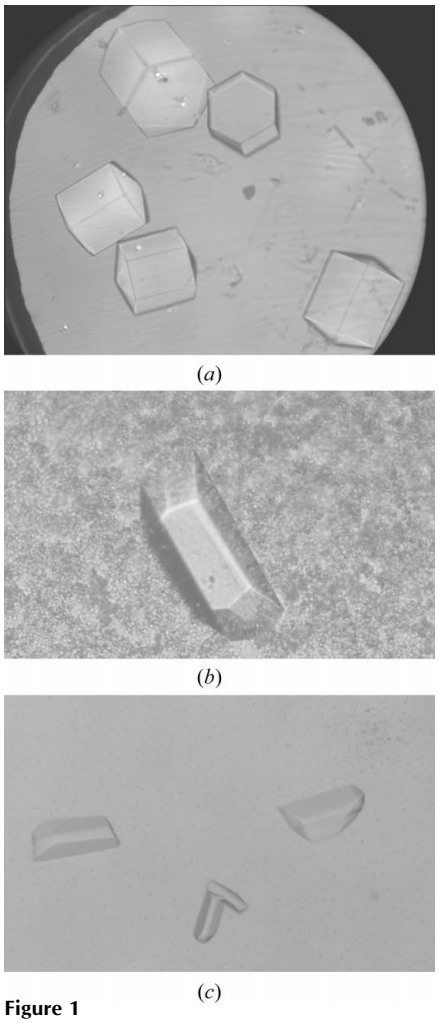

Figure 1
Multiple crystal forms of PrpD protein as described in Table 2. (*a*) Highly anisotropic hexagonal rods. (*b*) Twinned rhombohedral crystals. (*c*) Orthorhombic crystals grown with PEP 426 as the precipitant.

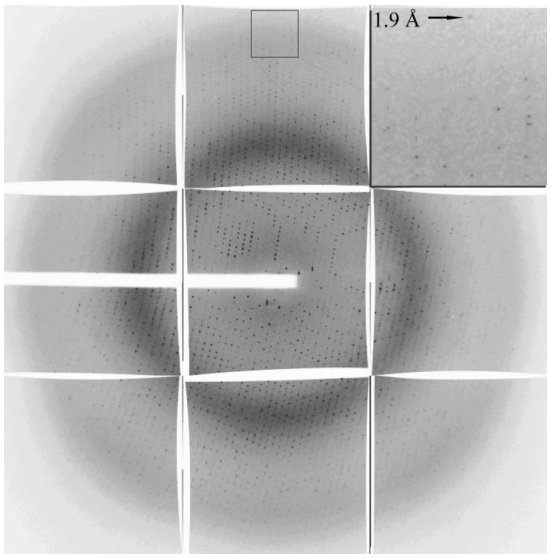

Figure 2
Diffraction pattern of a PrpD crystal taken at 113 K using synchrotron radiation. Data were collected at 0.9649 Å and processed with *HKL*2000 (Otwinowski & Minor, 1997). The maximum resolution spots shown here are 1.8 Å.

present at 40% and showers of small needles grew from a wide range of salt concentrations and pH values. Small needles grew from three conditions with PEE 797 and one condition with PEE 270. The PEP precipitants proved more effective at inducing crystal growth, with crystals appearing in eight of the 12 conditions with either PEP 629 or PEP 426. Generally, the crystals appeared within 7 d. After the initial crystallization survey, the conditions were refined; the best crystals were obtained by the hanging-drop method utilizing a precipitant containing 35–37% PEP 426, 0.2 *M* KCl, 50 m*M* HEPES pH 8.0 at 277 K (Fig. 1). The new crystals belong to the orthorhombic space group $C222_1$, with unit-cell parameters $a = 73.2$, $b = 216.4$, $c = 214.3$ Å, and diffract beyond 2.0 Å with synchrotron radiation.

One benefit of small organic precipitants is their inherent ability to serve directly as cryoprotectants. To test the suitability of the PEP 426 compound for fulfilling this function, solutions were made with 25–40% PEP 426 containing 0.2 *M* KCl and 50 m*M* HEPES. All solutions froze clear and gave no ice rings.

3.2. Data collection

In contrast to the earlier attempts to obtain a high-resolution data set of PrpD from conventional precipitants, a well defined data set was obtained from the crystals grown from PEP 426. Prior to data collection, a crystal was transferred directly from the hanging drop into a stream of nitrogen at 113 K for data collection. A preliminary data set was recorded on ID19 at the Structural Biology Center-CAT at the Advanced Photon Source, Argonne National Laboratory, where the crystals diffracted beyond 1.8 Å (Fig. 2). The data were processed with *HKL*2000 (Otwinowski & Minor, 1997) and *TRUNCATE* from the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994). The resultant data set was 98% complete to 2.1 Å resolution and gave an $R_{\rm merge}$ of 10.8% (15% in the outer shell). A self-rotation function was performed on the data with *CNS* version 1.0 (Brunger *et al.*, 1998), giving a twofold non-crystallographic rotation axis with an RF function of 21.8. This suggests there are two molecules in the asymmetric unit. Using a unit-cell volume of 3.38 × 10^6 Å^3 and a molecular weight of 53 795 Da, the $V_{\rm M}$ value for this crystal is 3.9 Å^3 Da^{-1} (Matthews, 1968). Attempts to reproduce these crystals with SeMet-labeled protein are currently under way.

Table 2
Crystal forms of PrpD investigated.

Crystallization conditions	Unit cell	Comments
0.5 *M* NaCl, 1–5% MPD, 50 m*M* MES pH 6.25, 277 K	*P*622; $a = b = 245.4$, $c = 84.9$ Å	Highly anisotropic
12% MePEG 2000, 50 m*M* $MgCl_2$, 100 m*M* NaCl, 10% MPD, 50 m*M* HEPES pH 8.0, 295 K.	*R*3; $a = b = 137.2$, $c = 505.6$ Å, $\alpha = \beta = 90$, $\gamma = 120^\circ$	Twinned
36% PEP 426, 250 m*M* KCl, 50 m*M* HEPES pH 8.0, 295 K	$C222_1$; $a = 73.2$, $b = 216.5$, $c = 214.0$ Å	

4. Conclusions

The new compounds described herein provide an additional tool in the crystallization of macromolecules. The compounds are similar to the lower molecular weight polyethylene glycols; however, the branched nature of the pentaerythritol backbones appears to provide them with considerably different properties. Their larger size also differentiates them from smaller organic compounds frequently used in crystallization experiments, such as ethylene glycol and 2-methyl-2,4-pentanediol. Similar to 2-methyl-2,4-pentanediol, these pentaerythritol-based compounds inherently contain chiral combinations of substitutents. It is unknown if specific isomers or more homogeneous mixtures might prove more useful for crystallization, but might this be an avenue worth pursuing in the search for improved reagents. Lastly, their use as a cryoprotectant in addition to their role as a crystallization agent may increase their utility to macromolecular crystallographers.

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References

Brunger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jiang, J. S., Kuszewski, J., Nilges, M.,

Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T. & Warren, G. L. (1998). *Acta Cryst.* D**54**, 905–921.
Carter, C. W. Jr & Carter, C. W. (1979). *J. Biol. Chem.* **254**, 12219–12223.
Collaborative Computational Project, Number 4 (1994). *Acta Cryst.* D**50**, 760–763.
Gerlt, J. A. & Babbitt, P. C. (1998). *Curr. Opin. Chem. Biol.* **2**, 607–612.
Horswill, A. R. & Escalante-Semerena, J. C. (1999*a*). *Microbiology*, **145**, 1381–1388.
Horswill, A. R. & Escalante-Semerena, J. C. (1999*b*). *J. Bacteriol.* **181**, 5615–5623.
Horswill, A. R. & Escalante-Semerena, J. C. (2001). *Biochemistry*, **40**, 4703–4713.
Jancarik, J. & Kim, S.-H. (1991). *J. Appl. Cryst.* **24**, 409–411.
McPherson, A. (1985). *Methods Enzymol.* **114**, 120–125.
McPherson, A. (2001). *Protein Sci.* **10**, 418–422.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.