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Antifreeze Protein-Induced Selective Crystallization of a New Thermodynamically and Kinetically Less Preferred Molecular Crystal

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Abstract

The formation of a new, dihydrate crystalline form of 5-methyluridine (m^5U) was selectively induced by a protein additive, antifreeze protein (AFP) in a highly efficient manner (in 10^{-6} molar scale, while known kinetic additives need 0.1 molar scale). The hemihydrate form (form I, the only previously known crystalline form of m^5U) and the dihydrate form of m^5U (form II) obtained herein were characterized using X-ray crystallography and differential scanning calorimetry (DSC). Compared to form I, remarkably, form II is thermodynamically and kinetically less preferred. The presence of AFP can selectively inhibit the appearance of form I and hence allows the growth of form II, the pure form of which cannot grow directly from m^5U supersaturated solutions under the same conditions. An explanation supported by both experimental and theoretical results was provided for the AFP-induced selection process. Implications on AFP-induced ice shape changes have also been discussed. Control of crystallization from supersaturated solutions is of great interest in both fundamental research and practical applications in fields like chemistry, pharmacology and materials science. These findings suggest that crystallization processes using AFPs is valuable for selective growth of hydrates and polymorphs of important pharmaceutical compounds.

Introduction

Controlling the crystallization of compounds is of great interests in various fields of applications, from chemistry to pharmacology, and from materials science and food science.^[1] For instance, changes in the size and shape of crystals of pharmaceutical compounds can impact on their bioavailability, chemical stability, and production efficiency of the drugs. Traditional methods to control crystallization usually include the alterations of temperature, solvent, supersaturation, and seeding conditions.^[2] A number of recent methods, such as using tailor-made additives,^[3] ledge-directed epitaxy,^[4] polymer microgel,^[5] polymer heteronuclei,^[6] capillaries,^[7] porous materials,^[8] and laser-induced nucleation,^[9] have been developed and employed in the crystallization process of certain

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compounds to select favored and/or discover new crystalline forms of the compounds. Although tremendous effort has been devoted to understanding the crystallization process and selective crystallization, the crystallization control process remains largely trial-and-error, experiencing substantial difficulties in exclusive production of the desired forms as well as the production of both thermodynamically and kinetically less favored forms.^[1a, 5] Moreover, much less progress has been made in additive-controlled organic crystallization than in additive-controlled inorganic crystallization and the selective production of organic single crystals with defined crystal phase and morphology is hard to achieve.^[10]

Antifreeze proteins (AFPs) are a group of structurally diverse proteins that are known in many cold-adapted organisms for their survival at subzero temperatures.^[11] AFPs can inhibit ice-crystal growth and modify the ice-crystal habit by binding to specific faces of ice crystals, providing one of the most notable examples of crystallization control in nature.^[12] The affinity of AFPs to ice depends on hydrogen bonding and/or hydrophobic interactions, which is unlike most protein-mineral interactions where ionic interactions often dominate.^[13] AFPs can also inhibit the growth of gas hydrates^[14] as well as the growth of other molecular crystals that are not ice-like.^[15] Nucleosides (and analogues) are important pharmaceutical compounds and it is of practical significance to obtain different forms of these compounds due to the different physicochemical properties and bioavailabilities of the different forms.^[16] The nucleoside, 5-methyluridine (m^5U), is an important compound in pharmaceutical industry.^[17] We have reported that DAFP-1, a beetle AFP from *Dendroides canadensis*, can efficiently inhibit the crystal growth of m^5U hemihydrate, the only previously known crystalline form of m^5U , where hydrogen bonding interactions between DAFP-1 and specific m^5U crystal surfaces may play an important role.^[15]

Crystal growth at the molecular level can be modeled as a process starting from prenucleation aggregate formation, and then followed by crystal nuclei evolution and macroscopic crystal development.^[18] The arrangements and shapes of at least some of these prenucleation aggregates and evolved nuclei are thought to resemble the final crystal structure, and control of which is a decisive step in controlling the final crystalline form. To further understand whether (and how) DAFP-1 influences polymorphism or crystalline form(s) of m^5U , we have made significant efforts on altering and controlling the crystallization conditions.

In this paper, we present the first case of using DAFP-1 as a novel, highly efficient additive to exclusively obtain a new crystalline form of m^5U , m^5U dihydrate, from supersaturated m^5U aqueous solutions. The structures of the two crystalline forms of m^5U obtained herein, m^5U hemihydrate (form I) and m^5U dihydrate (form II), were completely determined using X-ray crystallography and the crystals were also characterized using differential scanning calorimetry (DSC). Form II crystals with a block-like morphology are thermodynamically and kinetically less preferred than the needle-like form I crystals. It is known that crystal shape changes can be due to either the habit modification of the same crystalline form or more frequently the formation of a new crystalline form.^[1a, 2] In this study, the formation of the new, less thermodynamically and kinetically preferred m^5U dihydrate crystals was able to be induced by DAFP-1, demonstrating the latter case.

Few physical or chemical processes can happen when the product is neither thermodynamically nor kinetically favored.^[2, 19] Under the same conditions, pure form II cannot grow directly from m^5U supersaturated solutions, however, the presence of DAFP-1 can selectively inhibit the nucleation of form I and hence allows the crystal growth of form II. The AFP-induced selection process was depicted based on the experimental and theoretical results. Using AFPs in crystallization processes may be valuable for selective growth of hydrates and polymorphs of hydroxyl compounds.

Results and Discussion

The only previously known crystalline form of m^5U is its hemihydrate crystal, designated form I, which was crystallized exclusively by evaporation from the supersaturated aqueous solutions. By using DAFP-1 as an additive, remarkably, we were able to control the crystallization of m^5U and discover a novel, less thermodynamically and kinetically preferred form, m^5U dihydrate crystal, designated form II. Forms I and II crystals are readily distinguished from each other by their morphology and stability. Form I crystals are needle-like in appearance (Figure 1a) and there was no change (in the appearance, single crystal X-ray diffraction pattern, or DSC profile) of the crystals after being left in air at room temperature for more than 24 months. Form II crystals are block-shaped (Figure 1b) and became white opaque powders/blocks in 2 months. The resulting solid powders were not suitable for single-crystal X-ray diffraction. By using the denatured DAFP-1 as an additive (a control), no effect on the crystallization of m^5U was observed under the same conditions, that is only the needle-like m^5U form I crystals were obtained (Figure 1c).

Structure aspects

The structures of both forms were determined by single-crystal X-ray diffraction (Figure 2). Both forms crystallize in the orthorhombic crystal system. Form I was solved in the space group $P2_12_12$, whereas the new form is in $P2_12_12_1$ (Table 1). A high degree of torsional freedom of the N1-C6 bond is in the m^5U molecule. The overlap of m^5U molecules in form I and form II reveals a significant conformational difference in the ribose moiety (Figure 3). Such change may allow optimization of hydrogen bonds between the hydroxyl groups in the ribose moiety and neighboring groups in m^5U and water. Water molecules are hydrogen bound to m^5U molecules through N-H...O and O-H...O interactions. Comparing to those in form I, the number of strong intermolecular hydrogen bonds between two m^5U molecules decreases, whereas the number of hydrogen bonds between m^5U and water molecules increases in form II (Table 2). The strong intermolecular hydrogen bonds between two m^5U molecules in form I are disrupted upon the introduction of additional water molecules. Consequently, the packing motifs of the two forms of m^5U are different (Figure 4).

All the growing faces in form I crystal contain potential hydrogen bond donor or acceptor atoms (Figures 4a and 4b). On the (100) face, there are three oxygen atoms from the thymine moiety (O1), a hydroxyl group in the ribose moiety (O4), and water (O7), respectively. Two oxygen atoms from a hydroxyl group in the ribose moiety (O5) and water (O7), respectively, are on the face (010) (Figure 4a and Table 2). The oxygen atom in the thymine moiety (O1) is on the face of (001) (Figure 4b and Table 2). In contrast to form I, the three growing faces of form II are more hydrophobic. No potential hydrogen bond donor or acceptor atom is observed on the face of (010) or (001); and the oxygen atoms (O7 and O8) of the two water molecule are on the (100) face (Figures 4c and 4d; Table 2).

Hydrogen bonding interactions are known to be essential in some AFPs to recognize ice crystals and the distance of the side chain hydroxyl groups in the conserved repeat residues in the AFPs matches that of repeating oxygen atoms on the prism face of the normal ice crystal.^[15, 20] DAFP-1 is a repeat protein with conserved threonines as putative ice-binding residues in each repeat unit.^[21] It suggests DAFP-1 may recognize these faces of m^5U form I through hydrogen bonding interactions, while may not interact with any face of m^5U form II crystal due to the lack of match of possible hydrogen bond donor/acceptor atoms in DAFP-1 and the growing faces of form II (Supporting Information; Figure S3). Therefore, DAFP-1 can selectively inhibit form I crystallization, but allow the growth of form II crystal.

The interactions between DAFP-1 and m⁵U form I are highly efficient since only a tiny amount of DAFP-1 as additives is needed (the additive/m⁵U molar ratio was 5×10^{-6}). Moreover, such interactions need to be weak in order to guide the crystal growth. The selective crystallization of form II by using DAFP-1 cannot be obtained if the interactions between DAFP-1 and m⁵U are strong. This is because if the interactions between DAFP-1 and m⁵U were strong, some m⁵U molecules would bind to DAFP-1 completely in solution, leaving only pure m⁵U and finally resulting in form I only.

Thermodynamic stability analysis

Both forms of m⁵U were analyzed by DSC (Figure 5). Form I displays two endothermic events upon heating. The first endotherm associated with water loss is at 139.07 °C, whereas the second endotherm ascribed to the melting is at 185.65 °C. Form II exhibits two endotherms at 137.04 °C and 153.72 °C, respectively, for the loss of the two water molecules, followed by a recrystallization exotherm at 157.66 °C and then a melting endotherm at 183.41 °C. Form II starts losing water and then melts at relatively lower temperatures than those of form I. The enthalpy profiles for both forms are compared in Figure 6. Form I is more thermodynamically stable, which is in accordance with the previous observations.

The crystallization processes of both forms were carried out at ambient conditions. If we assume that the reaction of form II becoming form I by losing water at room conditions, i.e., m⁵U form II $\rightarrow$ m⁵U form I + 1.5 H₂O, then heat is needed to cause the dihydrate form II to lose water and become the hemihydrate form I (Figure 6). Therefore, the ΔH for the reaction is positive, i.e.

$$\Delta H > 0 \quad (1)$$

At room temperature, T , we have^[19a]

$$d(\Delta G/T) dT = -\Delta H/T^2 \quad (2)$$

For a tiny temperature increase, dT , the resulting temperature, T' can be expressed as $T' = T + dT$ and

$$T' > T \quad (3)$$

Assuming that ΔG is a constant for such a tiny change, we obtain

$$\Delta G(d1/T) = -\Delta H/T^2 dT \quad (4)$$

By integrating the above equation, we get

$$\Delta G(\ln T'/T) = \Delta H(1/T' - 1/T) \quad (5)$$

Combining Eqs. 1, 3, 5, we obtain $\Delta G < 0$. Therefore, the free energy change of the form II-to I transition at room containing is negative. That is form I crystal is more thermodynamically stable. The results are consistent with the observations that form I crystals were stable in air for at least 2 years, while form II crystals became white powders/blocks in 2 months in air.

Crystallization kinetics analysis

In the absence of DAFP-1, form I starts growing on day 3 and the crystallization completes on day 9 (Figure 7). DAFP-1 can inhibit the crystallization of form I. In the presence of DAFP-1, only form II can be grown and the crystallization starts on day 6 and finishes on day 15. The crystallization of form II cannot happen in the absence of DAFP-1, since the crystallization of form I has much faster kinetics than that of form II. However, the presence of DAFP-1 completely inhibits the fast growing form I and hence results in the exclusive formation of the new, slow growing form II. The average crystal growth rate of form II is estimated to be 10.5 % per day, which is slower than that of form I, 13.3 % per day.

Mechanism of AFP-induced selective nucleation and growth

Our approach with regard to the mechanism of the AFP-induced control of crystallization rests on the assumption that supersaturated solutions contain nuclei adopting similar arrangements to the final crystal structure. This assumption has been successful in designing tailor-made inhibitors, remarkably however, the efficiency of AFP is superior to most known additives.^[22] That is, the amount of AFP needed is significantly smaller than other additives. The molar ratio of AFP and m⁵U is in the range of 10⁻⁶, while in contrast, 5–10 % of additives are usually needed for crystallization control.^[22]

In the supersaturated solution of m⁵U, form I nuclei whose structure are similar to that of form I crystal are evolved around day 2 (Figure 7). In the absence of DAFP-1, form I crystal grows. As suggested by the match between the hydroxyl groups in the conserved threonines in DAFP-1 and the hydrogen bond donor/acceptor atoms on the growing faces of (100) and (010) in form I of m⁵U, DAFP-1 can recognize the fast growing face of (100) and the (010) face of form I through hydrogen bonding interactions. Hence, form I nuclei can be completely inhibited in the presence of DAFP-1 and then the extent of supersaturation increases during the following days until the crystallization of form II around day 5 (Figures 7 and 8). That is the higher degree of supersaturation attained in the presence of DAFP-1 is required for the formation of form II nuclei. In addition, due to the mismatch between the hydrogen bond donor/acceptor atoms in DAFP-1 and in the growing faces of form II, DAFP-1 cannot interact with the newly formed form II nuclei. Consequently, only form II grows, even though it is a thermodynamically and kinetically less preferred phase (Figure 8).

Theoretical Analysis

Both thermodynamic analysis and kinetics analysis show the possibility of the AFP induced selective crystallization of the less thermodynamically and kinetically preferred m⁵U dihydrate crystal and gives insights into this process.

Thermodynamic analysis—According to the Gibbs-Volmer theory of homogeneous nucleation, the overall free energy change, ΔG , between a small nucleus of m⁵U and m⁵U in aqueous solution is equal to the sum of the surface excess free energy, ΔG_s , and the volume excess free energy, ΔG_v .^[2] Therefore,

$$\Delta G_i(r_i) = \Delta G_{si} + \Delta G_{vi} \quad (6)$$

where i = I or II, representing form I or form II of m⁵U. ΔG_{si} is a positive quantity, while ΔG_{vi} is a negative quantity.^[2] For simplicity, a nucleus of m⁵U is assumed to be a sphere with a radius, r . Then,

$$\Delta G_i(r_i) = 4\pi r_i^2 \gamma_i + (4\pi r_i^3 \Delta G_{vi})/3 \quad (7)$$

where, γ_i is the interfacial tension between the developing crystalline surface and the supersaturated solution where the nucleus is located. The critical nucleus, $r_{ci} = -2\gamma/\Delta G_{vi}$, is obtained at $d\Delta G_i(r_i)/dr_i = 0$. At the critical m⁵U form I nucleus, we get

$$\Delta G_{ci} = (4\pi \gamma_i r_{ci}^2)/3 \quad (8)$$

DAFP-1 can bind to m⁵U form I nucleus.^[15] For the AFP attached form I nuclei,

$$\Delta G_{ci'} = F (4\pi \gamma_i r_{ci}^2)/3 \quad (9)$$

where i' is m⁵U form I nucleus with AFP attachment and F is a correct factor resulting from the AFP attachment of m⁵U form I nucleus. To inhibit m⁵U form I nucleation and crystal growth, we need $F > 1$ and hence $\Delta G_{ci'} > \Delta G_{ci}$.

At the critical m⁵U form II nucleus, we get

$$\Delta G_{cii} = (4\pi \gamma_{ii} r_{cii}^2)/3 \quad (10)$$

To make the less preferred m⁵U form II nucleation and crystal growth occur before m⁵U form I nucleation and crystal growth, we need $\Delta G_{ci'} > \Delta G_{cii}$, that is $F > (V_{ii} r_{cii}^2)/(V_i r_{ci}^2)$.

Similarly to the known structure of a beetle AFP from *Tenebrio molitor* (TmAfp), the two rows of solvent-accessible repeated threonines in DAFP-1 are putative ice binding residues and comparing to the side chain methyl groups of the threonines, the side chain hydroxyl groups play a more important role in the ice-binding of TmAfp and DAFP-1.^[11b, 21, 23] Although the ice-binding site of some other types of AFPs are reported to be more hydrophobic than the rest of the protein,^[11b, 24] the ice-binding site of TmAfp and DAFP-1 are suggested to be more hydrophilic.^[11b, 21, 23] Due to the remarkable diverse structures of AFPs, it would be possible that different ice-binding mechanisms may occur in different AFPs and both hydrophobic and hydrogen bonding interactions can be important for the binding of AFPs to the ice-water interface.^[25] Therefore, we speculate that the hydrophilic face of DAFP-1 can bind to the thermodynamically preferred, form I nuclei of m⁵U, while the hydrophobic face of DAFP-1 thus exposes to bulk solution^[15, 20–21, 23a] resulting in large repulsive interactions against incoming m⁵U molecules and hence inhibits form I crystallization, which leads $F > 1$. However, DAFP-1 cannot bind to m⁵U form II nuclei. The total contributions from F , interfacial tension and the radius of nuclei, finally lead to the nucleation of the metastable crystalline. At this point, the free energy of form I nuclei with AFP attachment is still higher than that of crystalline form II. This finally allows the thermodynamically less preferred dihydrate crystalline form to fully grow.

Kinetic analysis— J_i is the nucleation rate of form I crystal. The kinetic coefficient for form i crystal growth is k_i . For a system with two forms of potential crystals, i.e., m⁵U crystalline forms I and II, the following condition must be held to have m⁵U crystalline form II as the only crystallization product,^[26]

$$J_{ii} k_{ii}^3 \gg J_i k_i^3 \quad (11)$$

This suggests that the appearance of different structures may be influenced by additives designed to interfere selectively with either the nucleation, growth rates of a particular phase or both.

According to Arrhenius equation,^[2] the rate of nucleation can be expressed as

$$J_i = A_i \exp(-\Delta G_{ci}/kT) \\ = A_i \exp[-(16\pi\gamma_i^3 v_i^2)/(3k^3 T^3 \{\ln S_i\}^2)] \quad (12)$$

where A_i is Arrhenius pre-exponential factor for species i , k is the Boltzmann constant. T is the temperature in kelvins, v_i is the molecular volume for species i , S_i is supersaturation of the species i .

In the absence of AFPs, only m^5U form I crystals can form. Thus,

$$J_I k_I^3 \gg J_{II} k_{II}^3 \quad (13)$$

In the presence of AFPs, AFPs inhibit the form I nucleation.^[15] Therefore, $J_I \rightarrow 0$ and $J_{II} \gg J_I$, and we get

$$J_{II} k_{II}^3 \gg J_I k_I^3 \rightarrow 0 \quad (14)$$

Under this kinetic condition described in the above equation, we can exclusively obtain the kinetically less preferred m^5U crystalline form II.

Implications on AFP-induced Ice Habit Changes

AFPs can inhibit the ice growth by binding to specific faces of ice crystals and changing the ice crystal shape. For example, a Ca^{2+} dependent AFP^[27] and TmAFP^[23a] can change the ice crystal shape to hexagonal plates; type I fish AFP,^[24a, 28] type III fish AFP,^[29] and type IV fish AFP^[30] can change the ice shape to pyramids; and LpAFP^[31] and winter flounder AFP^[32] can alter the ice shape to hexahedrons. The resulting ice shapes by the same AFP can be different, which depend on the concentration of the AFP, the cooling rate, and other experimental conditions.^[33] Ice has been known with many polymorphs^[34] and studies have shown various ice crystal habits induced by AFPs, but few structures of these ice crystals with altered habits have been determined by x-ray diffraction or neutron diffraction. To date, only the ice crystal with a changed shape by a fish glycoprotein was reported to be ice I_h .^[12] Ice I_h is the most common form of ice, which is highly stable and the most dangerous to life.^[35] Some carbohydrates are known to be able to induce the crystallization of water into unstable cubic ice under certain conditions.^[36] Are these newly shaped ice crystals induced by AFPs new ice polymorphs or just habit modifications of the common ice I_h ? How does their stability compare to ice I_h ? Structure determination of these shape-changed ice crystals can be important to address the above questions and the answers will lead to a better understanding of the mechanism of antifreeze action of AFPs implied by this study.

Conclusion

An AFP-induced selective nucleation and growth of a new crystalline form has been demonstrated for the first time. Significantly, the newly identified form of m^5U is thermodynamically and kinetically less preferred and the exclusive crystallization of which is extremely difficult and could not be achieved previously. Moreover, compared to other

known additives for crystallization control, the AFP-induced selective crystallization is highly efficient, thus having great potential in applications such as new crystalline form screening.^[2, 37]

The mechanism of AFP-induced crystallization is discussed and analyzed based on both theoretical and experimental aspects. This study provides essential hints for the solution of the long-standing problem of the antifreeze mechanism of AFPs. Do AFPs may have similar actions on m⁵U and on ice in terms of selectively controlling crystallization? That is, could AFPs induce the exclusive crystallization of thermodynamically and kinetically less preferred ice polymorphs? Structure determination of the ice crystals with altered habits achieved by AFPs under different experimental conditions, particularly, under those close to physiological conditions, may improve our understanding in AFP action.

AFPs can be used as novel scaffolds to design effective additives for crystallization control, which are valuable in many fields including chemistry, materials science, and the pharmaceutical industry. Future studies on controlling crystallization by different types of AFPs and engineered AFPs, and on extending the findings to other crystal growth processes, should be of great interests to both basic research and practical applications.

Experimental Section

Materials

Chemicals were purchased from Sigma-Aldrich (St. Louis, MO) at ACS grade or better and used as received. Milli-Q water produced from a Synergy water system (Millipore) with a minimum resistivity of 18 M Ω -cm was used for making solutions. All the sample solutions were filtered through 0.2 μ m filters before use. Sample vials (10 mL, National Scientific) were used for crystallization. Glassware and stir bars were cleaned as previously described.^[15]

Synthesis

The syntheses of o,o'-bis(trimethylsilyl)thymine and 5-methyluridine used the following procedures modified from previous methods.^[38] Trimethylchlorosilane (6.51 g, 0.06 mole) and thymine (3.78g, 0.03 mole) were suspended in 100 mL dry benzene. The suspension was quickly stirred under N₂ and 8.25 mL triethylamine in benzene was added dropwise. The mixture was then refluxed for 2 hours. O,O'-Bis(trimethylsilyl)thymine was crystallized upon standing at room temperature. After recrystallization for three times, the pure o,o'-bis(trimethylsilyl)thymine (7.55 g, yield 93%) appeared as slightly yellow crystals. Then, the obtained o,o'-bis(trimethylsilyl)thymine (11.90 g, 0.044 mole, 10% excess) and β -D-ribofuranose 1-acetate 2,3,5-tribenzoate (20.18 g, 0.04 mole) were first mixed in 80 mL CH₃CN under N₂. The mixture temperature was kept at 0 °C and stirred. SnCl₄ (1.0 M, 41.34 mL) in methylene chloride was added dropwise. The reaction was run at 0 °C for 2 hours and warmed up to room temperature for another hour. KHCO₃ (60 g) in 60 mL water was added to remove the trimethylsilyl protected group. The solvents were removed under reduced pressure at 60 °C. The product was a white solid or crystals after silica gel chromatography using EtOAc in CH₂Cl₂ as eluent and the formation was revealed by TLC analysis (R_f = 0.60) using 20% EtOAc in CH₂Cl₂. Sodium ethoxide in ethanol (668 mL, 21% w/v) was used to remove the Bz protecting group. The solvents were removed under reduced pressure at 50 °C. The product was a white solid or crystals after silica gel chromatography using ethanol in CH₂Cl₂ as eluent and the formation was revealed by TLC analysis (R_f = 0.70) using 25% EtOAc in CH₂Cl₂ and LC/ESI-MS and NMR (Supporting Information; Figures S1–S3).

Preparation of antifreeze protein and control

DAFP-1 was expressed and purified as described previously.^[39] The purified protein was characterized using SDS-PAGE gel electrophoresis, MALDI-TOF mass spectrometer, circular dichroism (CD) spectrometry, and differential scanning calorimetry (DSC), respectively, as previously described^[21] and the identity of DAFP was confirmed. The concentration of stock DAFP-1 solution was determined using a Cary 100 Bio UV-Vis spectroscopy (Varian) and the extinction coefficient of $5.47 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ at 280 nm was used.^[40] The denatured DAFP-1 with completely reduced disulfide bonds was prepared following the previously reported methods^[41] and used as a control in this study. Purified DAFP-1 at around 1 mM was incubated in 0.10 M sodium citrate, pH 3.0, and 15.0 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP) at 60 °C for 30 min. The denatured DAFP-1 was then purified using a Sephacryl S-100 gel filtration column connected to an ÄKTA Purifier 10 (GE Healthcare).

Crystallization

Supersaturated m^5U aqueous solutions (0.6 M) were made at 30 °C and filtered as soon as possible. Crystals did not form in the first few days after the temperature of the supersaturated solution was lowered to room temperature. On day 1, 600 μL of 0.60 M m^5U solution was first added to each sample vial, and then 5 μL of water, 0.36 mM DAFP-1, or 0.40 mM denatured DAFP-1 solution was added into the specific vial, respectively. The vials were gently swirled after the additions. The resulting m^5U concentration was 595 mM in each vial and additive/ m^5U molar ratios ($\times 10^{-6}$) were either 0 or 5. The sample vials were left open in the air at room temperature and three observations at least were recorded per day (every 8 hours) until the solutions in all the vials were dry. The above experiments were repeated five times. Optical micrographs were taken under Nikon SMZ800 microscope with a Nikon Coolpix 5400 when the crystallization completed.

Crystallization kinetics

Under the above conditions, only form I crystalline m^5U was able to grow in the absence of DAFP-1; however, in the presence of DAFP-1, form II crystalline m^5U was grown exclusively. The kinetics of crystallization of m^5U form I and form II, respectively, was estimated based on the rate of the weight of the occurring crystals and the initial weight of m^5U in the specific vial. All the liquid in the vial was collected gently and the weight of the vial or the vial with crystals was measured. After the measurement, the collected liquid was put back into the vial and the crystallization continued. The weights were measured on an Ohaus Discovery semi-micro analytical balance at 9 am every day until the crystallization finished. The experiments were repeated on three vials and the average values were reported.

Single crystal X-ray diffraction

Colorless crystal of m^5U form I was mounted on a Cryoloop with Paratone-N oil and data was collected at 90K with a Bruker APEX I CCD using Cu K alpha radiation generated from a rotating anode. In a similar fashion, a colorless crystal of m^5U form II was mounted on a Cryoloop with Paratone-N oil and data was collected at 100K with a Bruker APEX II CCD using Cu K alpha radiation generated from a rotating anode. For both crystals data were corrected for absorption with SADABS and structures were solved by direct methods. All non-hydrogen atoms were refined anisotropically by full matrix least squares on F^2 . Hydrogen atoms on O and N atoms were found from Fourier difference maps and were refined isotropically with distances of O-H and 0.85(0.02) or 0.86 (0.02) Å and N-H distance 0.87(0.02) Å and at 1.20 or 1.50 Ueq parent atom. All other hydrogen atoms were placed in calculated positions and refined as riding models with C-H distances of 0.950 Å

(CHar), 1.000 Å, (CH), 0.990 Å (CH₂), 0.980 Å (CH₃) and at 1.20 or 1.50 Ueq of parent C atom. Flack parameters for m⁵U forms I and II were −0.0187 and −0.1358. Bruker suite of X-ray data collection (APEX2 and SAINT), and Shelrick's processing and refinement programs (SHELXS97, SHELXL97, SHELXTL) were used for these structural determinations.^[42] The crystallographic data of m⁵U forms I and II have been deposited in the Cambridge Database (CCDC) and the CCDC deposit numbers are 916820 and 916088, respectively.

Differential scanning calorimetry (DSC)

All experiments were performed with a DSC 1 (Mettler Toledo, OH). An indium standard (Mettler Toledo, OH) was used to calibrate the instrument. Samples weighed 3–4 mg and were hermetically encapsulated in standard aluminum sample pans with pierced lids to release any pressure build-up during the experiments. The samples were heated from 25 to 200 °C at a rate of 5 °C/min.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Optical micrographs of the finally achieved m^5U crystals: (a) needle-shaped m^5U hemihydrates (form I); (b) block-shaped m^5U dihydrates (form II) obtained in the presence of DAFP-1.(c) needle shaped m^5U hemihydrate (form I) obtained in the presence of denatured DAFP-1 (a control).

Figure 2.
Representative molecules in (a) m^5U form I (hemihydrate) and (b) m^5U form II (dihydrate).

Figure 3. Overlay of unique molecules in the crystal structure of m⁵U form I (all are in gray) and form II (all are in gray except that hydrogen atoms are in a lighter gray color).

Figure 4. Packing diagrams of m⁵U form I (a and b) and form II (c and d). Dotted lines represent intermolecular hydrogen bonding in the crystals.

Figure 5.
DSC thermograms (exo up) of form I (a) and form II (b) of m^5U .

Figure 6.
Comparison of relative enthalpies for the phase transitions of form I and form II m⁵U.

Figure 7.

Comparison of crystallization kinetics of form I and form II of m^5U . Mass increase of crystals during the crystallization processes was used to estimate relative crystallinity.

Figure 8.
Suggested mechanism for crystallization of m⁵U (a) and selective crystallization of m⁵U form II using DAFP-1 (b).

Table 1Crystallographic Data for the Hemihydrate (Form I) and Dihydrate (Form II) of m⁵U

Parameter	Form I	Form II
Formula	C ₁₀ H ₁₄ N ₂ O ₆ · 0.5H ₂ O	C ₁₀ H ₁₄ N ₂ O ₆ · 2H ₂ O
Formula weight	267.24	294.26
Temperature (K)	90(2)	100(2)
Crystal system	orthorhombic	orthorhombic
Space group	<i>P</i> 2 ₁ 2 ₁ 2	<i>P</i> 2 ₁ 2 ₁ 2 ₁
<i>a</i> /Å	13.953(2)	6.75400(10)
<i>b</i> /Å	17.201(3)	8.83730(10)
<i>c</i> /Å	4.8017(7)	20.9847(3)
<i>a</i> = <i>β</i> = <i>γ</i>	90°	90°
Cell volume/Å ³	1152.4(3)	1252.52(3)
Calc density/ g cm ⁻³	1.540	1.560
<i>Z</i>	4	4
Data / restraints / parameters	1941 / 5 / 184	2199 / 10 / 206
Final <i>R</i> indices for <i>I</i> > 2σ(<i>I</i>)	<i>R</i> ₁ = 0.0274 <i>wR</i> ₂ = 0.0730	<i>R</i> ₁ = 0.0282 <i>wR</i> ₂ = 0.0741

Table 2
Geometrical Parameters for Hydrogen Bonding Interactions in Forms I and II

	D-H...A [a]	d(D-H) (Å)	d(H...A) (Å)	d(D...A) (Å)	<(DHA) (°)
Form I [b]	O(4)-H(4O)...O(5) ⁱ	0.886(15)	1.881(15)	2.7658(15)	175.3(17)
	O(5)-H(5O)...O(2) ⁱⁱ	0.840(15)	1.887(15)	2.7158(15)	168.7(18)
	O(6)-H(6O)...O(1) ⁱⁱⁱ	0.833(15)	1.890(15)	2.7097(14)	167.6(19)
	O(7)-H(7O)...O(6)	0.860(14)	2.023(14)	2.8722(13)	169.2(18)
	N(2)-H(2N)...O(7) ^{iv}	0.889(14)	2.106(15)	2.9764(13)	166.0(17)
Form II [c]	O(4)-H(4O)...O(8)	0.838(16)	1.853(16)	2.6902(15)	177(2)
	O(5)-H(5)...O(7) ⁱ	0.86(2)	2.09(2)	2.8868(15)	154.2(19)
	O(6)-H(6O)...O(2) ⁱⁱ	0.865(16)	1.957(17)	2.7954(16)	163(2)
	O(7)-H(7WB)...O(5) ⁱⁱⁱ	0.846(15)	2.073(15)	2.9103(17)	170.0(19)
	O(8)-H(8WA)...O(5) ^{iv}	0.848(15)	2.041(16)	2.8510(16)	159(2)
	O(8)-H(8WB)...O(1) ^v	0.862(15)	1.926(15)	2.7783(16)	169.9(19)
	N(2)-H(2N)...O(7)	0.854(14)	2.049(15)	2.9016(16)	177.2(19)

[a] D = Donor, A = Acceptor.

[b] Symmetry transformations used to generate equivalent atoms: [i] $-x+1, -y+1, z$; [ii] $-x+1/2, y-1/2, -z+1$; [iii] $x-1/2, -y+3/2, -z$; [iv] $-x+1/2, y+1/2, -z+1$.

[c] Symmetry transformations used to generate equivalent atoms: [i] $-x+3/2, -y+1, z-1/2$; [ii] $-x, y+1/2, -z+1/2$; [iii] $-x+1, y+1/2, -z+1/2$; [iv] $x+1/2, -y+1/2, -z$; [v] $-x+2, y-1/2, -z+1/2$.