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The Study of Crystallization of Estrogenic 17β-Hydroxysteroid Dehydrogenase with DHEA and DHT at Elevated Temperature

Qing Han and Sheng-Xiang Lin[1]
Medical Research Council Group in Molecular Endocrinology and Oncology, CHUL Research Center and Laval University, Ste-Foy, Quebec, Canada, G1V 4G2

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Most crystallization experiments of macromolecules are carried out at a constant temperature. Room temperature (22°C) and 4°C are the most widely used settings in crystallization. In practice, crystal growth at relatively high temperatures has often been avoided for macromolecular crystallization. Human estrogenic 17β-hydroxysteroid dehydrogenase has been crystallized in complex with dehydroepiandrosterone or dihydrotestosterone. The crystallization experiments were carried out at 27°C. The 17β-HSD1 crystals were greatly improved at the elevated temperature. The effects of higher temperatures on crystal growth were studied. High temperatures stimulated the nucleation of 17β-HSD1, increased the rate of crystal growth, and higher occupancy of substrates was obtained in the crystal structure. This method also reduced the formation of twin crystals. Since temperature is the easiest factor to control in the laboratory, crystallization at elevated temperatures provides an efficient method to improve protein crystal growth. The mechanism of the effect of temperature and relative techniques are discussed.

17β-hydroxysteroid dehydrogenase type 1 (17β-HSD1) is widely distributed in human tissues. The enzyme principally catalyses the formation of estradiol (E_2) from estrone (E_1) and 5-androstene-3, 17-diol (Δ^5-dione) from dehydroepiandrosterone (DHEA). It also weakly catalyses the interconversion of dihydrotestosterone (DHT) and androstanedione (A-dione), testosterone (T) and androstenedione (Δ^4-diol), as well as 20α-dihydroprogesterone and progesterone (1–3). DHEA is the most abundant steroid precursor secreted by the adrenal gland in man and in some primates (4). It is converted into androgens and estrogens in the peripheral tissues. About 50% of androgens in men and 100% of the estrogens in postmenopausal women are synthesized from DHEA in peripheral intracrine tissues and in local tissues (5). DHT is the major active androgen in certain organs (prostate, seminal vesicles, skin, etc.). E_2 and DHT have important roles in the proliferation of breast cancer and prostate cancer cells, respectively (6, 7). Thus, a detailed study of the complex structures of 17β-HSD1 with DHEA and DHT and of the enzyme specificity toward these steroids is important to both endocrinology research and eventually to the therapy of hormone-sensitive cancers.

It is generally known that temperature is a major parameter in biological macromolecule crystallization (8). Most crystallization experiments are carried out at a constant temperature. Room temperature and 4°C are the most widely used settings in crystallization. In practice, crystal growth at relatively high temperatures has often been avoided for macromolecular crystallization. There are only limited examples of experiments performed at temperatures higher than room temperature. The Biological Macromolecule Crystallization database currently contains over 4000 crystallization conditions which includes 2000 different biological molecules, however only very few entries concern crystallization at relatively high temperature, and most of them use the slow cooling technique. In text books devoted to macromolecular crystallization, there are no detailed examples or comments about crystal growth at relatively high temperatures (8, 9). However, a few examples were found in the literature. In the case of lysozyme crystallization, two crystal forms were grown at two different temperatures (10). A tetragonal form was obtained in the presence of NaCl at pH 4.5 at room temperature, while an orthorhombic form was grown at 28°C with the same crystallization solution, thus indicating the important role of temperature on protein crystallization. Very recent publications reported that the subtilisin and myoglobin proteins and

[1] To whom correspondence should be addressed. Fax: (418) 654-2761. E-mail: sxlin@crchul.ulaval.ca.

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also a pokeweed antiviral protein from seeds were grown at temperatures above 30°C, thus indicating that protein crystals can be grown at relatively high temperatures, which can be a useful factor in macromolecular crystallization (11, 12).

The first diffraction quality crystals of 17β-HSD1 were grown at room temperature and reported in 1993 (13). DHEA and DHT are not cognate substrates of 17β-HSD1 since the enzyme has a low specificity towards these steroids (14). In addition, because of the hydrophobic nature of the steroid molecule, the solubility of DHEA and DHT in aqueous solution is only about 40 μM. Both of these factors have made it difficult to grow complex crystals with a high occupancy of steroids in the binding site. Several crystal structures of 17β-HSD1 complexed with different steroid hormones have been determined in our group, but in some noncognate complexes the electron densities associated with the steroids were too weak to define their binding positions. Therefore, we attempted to improve the quality of the crystals to achieve high occupancy levels through the use of high temperatures. Here we report the crystallization of 17β-HSD1 at the elevated temperatures, which resulted in significant improvements in crystal growth rate, nucleation, reproducibility and quality of complex crystal.

MATERIALS AND METHODS

Materials. DHEA (dehydroepiandrosterone), glycerol, β-OG (β-octyl glucoside), NAD^+, LiCl, NaCl, $NaHCO_3$-Na_2CO_3, PEG (polyethylene glycol) 3500, Tris-base [Tris–(hydroxymethyl) aminomethane], Hepes [*N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid], EDTA (ethylenediaminetetraacetic acid) and PMSF (phenylmethanesulfonyl fluoride) were all purchased from Sigma (St. Louis, MO). DTT (dithiothreitol) was obtained from Aldrich (Milwaukee, WI). Blue-Sepharose CL-6B and phenyl-Superose HR 10/10 columns were packed in our laboratory using media from Pharmacia Biotech (Montreal, Canada), and the Mono-Q HR 5/5 column was purchased from the same company. All reagents were of the best grade available. Centricon-30 and Centri-prep-30 concentrators were purchased from Amicon (Beverly, MA) and the incubator was from Sigma.

Preparation of 17β-HSD1. 17β-HSD1 was purified from human placenta using the procedure described previously consisting of three chromatographic steps: Q-Sepharose anion exchange, Blue-Sepharose affinity and phenyl-Superose hydrophobic interaction columns (15, 16). The chromatographic steps were carried out using a fast protein liquid chromatography (FPLC) system. The homogeneous preparation was concentrated to 10 mg/ml with Centricon in a buffer containing 20% glycerol, 1 mM EDTA, 0.4 mM DTT, 0.5 mM PMSF, 0.06% β-OG and 40 mM Hepes at pH 7.2. It was kept at room temperature prior to use.

Crystallization experiments. Crystallization experiments were carried out in a Linbro plate with the vapor-diffusion technique. The Linbro plates were kept in the incubators controlled at different temperatures. The protein solution contained 10 mg/ml 17β-HSD1 in the above mentioned enzyme buffer. The reservoir solution contained 0.1 M Hepes, 20% glycerol, 24–30% PEG, 0.1–0.18 M $MgCl_2$. Hanging drops were produced by mixing 2.5 μl protein solution and 2.5 μl reservoir solution.

Complex formation. It is well known that steroids have a very low solubility in aqueous solutions, for example, approximately 45 μM for DHEA. Crystallization of the enzyme with hydrophobic and noncognate steroid substrates has proven to be extremely difficult. Since the enzyme concentration used for crystallization is usually higher than 10 mg/ml, corresponding to more than 160 μM 17β-HSD1 and 320 μM in steroid binding sites, it was not possible to simply mix the enzyme and the substrate and proceed with the crystallization (17, 18). Therefore, special procedures were developed to increase the substrate concentration. This included first adding saturated DHEA or DHT solutions into the protein solution at low concentration, followed by gradually increasing the concentration of the protein-steroid solution via Centricon concentration (18). Then, new buffer saturated with DHEA or DHT was added into the protein solution at low concentration. Eventually, after several addition and reconcentration steps, 17β-HSD1 became fully saturated with the DHEA or DHT in the solution. Because DHEA and DHT could be more easily dissolved in 30% PEG, we were able to saturate the precipitant solution with about 1 mM DHEA or DHT. Using these approaches, the enzyme in complex with these two substrates (DHEA and DHT) was obtained and the occupancy of these steroids at their respective binding site was effectively increased.

Data collection. Single crystals of the DHEA-enzyme complex were mounted in a glass capillary tube for data collection using an R-axis II C image-plate area detector on a Rigaku RU-200B rotating-anode X-ray source. CuKa radiation (50 kV, 180 mA) with a normal focus of 0.5 mm $\times$ 10 mm filament at ambient temperature was used. The R-axis IIc detector was set at 100 mm from the crystal and the beam was collimated to 0.5 mm. The data for the DHT-enzyme complex were collected using a MAR 30 cm image plate with beam line X8C at the National Synchrotron Light Source (NSLS), Brookhaven Laboratory (Upton, NY). Data collection was performed at a temperature of 100 K and the detector was placed 197 mm away from the crystal. All the data sets were processed with the HKL Software package.

Structural refinement. The structure was built from a Difference Fourier map using the phases from the structure of native human estrogenic 17β-HSD (19) as an initial model. The refinement of the model was initiated with X-Plor at 10.0–3.0 Å resolution and then developed to high resolution data. For detailed information refer to the structural paper (14).

17β-HSD1 assay. The enzyme was assayed by monitoring the absorption increase at 340 nm from NAD reduction following the oxidation of estradiol at room temperature (22 $\pm$ 1°C). The reaction mixture contained 0.5 mM NAD and 25 μm estradiol in 50 mM diethanolamine buffer, pH 9.1. One unit of enzyme is the amount of enzyme required to catalyze the formation of 1 μmol of estrone in 1 min under the above conditions.

RESULTS

Significantly Higher Nucleation Rate and Crystal Growth Rate Can Be Achieved at Elevated Temperatures

17β-HSD1 is usually crystallized at room temperature under crystallization conditions previously determined in our laboratory (13). When experiments at 4°C were attempted, no crystals were obtained. However, when experiments were carried out at 25, 27, or 30°C, some surprising results were observed. The crystal growth rate at these temperatures was significantly higher than that observed at room temperature (22°C). Microcrystals could be observed in 4–7 h in those experiments in which the initial drop contained only 5

mg/ml 17β-HSD1, and 14.5% PEG 4K, 0.05 M $MgCl_2$, 0.03% β-OG. The well solution contained 0.1 M $MgCl_2$, 29% PEG 4K, and 0.1 M Hepes. In identical experiments performed at 22°C, the microcrystals usually appeared four days after the initiation of crystallization. The crystals also grew faster at the higher temperatures. In experiments carried out at 27°C, crystals with final volume of $0.3 \times 0.3 \times 0.2$ mm^3 were obtained after only four days, whereas we needed about two weeks to grow similar sized crystals at 22°C. Based on our experience, a fast growth of 17β-HSD1 crystals at room temperature with stronger crystallization conditions than usual will induce some defects in crystal shape and quality. However, crystals grown at higher temperatures with a fast growth rate do not have these problems. Perfect crystals could even be obtained in two or three days. We first grew crystals with the enzyme alone, and then we grew the crystals complexed with DHEA, DHT and inhibitors. Crystallization experiments were also carried out at 25°C and 30°C and in both cases similar results to those at 27°C were obtained.

High Temperature Increases the Reproducibility of 17β-HSD1 Crystals

We have been working on the crystallization of 17β-HSD1 since 1991. In our experiments, we have used 17β-HSD1 samples purified from human placenta and also recombinant protein from the baculovirus cell expression system later on. Although a very good purification procedure has been developed, some times the crystallization conditions can not be easily determined for a new batch of sample. In order to optimize crystallization conditions under which the stability of the protein is preserved and crystallization can be routinely achieved, a small screen of 16 different crystallization solutions was carried out. Based on a large number of experiments, we found that 17β-HSD1 crystallization became easier at higher temperatures. At 27°C we obtained crystals with all the batches of purified protein samples. When some droplets which would not crystallize at room temperature were moved into an incubator at 27°C, in many cases crystallization did start and proceed properly. These results indicate that the higher temperature facilitates nucleation and crystallization of 17β-HSD1. We studied this further and also found that the elevated temperatures can recover activity that 17β-HSD1 had lost during storage or purification. This phenomenon was reported in a biochemical study of 17β-HSD1 (20). There are multiple stages which could induce slow denaturation or nonspecific aggregation during the purification process or during storage. It has been proven that loss of activity of 17β-HSD1 is induced by aggregation (20, 21, Han *et al.*, unpublished). High temperature may thus recover or help maintain activity by preventing aggregation. This preserves protein homogeneity which is very important for proper crystallization, particularly for nucleation (22).

High Temperature Reduces Twin Crystals

Twin crystals are often formed during the 17β-HSD1 crystallization process and is an obstacle to the production of high quality crystals. One of the reasons why the microgravity experiments of 17β-HSD1 crystallization aboard MIR were carried out, was to help overcome this problem (23). We also developed new techniques to improve crystal growth (24, 25). It was found that higher temperatures can reduce the formation of twin crystal of 17β-HSD1. Even in fast growing crystals, the number of twin crystals was decreased while single crystal percentage was increased, particularly in the presence of 2.0–3.5% MPD in the protein solution. Figure 1 shows high quality crystals of DHEA and DHT complexes obtained at 27°C.

In those experiments where the crystals were grown in complex with steroid hormones at 27°C, we obtained high resolution and very low Rsym. The data of some crystals of the DHEA complex collected reached a high resolution of 2.0 Å. Using the home rotating anode source, a 2.3 Å 95% completed data set was collected at room temperature. One crystal complexing DHT gave perfect diffraction spots and a 2.2 Å data set was collected at beam line X8C at the NSLS (National Synchrotron Light Source), Brookhaven National Laboratory (Fig. 2, Table 1). Here the Rsym was only 3.8%, details of data statistics are given in Table 1.

High Temperature Is Beneficial for Increasing the Occupancy of Substrates in the Crystal Form with 17β-HSD1 Complexes

17β-HSDs can catalyze the conversion of estrogen and androgen hormones which have very different activities. The role of the different steroid hormones is very important and we are very interested in the mechanism of discrimination of 17β-HSD1 (14, 26). DHEA and DHT are poor substrates for 17β-HSD1. The K_m of 17β-HSD1 increases by more than a 1000-fold from estrone to DHEA, and 10-fold from estradiol to DHT (14). Using cocrystallization or soaking methods at room temperature, we have grown a number of 17β-HSD1 crystals complexed with different steroid substrates or inhibitors. Some of these ligands have a similar binding affinity to DHEA and DHT with 17β-HSD1. However, in several cases, as a result of the poor electron densities associated with the substrates, we could not define the precise location of the steroid. This was due to the low occupancy of steroids in the crystal. To overcome this problem, we carried out cocrystallization experiments at 27°C with the aim of improving the occupancy of the steroids. Currently,

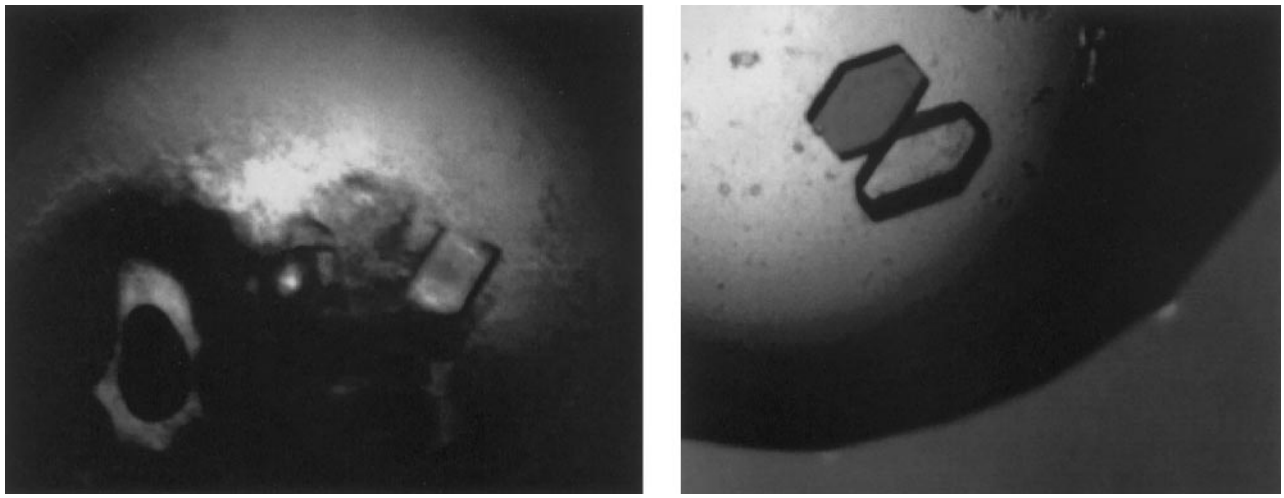

FIG. 1. The crystals of DHT and DHEA complexes. (a) The crystal was obtained in five days in the solution containing DHEA, 24% (w/v) PEG (3500), 0.12 M MgCl2, 0.08 M Hepes pH 7.5, and final protein concentration was 12 mg/ml at 27°C. (b) The crystals were obtained in four days in the presence of DHT, 29% (w/v) PEG (3500), 0.12 M MgCl2, 0.08 M Hepes pH 7.5, and final protein concentration was 9 mg/ml at 27°C. The crystal size is 0.2 mm × 0.2 mm × 0.15 mm.

two structures for the 17β-HSD1 complexed with DHEA or DHT have been solved. The electron density of DHEA or DHT in the refined structures are significantly stronger than any of the other structures that were grown at room temperature under similar conditions. The omitted F_O–F_C map of DHEA was well de-

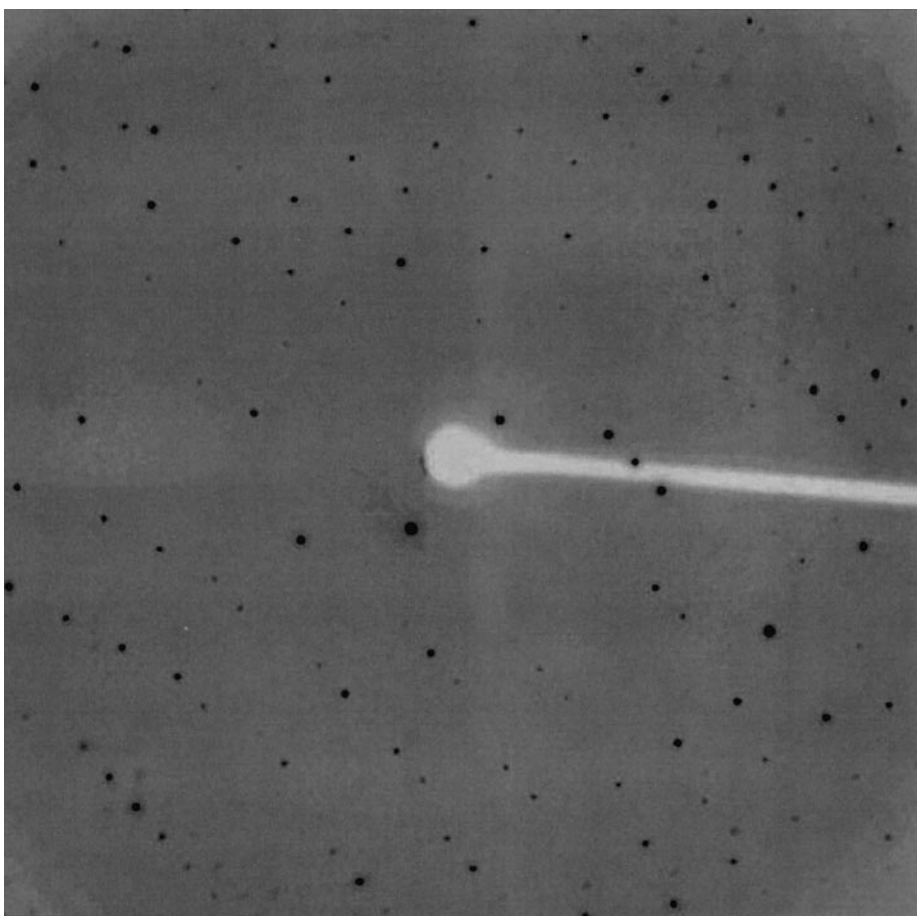

FIG. 2. Diffraction pattern of crystal of DHT complex that was grown at 27°C.

TABLE 1

Data-Collection Statistics for Crystal of DHT Complex

Resolution shell (Å)	Number of unique reflections	Completeness (%)	R_{merge} [†] (%)
100.00–4.85	1524	96.5	0.025
4.85–3.85	1480	97.4	0.025
3.85–3.36	1483	97.9	0.036
3.36–3.05	1482	98.1	0.042
3.05–2.83	1469	98.1	0.054
2.83–2.67	1473	97.8	0.062
2.67–2.53	1446	97.7	0.080
2.53–2.42	1476	98.3	0.090
2.42–2.33	1458	97.7	0.113
2.33–2.25	1401	94.0	0.133
All hkl	14692	97.4	0.038

[†] $R_{merge} = \Sigma_{hkl}\Sigma_i|I(hkl)_i - \langle I(hkl)\rangle|/\Sigma_{hkl}\Sigma_i\, I(hkl)_i$.

fined (14). In the structure of the DHEA complex, an important intermediate conformation of DHEA reduction was revealed. Thus, from both theory and experimental results, we may therefore consider that higher temperatures can enhance substrate and inhibitor occupancy in the crystals when crystallization is improved. Higher substrate occupancy is an essential condition if we are to define its binding position by crystallography.

The crystals of DHEA complexes were grown at 27°C using the vapor diffusion technique. Linbro plate and data collection was performed at room temperature. The crystals of DHT complex were grown at 27°C, but they were transferred to room temperature at the synchrotron site at Brookhaven National Laboratory just before data collection was performed at 120 K. We compared the 3D-structures of DHEA and DHT complexes with that of E2-17β-HSD1 crystals grown at room temperature (17). Superposition of these molecules showed a very good match for most atoms of backbones of the three structures. The conformation of most side chains located at the interior of the molecule also showed similar orientation. However, the side chains located at the solvent accessible surface showed a significant difference, indicating the flexibility of these chains.

A Critical Point for the Technique of Crystallization at High Temperature

Temperature has a great (great instead of important) effect on the rate of vapor diffusion. In our early experiments, when crystallization was performed in Linbro plates at a relatively high temperature in the incubator, the volume of the droplets often became bigger after vapor diffusion. In this case, it was difficult to obtain crystals because the protein could not reach supersaturation. This problem was carefully examined and we found that the temperatures between the bottom and the middle of the incubator were different. The temperature of the plates at the bottom, near the heat source, was higher than that at other places in the incubator. If the Linbro plates were put at the bottom of the incubator, the temperature of the reservoir would be 1–2°C higher than the temperature of the droplet, and this temperature difference was enough to increase vapor diffusion of the well solution. Thus, the volume of the droplets was increased by absorbing the water vapor. In conclusion, when the experiment is carried out in Linbro plates, the reservoir solution and the droplets should be at precisely the same temperature. Otherwise, the equilibrium between the droplet and the reservoir will not only depend on the supersaturation of the sample, but also on the temperature differences. In our later experiments the Linbro plates were placed in the middle of incubator to obtain uniform temperature. The adequate control of temperature is critical for the crystal growth process.

DISCUSSION

The homogeneity of the macromolecule conformation is of major importance to nucleation and crystal growth. The influence of macromolecular heterogeneities on the nucleation process was clearly demonstrated in several experiments (9, 27, 28). Proteins with a reduced biological activity can result from a conformation change of the active site, molecular denaturation, or aggregation. Enzyme molecules can exist in different conformations which are in equilibrium with one another. Therefore, the concept of purification in crystallization should include the conformational homogeneity of protein itself (22). The activity of 17β-HSD1 could be recovered at elevated temperatures after a period of cold inactivation, suggesting that the low activity form could be converted into the high activity form by reversing the aggregation (20). This preference for relatively high temperatures may be a reason why 17β-HSD1 is easier to crystallize at higher temperatures than at room or lower temperatures. Many enzymes show higher and stable activity at a relatively higher temperature. It may be advantageous to crystallize them under such conditions.

The creation of supersaturation is a necessary condition for crystal growth. The concentration of protein and precipitant in the initial droplet are normally below the supersaturation limit required for nucleation and crystal growth. High temperature increases the rate of vapor diffusion and shortens the time required for equilibration between the drop and the reservoir. Thus, with the vapor diffusion method, the rate of crystal growth will increase at elevated temperatures.

Heat denaturation will happen when the temperature is somewhat higher than the preferred tempera-

ture for the enzyme. Increasing the temperature may not be a favorable condition for those proteins which are sensitive to high temperatures. To decrease the effect of possible denaturation, the experiment should be designed to produce crystals in no more than several days.

Usually, proteins are not stable at high temperatures, and neither are the crystal lattices. Crystals stored for a long period of time become more easily damaged at elevated temperatures than at lower temperatures. Another potential problem is that the crystallization cells sealed with vacuum oil may become loose at high temperatures and this may induce drying of the well solution and of the droplet. Thus crystals grown at high temperatures should be used as soon as possible, moved to a lower temperature or should be frozen after they are fully grown.

Denaturation of the enzyme leads to heterogeneities, which in turn can induce strain in packing and defects during crystal growth or even lead to the cessation of crystal growth. Reagents which protect the proteins from inactivation should therefore be used in the crystallization solution. The structure-stabilizing agents that we currently use with 17β-HSD include glycerol, PEG, and DTT. The use of these agents is rapidly growing in crystallization experiments thus allowing more protein crystal growth at elevated temperatures.

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