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Crystal Growth of Human Estrogenic 17β-Hydroxysteroid Dehydrogenase

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Abstract

Estrogenic 17β-hydroxysteroid dehydrogenase from human placenta, an enzyme of low solubility, has been crystallized in the complex form with its cofactor $NADP^+$. These are the first crystals with X-ray diffraction quality for structure analysis from any human steroid-converting enzyme. The crystals were grown by vapor diffusion in the presence of 0.06% β-octylglucoside, using polyethylene glycol 4000 as the precipitant (27-28%) and one of several different salts at pH 7.5 and room temperature. Crystals grown with magnesium chloride diffract up to 2.4 Å. The most important steps leading to the rapid success of the crystallization of this labile enzyme were the following: preparation of a highly active and homogeneous enzyme protein using a rapid procedure; the choice of a suitable enzyme buffer system and a detergent favorable to maintaining high activity and solubility for the enzyme; and a combined screening procedure. The present study could be useful for the successful crystal growth of other hydrophobic or membrane-bound proteins.

Introduction

The crystallization of human 17β-hydroxysteroid dehydrogenase (17β-HSD) has been attempted since the 1970's (Chin, Dence & Warren, 1976). Because this enzyme plays an important role in estrogen conversion and breast cancer proliferation, determination of its three-dimensional structure is critical to drug design in breast cancer therapy.

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Chin and colleagues (Chin, Dence & Warren, 1976) obtained crystals of 17β-HSD with a technique of 'electrophoretic diffusion' using tris-barbituric acid buffer, pH 7.0, containing 20% glycerol. This technique combines the principles of zone electrophoresis and membrane dialysis. In fact, they had first attempted ammonium sulfate fractionation, equilibrium dialysis and vapor diffusion in their laboratory to crystallize estradiol 17β-HSD, but without success. They then reported obtaining 'a heavy crop of crystals' from the electrophoretically concentrated enzyme solution kept at 277 K overnight using electrophoretic diffusion. However, to our knowledge, no X-ray diffraction data were ever published.

It should be mentioned that 17β-HSD has been studied since the 1950's. Because of its liability, difficulties were encountered in the development of a satisfactory procedure for its homogenization under conditions commonly used for enzyme purification; the presence of its cofactors, 17β-estradiol or glycerol stabilizes the enzyme (Langer & Engel, 1958; Jarabak, Seeds & Talalay, 1966; Jarabak, 1969; Chin, Dence & Warren, 1976; Lin *et al.*, 1992). In fact, 17β-HSD is rapidly inactivated at 273 K in the absence of glycerol and is stable in the presence of 50% glycerol at this temperature for many months. A homogeneous and highly active 17β-HSD protein was successfully prepared from a modification of a rapid purification procedure (Lin *et al.*, 1992, and for comparison of the enzyme specificity from various preparations, please refer to the *Discussion* section of this reference). Contradictory opinions about the identity of the subunits of 17β-HSD have been reported. Jarabak & Street (1971) and Burns, Engel & Bethune (1971, 1972) proposed that the two subunits are probably identical, but Engel & Groman (1972) suggested the existence of three different monomers

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that can interact with each other to form six dimers. Our recent study, using a combined method of enzymology and molecular biology, concluded for the first time that 17β-HSD is formed by two identical 34.5 kDa subunits (Lin *et al.*, 1992). Besides the subunit structure, some studies on the enzyme active site or binding site using chemical modification have been reported (Chin, Murdock & Warren, 1982; Murdock, Chin & Warren, 1986). Nevertheless, the information above is still incomplete and determination of the enzyme structure is required for a proper understanding of its functions.

Thus, the crystallization and determination of the three-dimensional structure becomes critical for further study of this enzyme and for the synthesis of therapeutic inhibitors. We report here the following critical steps in 17β-HSD crystallization: stabilization of the enzyme, a detergent search to increase its low solubility, and combined screening for its crystallization.

Materials and methods

Chemicals

$NADP^+$, NAD^+, glycerol, β-OG (β-octyl glucoside), $MgCl_2$, LiCl, NaCl, Na, K tartrate, PEG (polyethylene glycol) 4000, Tris-base [Tris = tris-(hydroxymethyl)aminomethane], Hepes [*N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid], ADA [*N*-(2-acetamido)iminodiacetic acid], diethanolamine, EDTA (ethylenediaminetetraacetic acid) and PMSF (phenylmethanesulfonyl fluoride) were purchased from Sigma (St Louis, MO, USA); 17β-estradiol and DTT (dithiothreitol) were obtained from Aldrich (Milwaukee, WI, USA); Q-Sepharose Fast Flow and Blue-Sepharose CL-6B columns were packed in our laboratory using media from Pharmacia Biotech (Montreal, Canada), and the phenyl-Superose HR 10/10 column was from the same company. All reagents were of the best grade available. Centricon-30 and Centri-prep-30 concentrators were bought from Amicon (Beverly, MA, USA).

Methods

17β-HSD assay. The enzyme was assayed by monitoring the absorption increase at 340 nm from the NAD^+ reduction following the oxidation of estradiol. The reaction mixture contained 0.5 m*M* NAD^+ and 25 μ*M* estradiol in 50 m*M* diethanolamine buffer, pH 9.1. One unit of enzyme is the amount required to catalyze the formation of 1 μmol of estrone in 1 min under the above conditions [295 (1) K].

SDS–PAGE. Sodium dodecyl sulfate polyacrylamide gel electrophoresis was carried out in the Laemmli discontinuous buffer system (Laemmli, 1970) using the Bio-Rad Mini-PROTEAN II (or PROTEAN II). The samples contained 2–4 μg of each protein for Mini-PROTEAN II (or 4–10 μg for PROTEAN II), 0.5–1% SDS and less than 10% glycerol. The gels were stained with Coomassie blue (Bio-Rad, 1990) or 'high blue staining' from Pharmacia for higher sensitivity, in which 0.05% Coomassie blue, 10% methanol, 7% ammonium sulfate and 9% acetic acid were used for staining.

To check the protein integrity in the crystals, the crystals were washed rapidly several times in the reservoir solution with the help of capillaries before being dissolved and applied on the gel.

Protein concentration measurements. Protein concentration was determined by the optical method of Warburg & Christian (1942), expressed by protein concentration (mg ml^{-1}) = $1.55A_{280} - 0.76A_{260}$. The optical density was determined with a Beckman DU-70 spectrophotometer in which microcells of 50 μl were used. The bicinchoninic acid assay (Pierce Chemical Co., 1989) gave similar but slightly higher readings.

Preparation of 17β-HSD. The purification of 17β-HSD from the soluble subcellular fraction of human placenta was a modification of a procedure described previously (Lin *et al.*, 1992) that consisted of three chromatographic steps: Q-Sepharose anion exchange, Blue-Sepharose affinity, and phenyl-Superose hydrophobic interaction columns. All chromatographic steps were carried out using a fast-protein liquid chromatography (FPLC) system consisting of two P3500 pumps, a UV-M monitor, and an LCC-500 controller from Pharmacia Biotech. A Pharmacia peristaltic pump P50 was used instead of the p1 pump previously used during the sample loading in the Q-Sepharose chromatography. This resulted in a notable acceleration of the purification, as a sample nearly 2000 ml needs to be applied on this column from three placentas. $NADP^+$ replaced NAD^+ in the elution of the affinity chromatography, and the latter was employed in a previously reported procedure (Lin *et al.*, 1992). Our recent spectrometric and fluorometric studies indicated that the homodimer of 17β-HSD molecule binds two $NADP^+$ in the presence of the cofactor at 0.6 m*M* (unpublished results).

Crystallization of 17β-HSD. The crystallizations were carried out with the vapor-diffusion technique in hanging drops. The reservoir contained 0.9 ml of solution including the precipitant (PEG 4000), the salt and sodium azide (2 m*M*), at pH 7.5. The enzyme solution is prepared in a buffer containing 20% glycerol, 1 m*M* EDTA, 0.4 m*M* DTT, 0.5 m*M* PMSF, and 40 m*M* Tris-HCl, pH 7.5, hereafter referred to as buffer *A*. β-OG (0.06%) was added to the enzyme sample just before crystallization *via* centricon buffer change, and at the same time the protein was concentrated to 20–22 mg ml^{-1}. Finally, $NADP^+$ was added to the sample to 0.6 m*M* concentration, and equal volumes (2–3 μl) of the sample and the reservoir solutions were mixed to initiate the crystallization.

Results

Rapid preparation of homogeneous and highly active 17β-HSD

Our preparation was based on FPLC. The procedure is very rapid because of the high flow rate of FPLC media and also because of the optimal simplification of intermediate steps. Moreover, $NADP^+$ elution in affinity chromatography is very specific, thus improving both the homogeneity and the yield of the purification. The total recovery increased to about 40% from 30% using the former procedure (Lin *et al.*, 1992). The enzyme preparation has a high specific activity, which catalyzes the oxidation of more than 8 μmol estradiol per mg of 17β-HSD protein (*i.e.* > 8 U mg^{-1}), which could be due to the elimination of microheterogeneity caused *in vitro* by oxidation-reduction effects or partial proteolysis (Giegé *et al.*, 1986; Lorber *et al.*, 1987). This preparation showed a single band at 34.5 kDa on SDS-PAGE, similar to Lin *et al.* (1992) (Fig. 1*a*).

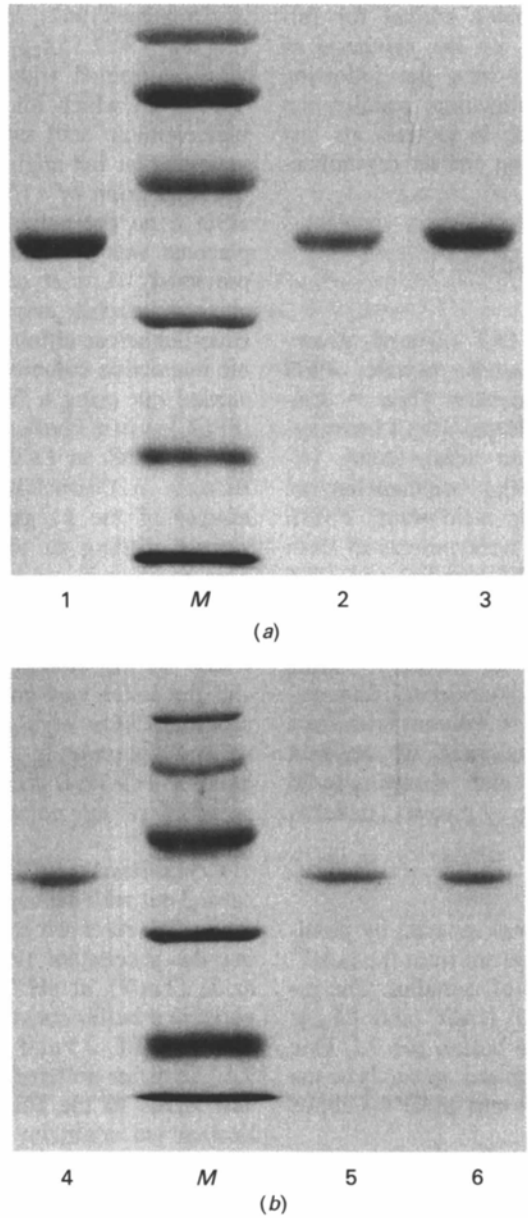

Fig. 1. SDS-PAGE of 17β-HSD directly purified from human placenta or from dissolved crystals. (*a*) 1,2,3: phenyl-Superose fractions from the purification. (*b*) 4,5,6: samples obtained from dissolved crystals of 17β-HSD-$NADP^+$ complex. In both (*a*) and (*b*) *M* indicates the protein standards of 97.4, 66.2, 45, 31 and 21.5 kDa from top to bottom.

Stabilization of 17β-HSD

As noted in the *Introduction*, the presence of 20% glycerol in the buffer system is critical to the stabilization of 17β-HSD. In fact, this glycerol content is necessary for crystallization (see *Screening and crystal growth of 17β-HSD–NADP⁺ complex*, below). In the repeated purification of the enzyme, we also found that inclusion of EDTA is important to yield a highly active preparation, as some metal ions have inhibitory effects on 17β-HSD (unpublished results). A principal buffer (buffer *A*, see *Materials and methods*), containing EDTA, glycerol and PMSF at pH 7.5, was used for the purification procedure and for handling the protein sample for crystallization. The near neutral pH is also important to the enzyme's stability as suggested in the literature, and verified in our experiments (Jarabak, 1969; Chin, Dence & Warren, 1976; Lin *et al.*, 1992). Enzyme activity is stable when stored in buffer *A* at room temperature for more than 10 d; within this period, 17β-HSD crystals are reproducible. Furthermore, when the crystals of 17β-HSD-NADP⁺ obtained up to 1 month earlier were washed several times with the reservoir solution, dissolved, and electrophoresed on SDS-PAGE, a single band was observed, indicating the protein integrity (Fig. 1*b*). Enzyme samples from the dissolved crystals were assayed at the same time, and no significant loss of activity was found. The above control showed that the enzyme is stable in its crystalline form in buffer A in the presence of NADP⁺ and β-OG (see *Screening and crystal growth of 17β-HSD–NADP⁺ complex*, below).

Detergent search to increase 17β-HSD solubility

Our highly active 17β-HSD preparation revealed a solubility of about 3 mg ml^{-1} in buffer *A* at 295 (1) K. This enzyme had a room-temperature solubility of about 1–2 mg ml^{-1} in 0.05 *M* potassium phosphate buffer containing 0.005 *M* EDTA and 20% glycerol, pH 7.0 (Chin, Dence & Warren, 1976).

When assayed with the inclusion of β-OG, a mild non-ionic detergent, at various concentrations, the specific activity of the enzyme remained unaffected in the presence of the detergent from 0.02 to 0.15% in buffer *A*. Within that range of β-OG concentration, 17β-HSD solubility was increased to >40 mg ml^{-1}, or more than tenfold higher than in the absence of the detergent.

Screening and crystal growth of 17β-HSD-NADP⁺ complex

As 17β-HSD has a low solubility that can be increased many fold in the presence of the detergent β-OG, we first used the PEG/NaCl/β-OG model system to screen the complex (Garavito & Picot, 1990). The initial conditions consisted of 5–15%(*w*/*v*) PEG 4000, 0.1–0.3 *M* NaCl, 0.02–0.15% β-OG, and 6–9 mg ml^{-1} as the final concentration for 17β-HSD. Tiny crystals were obtained, and the best ones were grown in the presence of 0.06% β-OG and 7–8% PEG. To complement the above screening, we performed a parallel search with the sparse matrix sampling method (Jancarik & Kim, 1991). Only those with pH > 6.5 of their 50 conditions were used, since 17β-HSD is unstable at lower pH levels. In the hanging drops, the final concentration of 17β-HSD was 15 mg ml^{-1}. Very fine, long needles were obtained in the presence of 1 *M* Na, K tartrate and 0.1 *M* ADA, pH 6.5. Some cubic but multiple crystals were obtained in the presence of 30% PEG 4000, 0.6 m*M* NADP⁺, 0.2 *M* $MgCl_2$ and 0.1 *M* Hepes, pH 7.5. These results were similar to those from the PEG/NaCl/β-OG system, confirming that we were close to optimal crystallization conditions. In all subsequent crystallizations 0.06% β-OG was included.

We call this approach a combined screening, as the parallel search in the two lines can increase the probability of success. In fact, in the case of 17β-HSD, the screening along the line of PEG/NaCl/β-OG and that of sparse matrix sampling complemented each other and improved the results. Based on the above preliminary data, further refinement was carried out with the PEG–$MgCl_2$ system. A series of crystals were obtained under similar conditions, but the best ones were obtained in the presence of 0.15 *M* $MgCl_2$ and 22 mg ml^{-1} protein as the final concentrations in the droplet after equilibrium. Although quite small (typically ~0.05 × 0.05 × 0.15 mm) (Fig. 2*a*), the crystals diffracted remarkably well, to a resolution of 2.7 Å. The best crystals using $MgCl_2$ as salt were recently obtained in the presence of 27–28% PEG, and they grew to a larger typical size of 0.07 × 0.12 × 0.25 mm (maximum 0.15 × 0.18 × 0.36 mm) than using other conditions (Fig. 2*b*). These crystals appeared 3–4 d after setting the drop and grew to the full size in 3 to 4 weeks. They were in monoclinic space group *C*2 with unit-cell parameters *a* =123.03, *b* = 45.03, *c* = 61.29 Å and β = 99.1° (Zhu *et al.*, 1993). The more recent crystals diffracted to 2.4 Å.

The glycerol content (20%) is necessary for 17β-HSD crystallization. No crystals have ever been obtained under similar conditions but with reduced glycerol concentration, *e.g.* at 15%. This requirement might be closely related to the stabilization of enzyme activity (see *Stabilization of 17β-HSD*).

Crystals obtained in the presence of different salts

Crystals of 17β-HSD-NADP⁺ were also obtained in the presence of LiCl (0.2 *M*) instead of $MgCl_2$, 26%(*w*/*v*) PEG 4000 and 40 m*M* Tris-HCl, pH 7.2. After setting up hanging drops, precipitates formed in 1–2 d, and crystals appeared in about 10 d (~0.05 × 0.06 × 0.08 mm) amidst precipitate. These crystals grew to a typical size of 0.2 × 0.2 × 0.3 mm in 3–5 weeks (Figs. 3*a*, 3*b*).

The same complex was also crystallized in the presence of NaCl (0.16 *M*), 26% PEG and 40 m *M* Tris-HCl, pH 7.2 (Fig. 3*c*). Crystal growth was similar to that observed in the presence of LiCl, but crystals appeared more slowly than in the presence of $MgCl_2$.

Discussion

The rapid preparation of homogeneous and highly active enzyme protein played an important role in the crystal growth of 17β-HSD. Our preparation can catalyze the oxidation of about 8 µmol estradiol in 1 min per mg enzyme protein, which is about two- to threefold higher than most reported values for 17β-HSD using conventional chromatography. A detailed comparison of various preparations of 17β-HSD was given by Lin *et al.* (1992).

17β-HSD has a much higher solubility (>40 mg ml^{-1}) in the presence of 0.06% β-OG than in its absence (~3 mg ml^{-1}) while maintaining its high specific activity. The somewhat strong hydrophobicity of 17β-HSD was also demonstrated in phenyl-Superose chromatography (Lin *et al.*, 1992), although the enzyme was isolated from the soluble subcellular fraction of human placenta. The β-OG concentration used here is well below its CMC (critical micelle concentration). This mild non-ionic detergent may play a role to reduce non-specific hydrophobic interactions and encourage electrostatic interactions. That may result in eliminating different states of aggregation of 17β-HSD and favor crystallization, similar to observations with a number of soluble proteins (McPherson *et al.*, 1986). We are studying 17β-HSD's overall cell localization and solubility further, to see whether it has hydrophobic interactions with membranes or other proteins. We have also found that the addition of a cofactor is critical to the enzyme's crystallization.

Using the steps above, crystals of this labile and relatively hydrophobic enzyme were readily obtained. The crystals diffracted to a high resolution of 2.4-2.5 Å. The preliminary X-ray diffraction analysis suggested that the enzyme's molecular twofold axis is coincident with the crystallographic twofold axis along the *b* axis, confirming our previous enzymology study (Zhu *et al.*, 1993; Lin *et al.*, 1992). Further screening for the crystallization of 17β-HSD complexed with various ligands, including substrates and inhibitors, is being carried out. The structure determination of enzyme-ligand crystals is important for a better understanding of 17β-HSD-ligand interactions and eventually will help in the design of efficient inhibitors. As mentioned above, 17β-HSD is essential for the synthesis of the most active estrogen, namely 17β-estradiol. This enzyme is present not only in the ovary, but also in peripheral target tissues, especially the mammary gland, where it is responsible for the local formation of estrogen (Labrie, 1991; Martel *et al.*, 1992). As another experiment, 17β-HSD samples have been sent for crystallization under microgravity on board the Russian MIR station, with the expectation of further improving the crystal quality under reduced convection.

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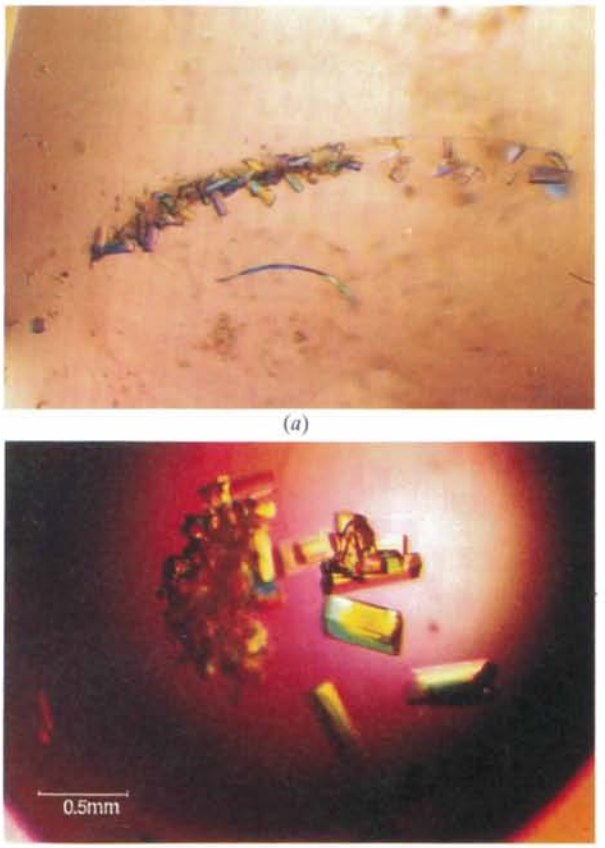
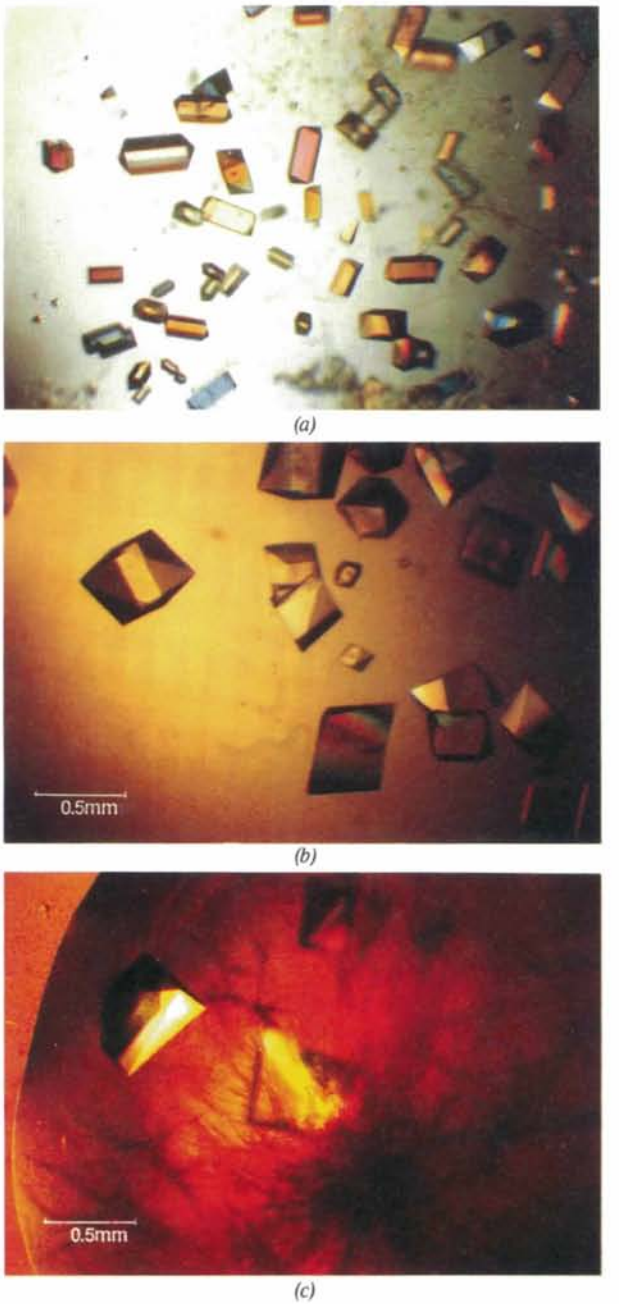


(a) (b)

Fig. 2. 17β-HSD-NADP$^+$ crystals grown in the presence of $MgCl_2$. (*a*) A large number of small cubic crystals were obtained in the presence of 30% PEG 4000, 0.15 *M* $MgCl_2$ and 0.1 *M* Hepes (final concentrations), pH 7.5. The final protein concentration was 22 mg ml^{-1}. The crystals have a typical size of 0.05 × 0.05 × 0.15 mm and diffract to 2.7 Å. (*b*) The same type of crystals grown under similar conditions but in the presence of 27–28% PEG. The typical size is 0.07 × 0.12 × 0.25 mm and the crystals diffracted to 2.4 Å.



(a) (b) (c)

Fig. 3. Crystals grown in the presence of LiCl and NaCl. The same complex crystals were obtained in the presence of 0.2 *M* LiCl or 0.16 *M* NaCl, 26%(*w*/*v*) PEG 4000, at pH 7.2. (*a*) Crystals with a size of 0.05 × 0.06 × 0.08 mm appeared in the presence of LiCl in about 10 d on the background of precipitants. (*b*) The above crystals grew to a typical size of 0.2 × 0.2 × 0.3 mm (maximal 0.25 × 0.40 × 0.45 mm) in 3–5 weeks. (*c*) Crystals grew to about 0.15 × 0.26 × 0.55 mm in the presence of NaCl in 3–5 weeks after drop setting.