CRYSTAL GROWTH AND CRYSTAL IMPROVEMENT STRATEGIES

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Extension of the Diffraction Resolution of Crystals

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Abstract

The diffraction resolution of crystals of the guanine nucleotide-exchange factor complex, EF-Tu-Ts, has been extended from 5.0 to 2.5 Å by lowering the solvent content in the crystals as well as the temperature of data collection. The common form of EF-Tu-Ts crystal belongs to space group $P2_12_12_1$ with a = 81.1, b = 109.9, c = 207.5 Å and has a solvent content of 61%. The crystals diffract to a resolution of 5.0 Å at 293 K and 4.0 Å at 273 K. When cryoprotective agents are slowly diffused into the crystals, the cell constants shrink to a = 74.4, b = 109.9, c = 198.7 Å and the solvent content falls to 55%. After the cryoprotective agent has been added, the crystals diffract to 2.7 Å resolution at 293 or 273 K and 2.5 Å at 250 K. X-ray diffraction data, collected before and after the transformation of individual EF-Tu-Ts crystals, demonstrate that a large percentage of the improvement in diffraction resolution is due solely to the addition of cryoprotective agents. The transfer procedures for the successful introduction of cryoprotective agents into EF-Tu-Ts crystals as well as the general applicability to other crystal systems will be discussed.

Introduction

Escherichia coli elongation factor (EF-)Tu (M_r = 43 643) is a guanine-nucleotide protein which regulates the elongation step of protein synthesis by a GDP/GTP exchange mechanism (Miller & Weissbach, 1977). The primary function of EF-Tu is to recognize, transport and position the codon-specified aminoacyl-tRNA onto the *A* site of the ribosome. EF-Tu exists in the cellular cytoplasm as an inactive GDP complex. A second elongation factor, EF-Ts (M_r = 30 257), effectively catalyzes the exchange of GDP for GTP to activate EF-Tu to a form which recognizes aminoacyl-tRNA. In the process, a binary complex, EF-Tu-Ts, is formed. A similar GDP/GTP mechanism appears to be common among other GTPase proteins, such as signal transduction G proteins and members of the p21 oncogene family. In signal transduction processes across membranes, an activated membrane receptor acts as the exchange factor (Bourne, Sanders & McCormick, 1991). In oncogenesis, soluble cytoplasmic factors serve as the guanine-nucleotide exchange factor (Crechet *et al.*, 1990). Neither the binding site of the exchange factor on the guanine nucleotide-binding protein nor the mechanism of exchange is well understood.

Two crystal forms of *E. coli* EF-Tu-Ts were first reported in 1981 (Leberman, Schulz & Suck, 1981). Both forms crystallized in the same space group, $P2_12_12_1$, with similar cell parameters, but only one form, which diffracted nominally to 5.0 Å, was reproducible. The second form diffracted to better than 3.0 Å and was observed sporadically. Improvement in EF-Tu-Ts complex purity (Yoder, Torres, Corelli & Jurnak, 1985) and extensive crystallization screens of ion type and concentration failed to influence the poor reproducibility of the high-resolution crystal form. Because both crystal forms were sensitive to radiation damage, with half lives of 2 h or less at 293 K, effort was refocused on increasing the crystal stability by low-temperature data-collection methods (Hope, 1990; Petsko, 1975). During the addition of cryoprotective agents to the low-resolution form of EF-Tu-Ts, the crystals were observed to undergo a physical transformation. The transformation was subsequently correlated with a shrinkage of cell parameters, a decrease in solvent content and an extension of the diffraction resolution. The findings are described herein and the applicability of the results to other crystal systems are discussed.

Materials and methods

E. coli EF-Tu-Ts was prepared as previously reported (Yoder, Torres, Corelli & Jurnak, 1985) and all reagents were purchased from Sigma unless otherwise stated. EF-Tu-Ts crystals which diffracted to 5.0 Å were grown by sitting-drop vapor-diffusion methods using Cryschem plates (Morris, Kim & McPherson, 1989) from the Supper Company, Natick, Massachusetts. The largest crystals were grown using microseeding techniques (Fitzger-

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Table 1. *Tested solvents for EF-Tu-Ts crystals*

All solutions are at pH 6.0. All % concentrations are %(*w*/*w*). MES = 2-(*N*-morpholino)ethanesulfonic acid. PEG = polyethylene glycol. pPEG = polyethylene glycol treated to remove impurities as described in Jurnak (1986).

	Solution	Morphology Changes	Cell constants (Å) *a*	*b*	*c*
A	20% PEG 4000 90 m*M* $(NH_4)_2SO_4$ 80 m*M* Na_3 citrate, pH 6.0	Initial solution No changes	81.1	109.9	207.5
B	28% PEG 8000 20% PEG 400 10 m*M* Na_3 citrate, pH 6.0	Orange–brown ordered cracks Reanneals	74.4	109.9	198.7
C	40% PEG 20000 3 m*M* MES, pH 6.0	Orange–brown ordered cracks Reanneals	74.8	110.1	199.8
D	40% PEG 8000 3 m*M* MES, pH 6.0	Orange–brown ordered cracks Reanneals	74.8	110.0	199.3
E	40% PEG 4000 3 m*M* MES, pH 6.0	Orange–brown ordered cracks Reanneals	74.6	109.5	199.4
F	40% pPEG 4000 3 m*M* MES, pH 6.0	Orange–brown ordered cracks Reanneals	74.7	109.8	199.5
G	40% PEG 1540 3 m*M* MES, pH 6.0	Orange–brown ordered cracks			
H	40% PEG 1000 3 m*M* MES, pH 6.0	Orange–brown ordered cracks			
I	40% PEG 400 3 m*M* MES, pH 6.0	Clear ordered cracks Dissolves			
J	40% PEG 200 3 m*M* MES, pH 6.0	Clear ordered cracks Dissolves			

ald & Madsen, 1986). One or more crystals of EF-Tu-Ts were immersed in 1 ml of 20%(*w*/*w*) polyethylene glycol (PEG) 4000 (J. T. Baker), 90 m*M* ammonium sulfate, 80 m*M* trisodium citrate, pH 6.0, and crushed with 20 strokes of a tissue homogenizer. The resulting microseed stock solution was then serially diluted until a 1 µl aliquot of the diluted solution produced one to three large crystals when placed in a 20 µl droplet containing 5 mg ml^{-1} EF-Tu-Ts, 6.4 m*M* Tris-HCl, 67 m*M* trisodium citrate, pH 6.0, 1.3 m*M* 2-mercaptoethanol, 20 m*M* guanine nucleotide monophosphate, 75 m*M* ammonium sulfate, 71 m*M* potassium chloride, 14%(*w*/*w*) polyethylene glycol (PEG) 4000 (J. T. Baker) and equilibrated against a solution containing 80 m*M* sodium citrate, pH 6.0, 90 m*M* ammonium sulfate and 20%(*w*/*w*) PEG 4000. Under these conditions, the microseeds grew to maximal dimensions of 0.4 × 0.5 × 1.0 mm within one week at 293 K.

To alter the diffraction properties, a procedure similar to that described by Ray *et al.* (1991) was used. Each crystal was individually transferred, at 277 K, to a series of 24 droplets in Cryschem plates containing 50 µl of decreasing concentrations of the equilibration solution and increasing concentrations of the cryoprotective mixtures listed in Table 1. A minimum interval of 5 min was used between the crystal transfer from one solution to another.

The diffraction resolution was initially recorded by 'still' photography at 293 K but, as a consequence of crystal degradation in the X-ray beam, only a limited amount of useful data could be collected. To measure cell constants, surviving crystals were mounted in the cryoprotective solution and wedged between fibers to prevent movement (Narayana, Weininger, Heuss & Argos, 1982). To determine cell parameters, three angular orientations of X-ray diffraction data were collected on a San Diego Multiwire System (SDMS) area detector as described below for the flow-cell experiments. To correlate changes in cell parameters and diffraction properties for the same crystal before and after immersion in the cryoprotective solution, crystals were individually mounted between fibers in a flow cell (Wyckoff *et al.*, 1967) in the equilibration solution. The capillary was mounted on a goniometer head and the open ends were attached to two Waters solvent-delivery systems controlled digitally with a Waters 680 Automated Gradient Controller. A partial data set for each single crystal was collected on a dual chamber SDMS area-detector system at 273 K using an FTS Systems dry-air cryostat. Three angular orientations of data were collected to align the crystal zones and to determine the cell parameters. Each orientation included 25 frames with a step size of 0.13° in ω and an exposure time of 45 s per frame using monochromatic Cu $K\alpha$ radiation from a Rigaku RU-200 rotating copper-anode X-ray generator operated at 45 kV and 175 mA. Once aligned, an additional orientation of 200 frames, centered upon a major zone, was collected at the same step size and exposure time. Subsequently, the crystal was converted to the high-resolution form by pumping in a gradient of the equilibration solution and the cryoprotective solution 1 at a rate of 0.02 ml min^{-1} over a 6–12 h interval. The same four orientations of data were recollected under the same conditions after transformation of the crystal to the high-resolution form. The total X-ray exposure for each crystal, before and after transformation, was approximately 4 h. All data were processed by the standard SDMS software (Howard,

Neilsen & Xuong, 1984) and reflections for the zero-level zones plotted to mimic a diffraction photograph using *XRSPACE* (McRee, 1992).

Results

Solutions tested for converting EF-Tu-Ts crystals from the low-resolution to the high-resolution form are summarized in Table 1. The initial solution (*A*) was serially diluted and the final solutions (*B–J*), serially concentrated over 24 steps. Using final transfer solutions *B–H*, the colorless crystals developed parallel pale orange-brown cracks in two of the three crystal faces during the ninth transfer step. The cracks were correlated with the crystal planes with the large changes in cell parameters; the color was apparently a result of optical effects. The colored cracks disappeared after the 21st transfer step when the final transfer solution contained 28% or higher PEG 4000, PEG 8000 or PEG 20 000. The appearance of the crystals during the initial, intermediate and final transfer steps using final transfer solution *B* containing 28% PEG 8000, 20% PEG 400 and 10 m*M* trisodium citrate is shown in Fig. 1. When either 40% PEG 400 or 40% PEG 200 was used as the final transfer solution, crystals developed colorless cracks before dissolving. Using precession photography at 293 K, it was observed that prior to the transfer procedure crystals diffracted to a resolution of 5.0 Å, and after the transfer procedure to a resolution of 2.7 Å. The results are reproducible and have been obtained for approximately 60 crystals.

Cell parameters were measured for those crystals which survived the transfer procedure intact and are summarized in Table 1. Before the addition of cryoprotective agents, the cell parameters were determined to be a = 81.1, b = 109.9 and c = 207.5 Å. After cryoprotective solutions containing PEG 4000, PEG 8000 or PEG 20 000 were diffused into crystals, the cell parameters refined to average values of a = 74.7, b = 109.9 and c = 199.3 Å. Cell parameters were not measured for crystals in the final transfer solutions containing either PEG 1540 or PEG 1000 because of severe cracking, or in solutions containing only PEG 400 or PEG 200 because of crystal dissolution. Similar changes in the cell parameters of the same crystal were observed in flow-cell experiments using a gradient containing the initial solution *A* and final transfer solution *B*. From prior results, the crystals were known to have an X-ray diffraction pattern consistent with space group $P2_12_12_1$. The unit-cell volume, V, is 1.85×10^6 Å^3 for the low-resolution form and 1.62×10^6 Å^3 for the high-resolution form. With two complex molecules in the asymmetric unit (M_r = 73 900) and eight

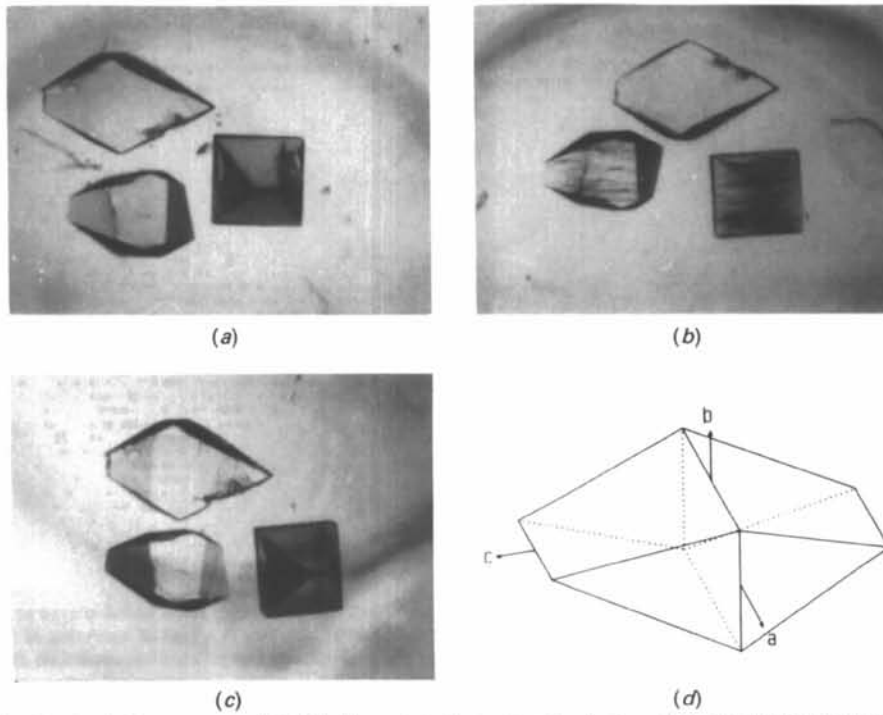

Fig. 1. Alterations in the physical appearance of EF-Tu-Ts crystals during the introduction of the PEG 400/PEG 8000 mixture as described in *Materials and methods*. (*a*) Initial appearance of EF-Tu-Ts crystals viewed down each cell axis. (*b*) Appearance of the same EF-Tu-Ts crystals as in (*a*) after the ninth transfer step. Ordered parallel cracks appear perpendicular to the *a* axis. (*c*) Appearance of the same EF-Tu-Ts crystals as in (*a*) after the 24th transfer step. The ordered cracks have disappeared. (*d*) Schematic drawing of the EF-Tu-Ts crystal morphology showing the relationship of the cell axes.

in the unit cell of $P2_12_12_1$, the value of V_m, the ratio of the V to protein mass (M_p), is 3.13 and 2.75 $Å^3\ Da^{-1}$ for the low- and high-resolution forms, respectively. Using a solvent content (V_s) of $1 - (1.23/V_m)$, V_s changes from 61 to 55% with the introduction of the cryoprotective agents (Matthews, 1968).

The maximum resolution of the data at 273 K was observed to be approximately 4.0 Å before and 2.7 Å after crystal transformation on 'stills' recorded on the area detector. As a consequence of crystal decay in the X-ray beam, it was not possible to collect complete data sets from a single crystal before and after the addition of the cryoprotective solution *B*. Therefore, only limited data sets were recorded to monitor the changes in the diffraction properties of a single crystal. Partial data for the *h0l* and 0*kl* zones are plotted in Fig. 2. A direct comparison of the zero-level zones before and after crystal transformation illustrates the alteration in cell parameters as well as the increase in diffraction resolution. Because the data were collected from the same crystal under identical conditions, the alterations in the diffraction properties are a result of the effect of cryoprotective solution 1 upon EF-Tu-Ts crystals rather than any other parameter such as crystal size or orientation. The flow-cell experiments were conducted reproducibly on several crystals, all with identical results.

Another indication of the change in diffraction properties of EF-Tu-Ts crystals before and after the addition of cryoprotective solution *B* is a plot of the I/σ ratio as a function of the resolution range. As shown in Fig. 3, the average I/σ ratio for the low-resolution crystal form is much lower at all resolution ranges than the ratio for the high-resolution form. The I/σ profile shown in Fig. 3 is typical of partial data sets collected from the same crystal as well as of complete data sets collected from different crystals.

Discussion

Previously, investigators have used cryoprotective agents to depress the freezing point (Hope, 1990; Petsko, 1975) and to improve flash freezing of macromolecular crystals

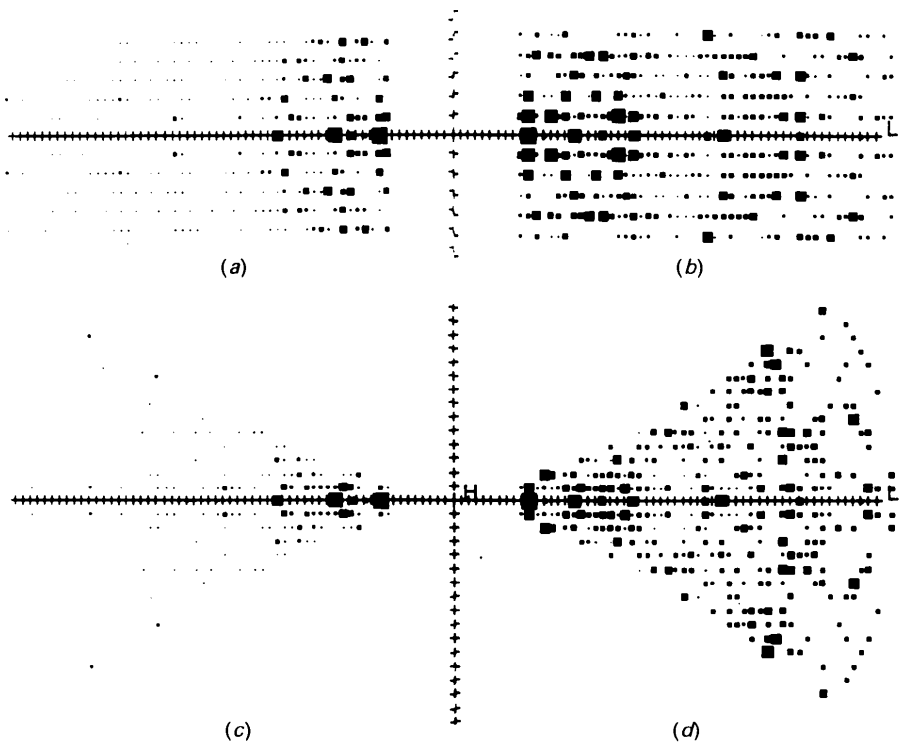

Fig. 2. X-ray diffraction data from a single crystal of EF-Tu-Ts collected on an SDMS area detector at 273 K and displayed as zero level reciprocal lattice zones. Because EF-Tu-Ts crystals are unstable in the X-ray beam, only partial data sets to a limited resolution of 3.4 Å were collected and, therefore, each zero-level zone is incomplete. All data shown in the figures were collected under identical conditions described in *Materials and methods* from the same crystal, before and after the introduction of solution *B via* a flow cell. The reciprocal lattice spacings, which change during the experiment, are indicated on the horizontal and vertical axes. (*a*) *h0l* zone of the low-resolution form of EF-Tu-Ts before addition of the cryoprotective agents. The maximum resolution of the strong diffraction is 8.6 Å. (*b*) *h0l* zone of the high-resolution form of EF-Tu-Ts after addition of the cryoprotective agents. The maximum resolution of the strong diffraction shown in the figure is 3.4 Å but diffraction to a resolution of 2.7 Å was observed. (*c*) 0*kl* zone of the low-resolution form of EF-Tu-Ts before addition of the cryoprotective agents. The maximum resolution of the strong diffraction is 8.6 Å. (*d*) 0*kl* zone of the high-resolution form of EF-Tu-Ts after addition of the cryoprotective agents. The maximum resolution of the strong diffraction shown in the figure is 3.4 Å but diffraction to a resolution of 2.7 Å was observed.

(Teng, 1990; Ray *et al.*, 1991). Many have noted an increase in the crystal lifetime and an improvement in the diffraction resolution of crystals. It is commonly believed that the improvement in resolution is due solely to the stabilizing effect of lower temperature and the ability to record the otherwise short-lived diffraction pattern at higher resolution. The present results are the first to demonstrate clearly that some cryoprotective agents, such as the higher molecular weight PEGs, also have an effect upon the diffraction resolution, independent of the temperature. At 277 K, the addition of the higher molecular weight PEGs to single crystals of EF-Tu-Ts caused an extension of the diffraction resolution from 4.0 to 2.7 Å. Additional, but smaller, improvements of 1 Å or less in the diffraction resolution of both crystal forms could be attributed to a lower temperature. Because a liquid-nitrogen apparatus was not available, it is not known whether further improvements in the diffraction properties could be achieved by flash-freezing techniques.

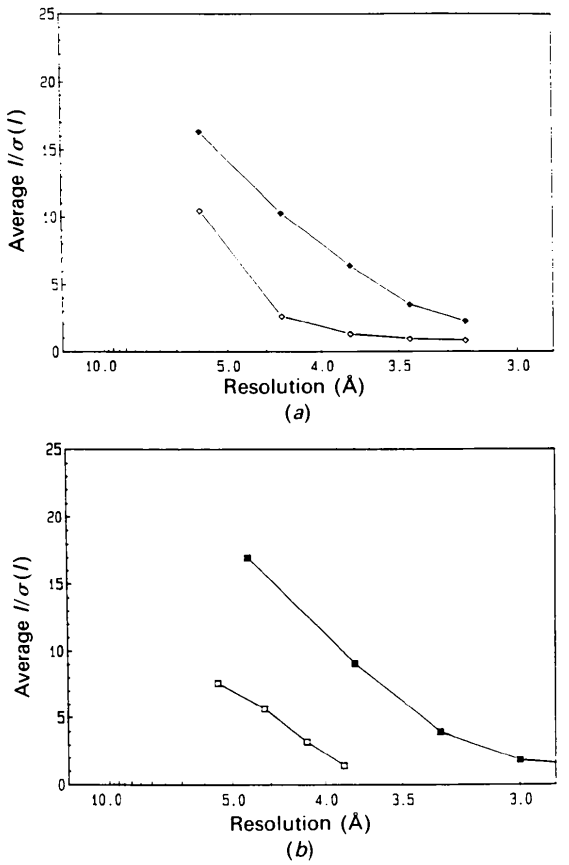

Fig. 3. Plot of I/σ as a function of resolution for the low- and high-resolution forms of EF-Tu-Ts. (*a*) Partial diffraction data set from a single EF-Tu-Ts crystal (◊) before and (♦) after addition of the cryoprotective solution *B*. (*b*) Average of complete diffraction data sets for the low-resolution form (□) of four EF-Tu-Ts crystals and the high-resolution form (■) of seven EF-Tu-Ts crystals.

Interestingly, the extension of the diffraction resolution of EF-Tu-Ts crystals was also accompanied by a decrease in solvent content. Many investigators have noted a similar inverse relationship between diffraction resolution and solvent content in other macromolecular crystal systems. The observed decrease in solvent content of the crystals is due to the use of high concentrations of the higher molecular weight PEGs in the cryoprotective mixture. The reduction in cell size is not due to contaminants in the PEG, because shrinkage also occurs in highly purified PEG 4000 (Jurnak, 1986). The reduction in cell size is not due to the removal of components of the mother liquor, because shrinkage does not occur in solution *A*, which contains only PEG 4000, ammonium sulfate and trisodium citrate, but does occur in solution *B*, which contains trisodium citrate, *E*, which contains PEG 4000, and a solution nearly identical to solution *B* in which 90 m*M* ammonium sulfate was substituted for trisodium citrate (data not shown). The high molecular weight PEGs are well known for their water-withdrawing properties, with PEGs of larger molecular weight being more effective than those of smaller molecular weights (Lee & Lee, 1981; Hermans, 1982). The inability of PEGs 1000 and 1540 to complete the crystal transformation suggests that water activity is a factor in the solvent reduction as well. Another critical factor in the experiments was the 24-step transfer of crystals into the cryoprotective mixtures. Most recently, Ray *et al.* (1991) demonstrated the importance of very gradual changes in the concentrations of additives to crystals to prevent internal osmotic shocks which caused irreversible damage. Because PEG was introduced by gradual transfer techniques, the solvent content was lowered by 6% without crystal damage. Experiments are underway to determine the general applicability of cryoprotective agents to the systematic extension of the diffraction resolution of other macromolecular crystal systems.

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