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Isolation and Crystallization of a Chimeric Qβ Replicase Containing *Thermus thermophilus* EF-Ts

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Abstract—Qβ replicase is a protein complex responsible for the replication of the genomic RNA of bacteriophage Qβ. In addition to the phage-encoded catalytic β subunit, it recruits three proteins from the host *Escherichia coli* cell: elongation factors EF-Tu and EF-Ts and ribosomal protein S1. We prepared a chimeric Qβ replicase in which the *E. coli* EF-Ts is replaced with EF-Ts from *Thermus thermophilus*. The chimeric protein is produced in *E. coli* cells during coexpression of the genes encoding the β subunit and thermophilic EF-Ts. The developed isolation procedure yields a substantially homogeneous preparation of the chimeric replicase. Unlike the wild-type enzyme, the S1-less chimeric replicase could be crystallized. This result facilitates studies on the structure of Qβ replicase and the mechanism of recognition of its templates that can replicate *in vitro* at a record rate.

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Qβ replicase (RNA-dependent RNA polymerase of bacteriophage Qβ) is able to amplify RNA *in vitro* at a record rate, producing up to 10 billion copies of a template RNA during a 10-min incubation at 37°C [1]. Like PCR, the amplification proceeds exponentially: in each cycle of replication, the number of RNA molecules increases by a factor of two as long as the replicase remains in molar excess over RNA [2]. However, Qβ replicase amplifies a very restricted category of RNAs, including the genomic RNA of bacteriophage Qβ and short-chained RQ RNAs (from "Replicable by Qβ replicase") often accompanying the phage as its satellites, but it does not amplify any of the cellular RNAs [1]. Upon selection by the SELEX procedure starting with the diversity of a trillion random sequences of 50-77 nucleotides in length, flanked with the 5′-GGG and 3′-CCC terminal stretches shared by all natural Qβ replicase templates, only a few RNA species appeared to be replicable, and only to a limited extent [3].

Elucidation of the mechanism of RNA replication and, in particular, the template specificity of Qβ replicase is not just of academic interest, but it is also of great practical importance because it would make it possible to design RNA molecules with extremely high ability to replicate *in vitro*. However, despite the fact that Qβ replicase was discovered and isolated in a pure state more than 40 years ago [2], earlier than any other RNA-dependent RNA polymerase, its ability to distinguish between "allied" and "alien" templates is still not understood.

Recently we have found that, while Qβ replicase is extraordinarily selective in RNA amplification, it is very indiscriminate in a one-round template copying. It was found that Qβ replicase recognizes and begins to copy both allied (legitimate) and alien (illegitimate) templates, but only the legitimate templates are capable of triggering the replicase transition into a closed conformation (in the presence of GTP) in which RNA synthesis continues even in the presence of aurintricarboxylic acid, a strong inhibitor of RNA–protein interactions [4]. Furthermore, it turned out that during the initiation of legitimate tem-

Abbreviations: Eco EF-Tu and Eco EF-Ts, *E. coli* elongation factors EF-Tu and EF-Ts, respectively; Tth EF-Ts, *T. thermophilus* elongation factor EF-Ts.

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plates replicase recognizes not only the initiator 3′-terminal, but the 5′-terminal sequence of the RNA as well. In other words, a legitimate template of Qβ replicase has the property of functional circularity [5].

To identify the structural elements of the legitimate templates and the replicase responsible for the recognition and the transition into the closed conformation, one must know the three-dimensional structure of Qβ replicase and its functional states corresponding to the initiation and the post-initiation complexes. But so far the only work devoted to the three-dimensional structure of Qβ replicase has been our work studying the overall morphology of this enzyme using electron microscopy [6]. X-Ray diffraction studies of Qβ replicase were impossible because of the lack of crystals.

The Qβ replicase holoenzyme is a four-subunit protein. One of the subunits (β), bearing the active site of the enzyme, is encoded by the phage genome, whereas the three others are supplied by the host, *E. coli*; these are elongation factors EF-Tu and EF-Ts, as well as ribosomal protein S1, usually involved in protein synthesis. EF-Tu and EF-Ts are firmly bound with the β subunit and form with it a stoichiometric complex, while protein S1 is bound loosely and is not present in every molecule of the enzyme [7], which results in a heterogeneity of the enzyme preparations preventing the crystal formation. Another peculiarity of protein S1 that hinders crystallization is the absence of a rigid compact structure. This, in particular, is the cause of the unusually low sedimentation rate of the four-subunit holoenzyme [8]. While EF-Tu and EF-Ts are required for the synthesis of RNA, protein S1 is only needed for the recognition of the (+)-strand of the genomic Qβ RNA. The core enzyme obtained upon the removal of protein S1 retains the functional activity [9].

Recently, we found that the *E. coli* elongation factor EF-Ts (Eco EF-Ts) in the Qβ replicase molecule can be replaced by its homolog from the thermophilic bacterium *T. thermophilus* (Tth EF-Ts). The resulting chimeric replicase has the same functional characteristics as the wild-type replicase, but it is more thermally stable (N. N. Vasiliev, Z. K. Tnimov, V. I. Ugarov, H. V. Chetverina, and A. B. Chetverin, in preparation).

Here we describe a method for the isolation of a nearly homogeneous chimeric Qβ replicase, both the holoenzyme and the core enzyme, and show that the chimeric core enzyme produces crystals capable of X-ray diffraction.

MATERIALS AND METHODS

Growing cells producing chimeric Qβ replicase. *Escherichia coli* cells BL21(DE3), transformed with plasmids pET-Tth-Ts (prepared by Z. K. Tnimov of the Institute of Protein Research from plasmid pET22b by inserting gene *tsf* from *T. thermophilus* HB8 between restriction sites *Nde*I and *Eco*RI, under the control of the T7 promoter) and pBAD-REP (prepared by V. I. Ugarov of the Institute of Protein Research from plasmid pBAD by inserting the gene encoding the β subunit of Qβ replicase between restriction sites *Nde*I and *Eco*RI, under the control of the arabinose promoter), were grown in two 5-liter flasks containing 1 liter of medium LB, as well as 100 μg/ml ampicillin, 100 μg/ml kanamycin, 25 mM Na_2HPO_4, 25 mM KH_2PO_4, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.5% glycerol, 0.05% D-glucose, 0.2% D(+)-lactose, and 0.2% L(+)-arabinose, during 12-14 h at 37°C with continuous shaking (200 rpm). After pelleting in a centrifuge, cells were stored frozen at −20°C. The yield was about 10 g of cells per liter of culture medium.

Preparation of cell lysate. Cells (20 g) were suspended in 40 ml of lysis solution (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 50 mM $MgCl_2$, 1 mM EDTA, 7 mM β-mercaptoethanol, 0.1% Triton X-100, 0.2 mg/ml lysozyme, 1 mM phenylmethylsulfonyl fluoride), stirred on ice for 30 min, and then disrupted with an Ultrasonic Homogenizer 4710 (Cole-Parmer, USA) in a beaker continuously cooled in an ice bath. Four rounds of sonication were performed with the power switch set to 7, each round being 30-40 sec with 1.5-2.0 min intervals in between. To the lysate, 1.8 ml of 10% polyethyleneimine-HCl (pH 7.5) was added dropwise with vigorous stirring on a magnetic stirrer, which was then continued for another 20 min. The precipitate formed and cell debris were removed by centrifugation for 60 min at 4°C and 10,000*g* (r_{av} 5.75 cm).

Qβ replicase activity was assayed as the synthesis of poly(G) on poly(C) template in the presence of the antibiotic rifampicin, an inhibitor of the cellular RNA polymerase, with the unit of activity being defined as the enzyme amount that can convert 1 nmol of [^{3}H]GTP into an acid-insoluble product during 10 min at 30°C [10]. The protein concentration in lysates was determined according to Lowry et al. [11].

First ion-exchange chromatography. The supernatant was diluted 5-fold with buffer TEG (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10% glycerol, 7 mM β-mercaptoethanol) and applied to a 40-ml column of Q Sepharose Fast Flow (GE Healthcare, Austria) equilibrated with buffer TEG-100 (TEG buffer containing 100 mM NaCl). After washing the column with buffer TEG-100 until the effluent absorption at 280 nm reached the base level, the sample was eluted with 400 ml of a linear gradient of NaCl (100-400 mM) in buffer TEG collecting 8-ml fractions. The flow rate was 4 ml/min.

Hydrophobic chromatography. Fractions in which the replicase β subunit was detected by SDS-PAGE were combined, and 4 M $(NH_4)_2SO_4$ was added to a final concentration of 0.8 M. The sample was applied to a 10-ml column of Butyl Toyopearl 650S (Tosoh Bioscience, Germany) equilibrated with buffer TEG containing 1 M $(NH_4)_2SO_4$. The column was washed with the same buffer, and the sample was eluted with 150 ml of a linear gradient of 1 M

$(NH_4)_2SO_4$ – 0.1 M NaCl in buffer TEG, followed by 20 ml of buffer TEG-100, collecting 2-ml fractions. The flow rate was 1 ml/min. Fractions containing all four replicase subunits (holoenzyme) and fractions lacking protein S1 (core enzyme) were separately pooled. Then the holoenzyme and the core enzyme were purified separately.

Second ion-exchange chromatography. The preparations obtained from the hydrophobic interaction chromatography were diluted with buffer TEG to the conductivity of buffer TEG-100 and applied at a flow rate of 2 ml/min to a 6-ml column of RESOURCE Q (GE Healthcare) equilibrated with buffer TEG-100. The column was washed with the same buffer, and the sample was eluted at a flow rate of 1 ml/min with 150 ml of a linear gradient of NaCl (100-400 mM) in buffer TEG collecting 3-ml fractions. Fractions containing the replicase were combined, supplemented with glycerol to 50% (w/w), and stored at –20°C.

Gel filtration. Before crystallization experiments, samples of Qβ replicase were subjected to gel filtration at a flow rate of 0.2 ml/min through a 1 × 30 cm column of Superdex 200 (GE Healthcare) in buffer containing 20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 5% glycerol while collecting 0.5-ml fractions. Fractions whose absorption at 280 nm exceeded 40% of the peak value were pooled and concentrated with a Centricon 30 ultrafilter (Millipore, USA) until the protein concentration reached 5.0-7.5 mg/ml. The protein concentration was determined using the extinction coefficients ($A_{0.1\%}$ at 280 nm) of 0.749 for the holoenzyme and 0.767 for the core enzyme, as calculated from the amino acid composition of the proteins using the ProtParam program tool (http://au.expasy.org/tools/protparam.html).

Protein crystallization. Search for conditions of Qβ replicase crystallization were performed using the robotic platform Cartesian Honeybee 8+1 robot (IGBMC Crystallization Platform; France) at 4 or 24°C. Immediately prior to crystallization, a replicase preparation was passed through a filter with pore size of 0.22 μm (Millipore). Crystallization was carried out in "sitting" drops, using the Qiagen (USA) and Hampton Research (USA) kits. Drops were obtained by mixing 0.5 μl of a concentrated replicase preparation in the gel filtration buffer and 0.5 μl of a solution from a crystallization kit, which also filled a 50-μl reservoir. When the kit "Silver Bullets" (Hampton Research) was used, the drops were obtained by mixing 0.4 μl of replicase, 0.2 μl of a "Silver Bullet" reagent, and 0.2 μl of a 50% Tacsimate® pH 7.0 solution, which also filled the reservoir. For manual screening of the crystallization conditions, 2 μl of a replicase preparation were mixed with 2 μl of the reservoir solution, with the reservoir volume being 400 μl.

Crystal studies. The crystals were placed in a cryoprotective solution prepared by mixing 100 μl of the reservoir solution and 30 μl of 87% glycerol, and their diffraction was examined at cryogenic temperature at the PX06 beam line at the Swiss Light Source synchrotron (Switzerland) using a typical exposure time of 5 sec for an oscillation range of 0.5°.

RESULTS

Production of chimeric Qβ replicase in *E. coli* cells. The chimeric replicase was obtained with the use of a two plasmid expression system, similar to one previously described for a non-chimeric replicase [12]. For expression of the genes encoding Tth EF-Ts (under the control of the T7 promoter) and the catalytic β subunit of Qβ replicase (under the control of the arabinose promoter), a modified auto-induction method [13] was used. With this method, inducers (in this case, lactose and arabinose, respectively) are present in the culture medium from the very beginning, and the induction occurs after glucose has been fully utilized by the cells. This method allowed the yield of the biomass to be increased 4-5-fold as compared with the standard procedure in which the inducers are added to the medium in the late exponential phase of the cell multiplication. Still, the content of the replicase β subunit in the cells was at least not reduced. Further, it turned out that production of the β subunit did not depend on whether Tth EF-Ts was also synthesized in the cells or not (Fig. 1, b and c). At the same time, in the

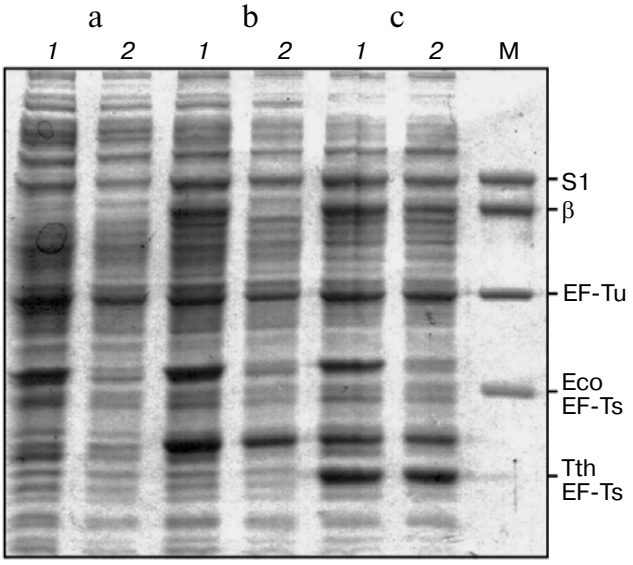

Fig. 1. Synthesis of the β subunit of Qβ replicase and Tth EF-Ts in the *E. coli* cells. SDS-PAGE analysis of proteins in the *E. coli* lysates before (lanes *1*) or after (lanes *2*) removal of the cell debris. a) Untransformed *E. coli* BL21(DE3) cells. b) Same cells transformed with plasmid pBAD-REP encoding the β subunit of Qβ replicase. c) Cells transformed with pBAD-REP, as well as with plasmid pET-Tth-Ts encoding Tth EF-Ts. M, 1 μg of a purified wild-type Qβ replicase holoenzyme containing Eco EF-Ts. The gel was stained with Coomassie blue R-250.

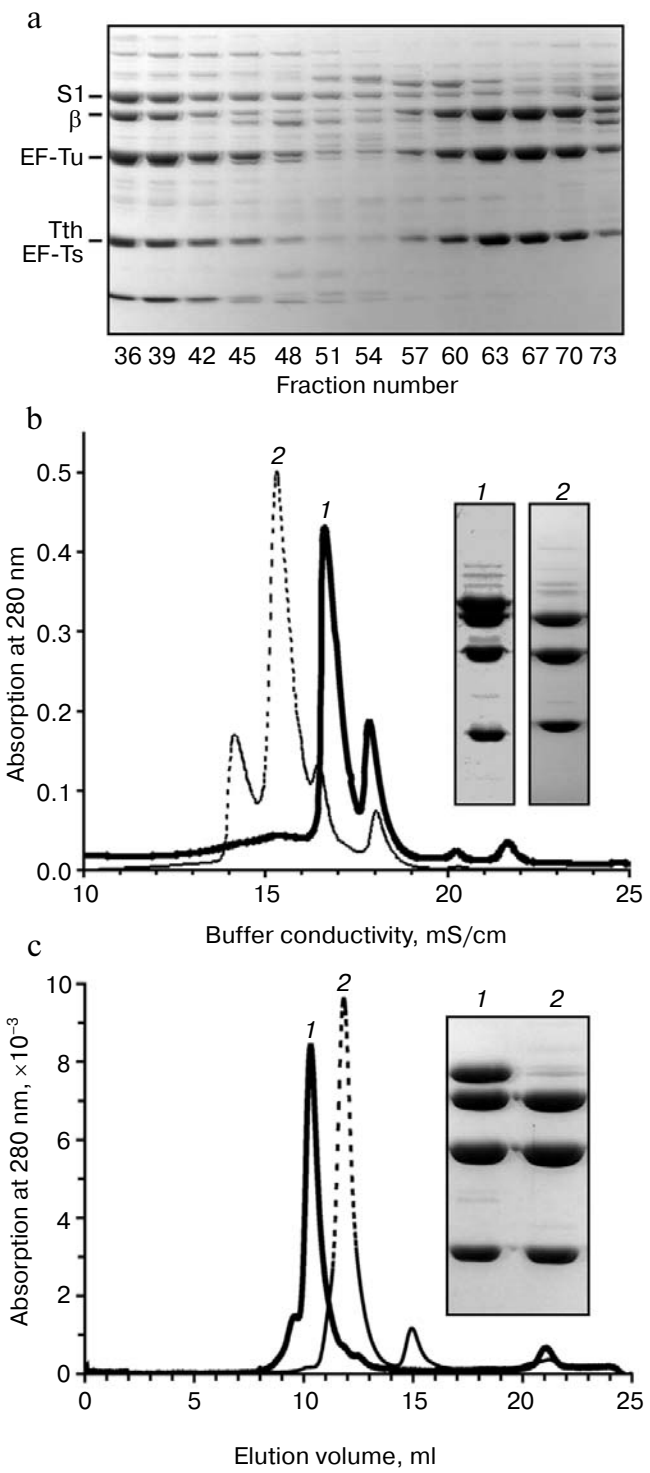

Fig. 2. Isolation of the holoenzyme (*1*) and the core enzyme (*2*) of the chimeric Qβ replicase. a) SDS-PAGE analysis of fractions obtained by hydrophobic chromatography on a Butyl Toyopearl column. b) Ion-exchange chromatography of the holoenzyme and the core enzyme on a RESOURCE Q column and SDS-PAGE analysis of the preparations obtained. c) Gel filtration of the holoenzyme and the core enzyme through a Superdex 200 column and SDS-PAGE analysis of the preparations obtained. Gels were stained with Coomassie blue R-250.

absence of Tth EF-Ts synthesis, the bulk of the β subunit was rendered insoluble and was lost on clarification of the cell lysate (Fig. 1b, lane *2*), whereas in the presence of Tth EF-Ts, solubility of the β subunit (apparently, within the Qβ replicase complex) significantly improved (Fig. 1c, lane *2*). The better solubility of the replicase correlated with its activity: in the experiment shown in Fig. 1, the specific activity of lysates was 58 and 16 units per mg of protein in the presence and absence of Tth EF-Ts in the cells, respectively.

Preparative isolation of chimeric Qβ replicase. A crude Qβ replicase preparation was obtained using a modified procedure, which includes precipitation of the bulk of nucleic acids and nucleoproteins with polyethyleneimine in the presence of 500 mM NaCl, followed by anion-exchange chromatography on a Q Sepharose column [14]. We introduced a step of hydrophobic chromatography on a Butyl Toyopearl column (Fig. 2a), which allowed us to separate the crude preparation into the core enzyme (containing the β subunit, Eco EF-Tu and Tth EF-Ts) and the holoenzyme (also containing ribosomal protein S1).

After a high-efficiency anion-exchange chromatography of the obtained fractions on a RESOURCE Q column (Fig. 2b), the yield of chimeric replicase was approximately 14 mg of the holoenzyme and 7 mg of the core enzyme from 20 g of the cell paste (Fig. 2b, inset). For comparison, the previously published maximum yield of the wild type replicase of a comparable purity was 17.7 mg of unseparated holo- and core enzyme from 50 g of *E. coli* cells [15]. These preparations are devoid of other RNA polymerases and are well suited for functional experiments. However, for the crystallization experiments we carried out an additional gel filtration through a Superdex 200 column, which allowed us to obtain nearly homogeneous preparations of the chimeric holo- and core enzyme (Fig. 2c).

Crystallization of chimeric Qβ replicase. In view of the absence of publications on the crystallization of Qβ replicase or replicases of related bacteriophages, we initially carried out a large-scale screening of crystallization conditions using a robotic device. As a result of testing 384 different conditions at each of the chosen temperatures (4 and 24°C) no crystals of the chimeric holoenzyme, as well as those of the wild type holo- and core Qβ replicase, were found. However, the chimeric core enzyme sometimes did produce crystals at 24°C when salts of organic acids were used as precipitants. The best results were obtained with a Tacsimate® cocktail, which includes seven salts of various organic acids. Crystallization conditions were further optimized in a manual mode using sodium malonate as a precipitant (Fig. 3), which is the major component of Tacsimate®.

Under optimal conditions, hexagonal crystals appeared within the first 24 h of incubation at 24°C and continued to grow during the next three to four days,

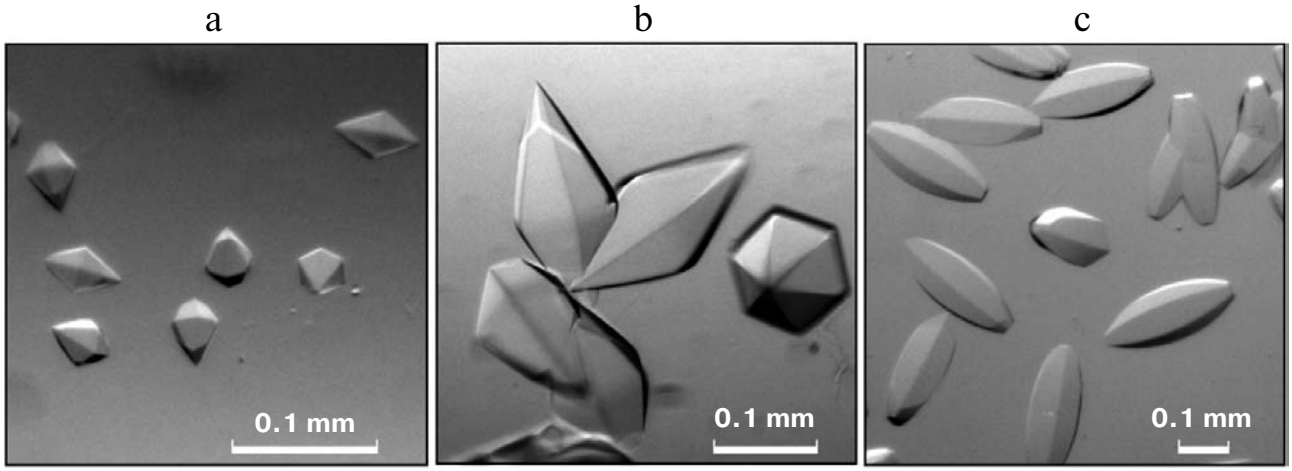

Fig. 3. Crystals of the chimeric Qβ replicase core enzyme grown as hexagonal bi-pyramids at 24°C in the presence of the following concentrations of sodium malonate: a) 0.35 M; b) 0.4 M; c) 0.6 M.

reaching the maximum size of about 300 μm (Fig. 3c). Such Qβ replicase ligands as Mg^{2+} ions (20 mM) and GTP (6 mM) did not significantly affect either the shape or size of the crystals. However, the presence of 5% glycerol in the gel filtration buffer was a prerequisite of successful crystallization.

Preliminary crystallographic study. Crystals of the core enzyme grown in the presence of 0.6 M sodium malonate were tested for their ability to diffract X-rays at the synchrotron. When placed in a cryoprotecting solution obtained by mixing 100 μl of the reservoir solution (100 mM Tris-HCl, pH 7.5, 1.2 M sodium malonate) and 30 μl of 87% glycerol, the crystals diffracted to a maximum resolution of 7 Å. A slightly better resolution (6.5 Å) was achieved by changing the cryobuffer to 50 mM Tris-HCl, pH 7.5, 2 M sodium malonate, and 10% xylitol. A preliminary data set was collected at 7.8 Å resolution, and the space group was determined to be $P6_122$ with the following unit cell parameters: a = 200 Å, b = 200 Å, c = 577 Å, $\alpha = \beta = 90°$, $\gamma = 120°$.

DISCUSSION

Qβ replicase occupies a special position among the RNA-dependent RNA polymerases. It was the first RNA-dependent RNA polymerase to be purified and studied *in vitro* [2]. In the late sixties, the journal *Proceedings of the National Academy of Sciences of the USA* published articles on Qβ replicase almost every month. It was studied by such prominent scholars as Sol Spiegelman and Charles Weissmann, and a large part of the 1968 Cold Spring Harbor Symposium was devoted to it. The extraordinary interest in Qβ replicase is due to the fact that it was and remains one of the most mysterious enzymes.

Qβ replicase very specifically recognizes its templates without the use of promoters or primers [1], but how this happens is still unknown. It is the only polymerase for which the lack of double stranded replicative intermediates was clearly shown. Moreover, it cannot use a double stranded RNA as a template, and does not employ a helicase or a gyrase to unwind the double helix that must inevitably form in the enzyme active site during the synthesis of a product complementary to the template. It is Qβ replicase, rather than the intramolecular secondary structure of the RNA strands, that prevents the unwound template and the product strand from collapsing back into duplex: any agent inactivating Qβ replicase (phenol, SDS, pronase) leads to immediate formation of the double helix [16]. Deciphering the mechanism for maintaining the replicative intermediate in the single-stranded form would help to imagine how a similar problem might have been solved in the RNA world [17]. The Qβ replicase reaction is the most efficient cell-free system for nucleic acid amplification, far more efficient than PCR or primer-dependent isothermal systems [4]. However, nobody has succeeded in employing it for the amplification of desired sequences. Finally, it is not known what the three proteins normally serving the ribosome are doing in the Qβ replicase. Elucidation of the role of these proteins in RNA replication might provide for better understanding of their role in protein synthesis.

All of the above problems require knowledge of the structure of Qβ replicase and its functional complexes. The present study demonstrates that achieving this goal is feasible. It is shown here that the core enzyme, which can perform all the known Qβ replicase functions except for the recognition of the (+)-chain of the genomic Qβ RNA, can be crystallized. This success was made possible, firstly, owing to replacement of the *E. coli* EF-Ts with its homolog from *T. thermophilus*. Unlike previous stud-

ies, in which a chimeric Qβ replicase molecule was obtained by renaturation of fully denatured proteins [18], here the chimeric molecules are formed under physiological conditions, directly in the *E. coli* cells, thereby ensuring their intactness. Introduction of a thermophilic protein into the enzyme resulted in the improvement of its solubility, probably due to the stabilization of its native state. This circumstance might have contributed to the crystallization as well. Secondly, the crystallization was facilitated by having the core enzyme isolated under non-denaturing conditions, with the use of hydrophobic chromatography. Previously, the core enzyme, i.e. the enzyme without protein S1, lacking a compact tertiary structure [19] and thereby preventing the crystal formation, could only be isolated in the presence of 4 M urea [9, 12].

At present, the quality of crystals does not allow the structure of the Qβ replicase core enzyme to be determined at high resolution. However, the results obtained here suggest that crystal quality can be improved by further stabilizing the protein molecule and increasing its rigidity. This can be achieved, for example, by introducing covalent bonds between the subunits of Qβ replicase [12] or by forming a complex with a ligand, for example, with an oligonucleotide mimicking the template.

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