Renaturation of a Multisubunit Multiactivity Enzyme Complex: Recovery of Phage Qβ RNA Replicase, EF-Tu, and EF-Ts Activities after Denaturation in Urea†

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ABSTRACT: Phage Qβ RNA replicase consists of four nonidentical subunits three of which are required for poly(C)-directed synthesis of poly(G): a phage-coded polypeptide and the two host-supplied protein biosynthesis elongation factors EF-Tu and EF-Ts. After denaturation of the enzyme in 8 M urea, poly(G) polymerase activity can be renatured by dilution of the denatured subunits into a high ionic strength buffer with glycerol. The renaturation reaction has a broad temperature optimum between 11 and 21°. The extent of renaturation is dependent on enzyme concentration: at low enzyme concentrations and 21° renaturation proceeds for more than 3 h with greater than 40% recovery of activity, whereas at high enzyme concentrations the reaction is complete by 1 h with less than 10% of the poly(G) polymerase activity regained. Activities catalyzed by the elongation factors can be measured while they are part of the replicase complex. Study of rates of renaturation of EF-Tu and EF-Ts dependent activities alone and in the replicase complex revealed that virtually 100% of the EF-Ts activity was recovered more rapidly than could be assayed at temperatures as low as 2°, while the rate of recovery of EF-Tu activity was comparable to that of the poly(G) polymerase activity and was independent of either EF-Tu concentration or the presence of other enzyme subunits. The rate of recovery of the poly(G) polymerase activity was found to be limited by the renaturation of EF-Tu, since the rate was dramatically increased by the addition of undenatured EF-Tu.

A full elucidation of the mechanism of a multisubunit enzyme requires a number of probes for examining the role of the individual subunits in the overall reaction. Chemical or genetic modification of the enzyme provides one approach to such problems. Modification techniques provide much more detailed information when the complex can be dissociated into its parts, modified, and reconstituted. To use this strategy in our studies of the RNA replicase [nucleosidetriphosphate:RNA nucleotidyltransferase (RNA dependent), EC 2.7.7.6] assembled by the *Escherichia coli* RNA phage Qβ, we have studied some of the parameters of the renaturation of the enzyme from solutions containing 8 M urea. The enzyme is composed of one each of four different subunits (Kamen, 1970; Kondo et al., 1970; Young and Blumenthal, 1975). The largest, subunit I (mol wt 70000) is the *Escherichia coli* 30S ribosomal protein S1 (Wahba et al., 1974; Inouye et al., 1974). It is not required for poly(G) polymerase activity (Kamen et al., 1972) and is not considered in this paper. The smallest two, subunits III and IV (mol wt 45000 and 35000, respectively), are *E. coli* protein synthesis elongation factors EF-Tu and EF-Ts[1] (Blumenthal et al., 1972). They appear to play a role in the initiation of polymerization (Landers et al., 1974). Subunit II (mol wt 65000) is the only one of the four encoded by the phage RNA and carries the catalytic site for RNA polymerization (Landers et al., 1974).

Under appropriate conditions the replicase can be separated into two inactive complexes: one composed of the two larger subunits, the other of EF-Tu·Ts (Kamen, 1970). A small fraction of the original polymerase activity is regained upon mixing of the two complexes (Kamen, 1970); however, as much as 50% activity can be regained if the two complexes are denatured in 8 M urea, mixed, and then renatured by dilution into a high salt buffer (Blumenthal et al., 1972). Using the latter technique both EF-Tu and EF-Ts were found to be required for maximal polymerase activity recovery (Blumenthal et al., 1972) and must be added at the start of the renaturation of subunits I + II from urea (Landers et al., 1974). Here we describe the kinetics of renaturation of some of the activities of EF-Tu, EF-Ts, and whole Qβ replicase. We have found that when all subunits are denatured, the rate-limiting step in the reappearance of polymerase activity is the renaturation of EF-Tu. Distinctly different kinetics are observed when polymerase is renatured in the presence of undenatured EF-Tu.

Materials and Methods

Qβ replicase was purified from Qβ*am*B86-infected *Escherichia coli* K12 strain Q13 by the method of Kamen (1972) with the modifications described previously (Blumenthal et al., 1972). The enzyme was at least 95% pure as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis. It was judged to be approximately 80% active in initiation of synthesis with poly(C) as template. This was calculated from the quantity of Qβ replicase estimated from polyacrylamide gels compared with the number of chains initiated with [γ-^{32}P]GTP. Protein synthesis elongation factor EF-Tu·GDP was made by the method of Arai et al. (1972). EF-Ts was a gift of Drs. J. Hachmann, D. Miller, and H. Weissbach of the Roche Institute of Molecular

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[1] Abbreviations used are: EF-Tu and EF-Ts, protein synthesis elongation factors Tu and Ts, respectively; EF-Tu·Ts, the complex of EF-Tu and EF-Ts.

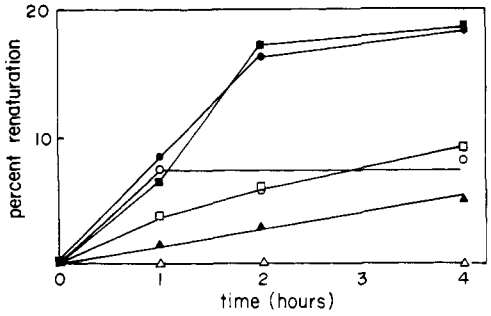

FIGURE 1: Renaturation of poly(G) polymerase activity from urea as a function of temperature. Qβ replicase was denatured and renatured at a final concentration of 0.36 μM as described in Materials and Methods except that renaturation incubation was at the following temperatures: (▲) 2°; (□) 7°; (■) 11°; (●) 21°; (○) 30°; (△) 37°. Percent renaturation was calculated from control tubes in which nondenatured enzyme was diluted to the same concentration in the same buffer, incubated at the same temperatures, and assayed at 4 h.

Biology (Nutley, N.J.). Urea "Absolute Grade" was purchased from R-Plus Laboratories (Denville, N.J.). All other chemicals were reagent grade.

Denaturation and Renaturation of Qβ Replicase. Pure Qβ replicase was denatured by the addition of crystalline urea (0.8 mg of urea/μl of enzyme solution, ca. 8 M), and 2-mercaptoethanol to 0.2 M. The mixture was then incubated at room temperature (approximately 21°) for 2 h. Renaturation was initiated by dilution into 50 mM Tris-acetate buffer (pH 8.0), 10 mM $Mg(OAc)_2$, 1 mM EDTA, 0.15 M $(NH_4)_2SO_4$, 20% glycerol, and 70 mM 2-mercaptoethanol. Final urea concentration was approximately 1.1 M. Each volume of enzyme used originally was diluted with ten volumes of renaturation buffer. The renaturation mixture was incubated at 21° unless otherwise stated. Protein concentrations given are after final dilution.

Poly(G) Polymerase Assays. Five-microliter samples of the renaturation mixtures were added to 0.1 ml of assay mixture containing: 50 mM Tris-HCl buffer (pH 7.5), 10 mM $Mg(OAc)_2$, 1 mM EDTA, 1 mM phosphoenolpyruvate, 1 mM dithioerythritol, 10% glycerol, 10 μg/ml of pyruvate kinase, 125 μg/ml of poly(C), and 25 μM [^{3}H]GTP (200 cpm/pmol), and incubated at 37° for 10 min before precipitating with 10% trichloroacetic acid, filtering, and counting as described by Kamen (1972).

Assay for EF-Tu. EF-Tu was assayed by its binding of [^{3}H]GTP to nitrocellulose filters; 25-μl samples of the renaturation mixtures were added to 0.1 ml of assay mixture containing 50 mM Tris-HCl buffer (pH 7.5), 5 mM $Mg(OAc)_2$, 1 mM EDTA, 0.1 mM dithioerythritol, and 1.8 μM [^{3}H]GTP (556 cpm/pmol). This was incubated for 5 min at 30°, and filtered through 6-mm discs punched from Schleicher and Schuell B6 nitrocellulose filters. The filters were then washed twice with 0.1 ml of the same buffer without GTP, dried and counted.

Assay for EF-Ts. EF-Ts was assayed by its catalysis of [^{3}H]GDP exchange with EF-Tu·GDP at 0°. To 0.1 ml of a solution containing 50 mM Tris-HCl buffer (pH 7.5), 5 mM $Mg(OAc)_2$, 1 mM EDTA, 0.1 mM dithioerythritol, and 2.5 μM [^{3}H]GDP (128 cpm/pmol), 5 μl of a 1/10 dilution of renaturation mixture was added at 0°. The reaction was initiated by the addition of 5 μg of EF-Tu·GDP, incubated for 5 min at 0°, and filtered and counted as in the GTP binding assays. The degree of renaturation was determined by comparison with the assay of an equivalent amount of nondenatured Qβ replicase.

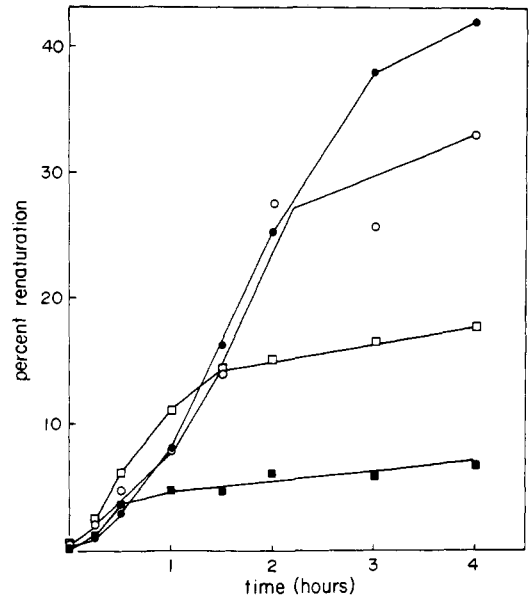

FIGURE 2: Effect of enzyme concentration on renaturation of poly(G) polymerase activity. Dilutions of a stock solution of Qβ replicase in 50 mM Tris-HCl (pH 7.5), 5 mM $Mg(OAc)_2$, 1 mM EDTA, and 0.2 M NaCl were denatured and renatured as described in Materials and Methods. Final enzyme concentrations after dilution with renaturation buffer: (●) 0.03 μM; (○) 0.14 μM; (□) 0.36 μM; (■) 0.72 μM. Percent renaturation was determined as in Figure 1.

Results

Conditions for Maximal Renaturation of Poly(G) Polymerase Activity. After denaturation of Qβ replicase in 8 M urea and 0.2 M 2-mercaptoethanol, optimal recovery of the poly(G) polymerase activity is approximately 50%. We have found little variation in the rate or extent of recovery of activity in buffers ranging from pH 7.5 to pH 8.5. High ionic strength favors both rate and extent; KCl, $(NH_4)_2SO_4$, and NH_4Cl are equally effective. Glycerol (or dimethyl sulfoxide) is required: 20–30% glycerol is essential for maximal rate and extent of renaturation. Although 10 mM $Mg(OAc)_2$ is present in our renaturation buffer, the Mg^{2+} is not required for optimal recovery of activity (unpublished observations). The final extent of renaturation is not affected by varying the temperature between 2 and 21° (for the 2 and 7° curves the reactions were allowed to proceed for 24 h). However, as shown in Figure 1, the rate of the renaturation reaction increases with increasing temperature up to 11°, but above 21° the extent of renaturation is inhibited until at 37° no activity is recovered. The renatured enzyme appears to function normally in the Qβ RNA directed "replicase" reaction (S. Brown, personal communication).

Effect of Replicase Concentration on Renaturation. The enzyme concentration dependence of the kinetics of renaturation of a multisubunit enzyme might be expected to reveal information on the mechanism of reassembly of the complex. We find that increasing concentrations give only a slight stimulation of the rate of renaturation at low concentration and that the extent of renaturation begins to decrease because of apparent irreversible aggregation of one or more of the subunits (Figure 2). At the highest concentrations a visible precipitate forms. The sigmoidal nature of the curves in Figure 2 suggests that the reappearance of the poly(G) polymerase activity involves more than just simple interactions of the three essential subunits: II, EF-Tu, and EF-Ts. Similar cases of protein aggregation and complex

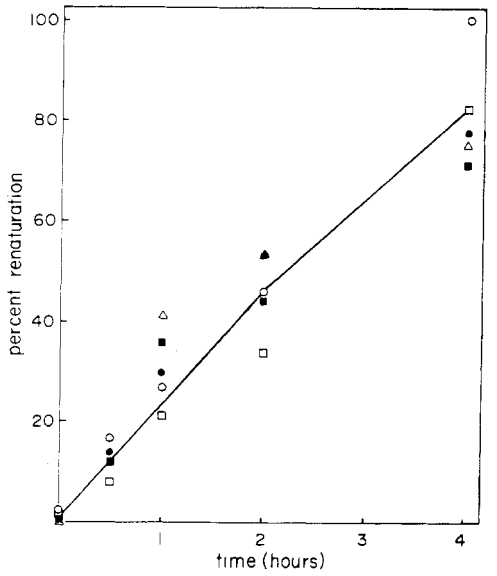

FIGURE 3: Renaturation of GTP binding activity of EF-Tu, EF-Tu·Ts, and Qβ replicase. The proteins were denatured and renatured as described in Materials and Methods. Samples were removed at the times shown and assayed for GTP binding as described in Materials and Methods. Proteins used and final concentrations in renaturation buffer: (△) EF-Tu, 22 μM; (●) EF-Tu·Ts, 1.0 μM; (□) Qβ replicase, 0.72 μM; (○) Qβ replicase, 0.14 μM; (■) EF-Tu·Ts isolated from Qβ replicase by the method of Kamen (1), 0.06 μM. Percent renaturation was determined as in Figure 1.

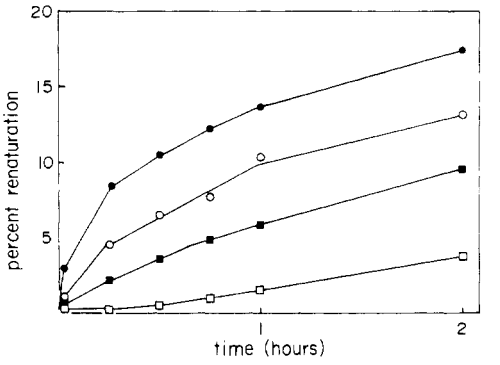

FIGURE 4: Stimulation of the rate of renaturation of poly(G) polymerase activity by nondenatured EF-Tu. Qβ replicase was denatured as described in Materials and Methods. It was diluted to a final concentration of 0.36 μM in renaturation buffer containing: (□) no added EF-Tu; (■) 0.26 μM EF-Tu; (○) 1.3 μM EF-Tu; (●) 5.2 μM EF-Tu. Percent renaturation was determined as in Figure 1.

kinetics have been observed by Ullman and Monod (1969) and Teipel and Koshland (1971a).

Renaturation of EF-Tu and EF-Ts. Both EF-Tu and EF-Ts activities are found in Qβ replicase preparations (Blumenthal et al., 1972). EF-Tu in Qβ replicase can be assayed by the binding of [^{3}H]GDP or [^{3}H]GTP to nitrocellulose filters, and the EF-Ts in Qβ replicase by catalysis of the exchange of [^{3}H]GDP with EF-Tu·GDP at 0°. In order to determine the relationship between the rate of renaturation of these subunits and the reappearance of polymerase activity, recovery of the elongation factor activities from denatured Qβ replicase or the separated subunits was measured. We found that the rate of recovery of EF-Ts activity at 2° is too fast to be measured by the GDP exchange assay. All of the input EF-Ts activity is recovered immediately after dilution into the renaturation buffer. From these

Table I: Effect of Added Elongation Factors on Rate and Extent of Recovery of Poly(G) Polymerase Activity from Denatured Qβ Replicase.[a]

Additions	% Renaturation	
	15 min at 2°	120 min at 21°
None	0.02	21.1
In denaturation mix		
EF-Tu	<0.02	9.2
EF-Ts	<0.02	11.0
EF-Tu + EF-Ts	<0.02	4.2
In renaturation mix		
EF-Tu	5.2	23.7
EF-Ts	0.04	23.0
EF-Tu + EF-Ts	7.8	25.8

[a] Qβ replicase (0.36 μM) was denatured as described in Materials and Methods. When the elongation factors were present in the denaturation mixture, EF-Tu (2.6 μM), EF-Ts (3.5 μM), or both were added before the 2-h 21° incubation with 8 M urea. When they were added in the renaturation mixture, they were mixed with renaturation buffer at the same final concentration before dilution of the denatured Qβ replicase. All tubes were incubated at 2° for 90 min and then moved to 21° and incubated for an additional 2 h. Samples were removed for assay after 15 min at 2° and again at the end of the incubation.

experiments we estimate that either the half-time for EF-Ts renaturation is less than 30 s, or EF-Ts is not inactivated by 8 M urea. This problem has not been investigated further.

The rate of renaturation of EF-Tu as judged by GTP binding activity is at least 1000-fold slower than for EF-Ts (Figure 3) with a half-time on the order of 2 h at 21°. The rate of renaturation is not affected by the source of denatured EF-Tu (pure EF-Tu·GDP, Qβ replicase, or EF-Tu·Ts) or the EF-Tu concentration (0.06–22 μM).

The Rate of Reappearance of Polymerase Activity is Limited by the Amount of Renatured EF-Tu. The close correspondence of the rate of appearance of poly(G) polymerase activity to the renaturation rate of EF-Tu (cf. Figures 2 and 3) suggested that the rate-limiting step in the recovery of replicase is the renaturation of EF-Tu. Such a model predicts that the presence of undenatured EF-Tu would increase the rate of appearance of polymerase activity. We found that when undenatured EF-Tu is included in the renaturation buffer the rate of renaturation of polymerase activity at 21° is at the upper limit of what we can readily measure. Native EF-Tu·Ts has a similar rate enhancing effect but EF-Ts alone does not stimulate polymerase renaturation (Table I, column 1). The stimulation of the rate is not simply due to the presence of excess EF-Tu since addition of an equivalent amount of denatured EF-Tu or EF-Tu·Ts to the denatured replicase mixture does not stimulate the rate of renaturation (Table I, column 1). The presence of excess elongation factors in the renaturation mixture does not significantly alter the extent of renaturation (Table I, column 2). We cannot account for the depression in extent of recovery observed when elongation factors are added during the denaturation, although this phenomenon might be related to the inhibitory effect of high enzyme concentrations on extent of renaturation (Figure 2).

By performing the experiments at 2°, the renaturation proceeds slowly enough that the effect of native EF-Tu concentration on the rate of appearance of polymerase activity can be examined more closely. As seen in Figure 4 increasing EF-Tu concentration dramatically stimulates initial

rates of polymerase recovery. All the curves of Figure 4 ultimately plateaued at the same percent renaturation, approximately 20%.

Discussion

The studies reported here on the kinetics of renaturation of the various subunits of Qβ RNA replicase required for the poly(C)-directed synthesis of poly(G) demonstrate: (a) the EF-Ts renatures quickly and completely (assuming it is in fact denatured in 8 M urea solution); (b) EF-Tu also renatures completely but more slowly, independently of concentration or the presence of other enzyme subunits; (c) the poly(G) polymerase activity is recovered at approximately the same rate as the GTP binding activity. In the presence of undenatured EF-Tu the rate of poly(G) polymerase renaturation increases markedly and changes from sigmoidal to hyperbolic kinetics, indicating that the slow rate of recovery of poly(G) polymerase activity is due to the slow rate of EF-Tu renaturation and possibly suggesting a catalytic role for EF-Tu in renaturation of the complex enzyme. The extent of recovery of the whole enzyme is much less than that of the two elongation factors. Furthermore, the extent decreases with increasing enzyme concentration. Since we have observed visible precipitates when renaturing high concentrations of Qβ replicase, we suggest that the reduced levels of renaturation are due to the formation of complexes of subunits which are not active in the poly(G) polymerase reaction.

The renaturation behavior of Qβ replicase might constitute an extreme case of the kinetically determined renaturation behavior described by Teipel and Koshland (1971a,b). According to this model, native EF-Tu or EF-Tu·Ts would act as a cofactor which stabilizes "productive" intermediates in the refolding of the phage-coded polymerase subunit. In the absence of EF-Tu, renaturation of subunit II apparently leads to a "kinetically trapped" or metastable intermediate which does not readily convert to an active form when EF-Tu·Ts is added (Landers et al., 1974). The same or a similar inactive metastable conformation may also be accessible to native subunit II after EF-Tu·Ts is removed by the low salt sedimentation method of Kamen (1970) since the resulting complex of subunits I + II is not readily reactivated by EF-Tu·Ts (Kamen, 1970). Further studies of the renaturation requirements of subunit II should clarify the roles of EF-Tu and EF-Ts in the structure and functioning of Qβ replicase.

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