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Articles

Kinetic and Thermodynamic Characterization of the Interaction between Qβ-Replicase and Template RNA Molecules†

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ABSTRACT: The specific binding of the RNA polymerase Qβ-replicase to some of its RNA template molecules, the single-stranded RNA variant MDV and also Qβ-RNA, was studied under various conditions by using a gel-retardation assay as well as filter retention. The dissociation of the replicase–RNA complex proceeds with first-order kinetics. The dependence of the dissociation rate constant on the concentration of monovalent ions suggests that there are three ionic contacts between the midivariant (MDV) RNA and the replicase. Through analysis of the temperature dependence of the dissociation rate constant, values of 35 and 43 kJ/mol were obtained for the activation energies of complex dissociation between Qβ-replicase and the minus (−) and plus (+) strands of MDV, respectively. The bimolecular association is of second order with high rate constants that increase when the temperature is raised and decrease at higher salt concentrations. The equilibrium constants vary between $4 \cdot 10^{11}$ M^{-1} and $5 \cdot 10^{7}$ M^{-1}, according to the reaction conditions. The temperature dependence of K_a gives $\Delta H = -39$ kJ/mol for MDV− and −47 kJ/mol for MDV+. Under nearly all conditions, distinct differences in the association and dissociation rates of plus and minus strands of MDV are observed. The binding of the small variant MDV to Qβ-replicase is three orders of magnitude stronger than the binding of the natural template Qβ-RNA.

Qβ-replicase is an RNA-dependent RNA polymerase that is synthesized during the infection of *Escherichia coli* cells with the phage Qβ. It specifically replicates the phage RNA and ignores the host RNA. In vitro, Qβ-replicase also multiplies some smaller single-stranded RNA molecules with a size in the range 30–220 nucleotides very efficiently. Some of these variants were generated in in vitro evolutionary experiments starting with Qβ-RNA (Mills et al., 1967). Others, the midi-, mini-, and nanovariants, were synthesized in vitro without the addition of an exogenous template (Kacian et al., 1972; Sumper & Luce, 1975; Mills et al., 1975; Schaffner et al., 1977). The 6S RNA was isolated from *E. coli* cells infected with Qβ-phages (Banerjee et al., 1969; Kacian et al., 1971). While Qβ-RNA and the midivariant RNA have a common sequence motif (Nishihara et al., 1983), most of the replicable RNA variants do not show sequence similarities. Most likely the replicase recognizes structural features. Biebricher et al.

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(1982) examined several of the single-stranded RNA variants and showed that all of them display significant secondary structure in solution. Only single-stranded RNA is accepted as a template (Spiegelman et al., 1968; Weissmann et al., 1967; August et al. 1968). The complementary RNA, which is synthesized from the 5′ to 3′ end, forms double-stranded RNA with the template only to a minor extent. This is essential since double-strand formation between template and replica RNA inhibits the replication, as was found by Priano et al. (1987) for some of the small RNA variants. To avoid double-strand formation, strong folding of the single strands is required.

The replication kinetics of the short RNA variants were examined intensively (Mills et al., 1978; Dobkin et al., 1979; Biebricher et al. 1981; Priano et al., 1987). The crucial step in the RNA replication by Qβ-replicase is the recognition and binding of the template. The formation of the replicase–template complex is followed by the binding and joining of two molecules of GTP. These processes constitute the *initiation*. The replicase binds close to the 3′ end of the template and moves to the 5′ end in the course of the *elongation*, leading to the formation of the complementary strand. Nishihara et al. (1983) localized the binding site of Qβ-replicase to midivariant RNA at an internal sequence of the molecule, although the 3′ end is required for the initiation of the replication. During *termination* the product strand is first released from the replication complex, and then the template and the enzyme dissociate (Dobkin et al., 1979). In a detailed model of the RNA replication mechanism formulated by Biebricher et al. (1983; 1984; 1985), the replication is divided into several steps, each of which is assigned a rate constant. This model makes a computer simulation of the replication kinetics feasible.

Here the experimental determination of the kinetic rate constants and thermodynamic parameters of the binding reaction between Qβ-replicase and different template RNA molecules is reported. The minus (−) and the plus (+) strands of the 221 nucleotide midivariant (MDV), as well as Qβ-RNA (4200 nucleotides) were used for these studies. The binding rates were measured at different temperatures and salt concentrations. The dependence of the reaction parameters on experimental conditions provides clues to the molecular mechanism of the binding reaction. The use of the experimentally determined parameters allowed the computer simulation of the replication kinetics to be made more realistic.

Materials and Methods

Purification of Qβ-Replicase. Qβ-replicase was isolated from transformed *E. coli* cells carrying a plasmid with the gene of the β-subunit; the strain was a gift from M. A. Billeter, University of Zürich. The purification procedure was performed as described by Kamen (1972) and modified by Bauer et al. (1989).

Preparation and Radioactive Labeling of MDV RNA. Midivariant (MDV) RNA was provided by C. K. Biebricher, Max Planck Institute, Göttingen. MDV RNA was multiplied by incubating it for 4 h at 30 °C with Qβ-replicase (0.4 mM) in the presence of 1 mM of each of the four nucleoside triphosphates. The enzyme was removed by chloroform extraction and the RNA precipitated with ethanol. Single-stranded RNA was obtained by heating for 2 min at 115 °C in sodium citrate buffer containing 50% formamide and afterward rapidly cooling to −70 °C. The single strands were separated on a 18% polyacrylamide gel with 50 mM tris borate and 1 mM $MgCl_2$ (Mills et al., 1978). The bands were cut out from the gel and the RNA purified by electroelution and ethanol precipitation. MDV RNA was stored in distilled water (HPLC pure, Baker) at −70 °C. The concentration was determined by measuring the optical density at 260 nm.

5′ end labeling with [γ-^{32}P] was performed according to Donis-Keller et al. (1977). The nonincorporated ATP was removed by gel filtration. Labeling with ^{35}S was achieved by incubating template RNA with Qβ-replicase as described above, but in the presence of 0.5 mCi [α-^{35}S]thio-UTP. Melting of the double strands, separation of the single strands, and purification was carried out in the same way as with nonradioactive RNA. Radioactive MDV was stored in the presence of 1 mM mercaptoethanol.

Isolation of Qβ-RNA from Phages. Qβ-phage lysate was provided by M. Gebinoga and A. Schwienhorst, Max Planck Institute, Göttingen. Qβ-RNA was isolated from 50 μL of concentrated lysate of Qβ-phages (10^{16} plaque forming units/mL) by incubating for 20 min with proteinase K at 37 °C and extracting five times with 1:1 phenol/chloroform and once with chloroform (Gebinoga, 1990). The RNA was precipitated with isopropanol and dissolved in 20 μL of HEPES–EDTA buffer containing RNase inhibitor. The concentration was determined by measuring the optical density at 260 nm, the purity being monitored by the ratio of the optical densities at 260 and 280 nm. Qβ-RNA was radioactively labeled by ligation of [α-^{32}P]CTP to the 3′ end with T_4 RNA ligase (England et al., 1980); the RNA was stored in the presence of RNase inhibitor and 1 mM mercaptoethanol at −70 °C. The condition of the RNA samples was checked by electrophoresis on a 1% agarose gel with tris borate–EDTA buffer.

Determination of the Degree of Binding between Qβ-Replicase and Single-Stranded MDV. The buffer for binding assays under standard conditions contained 50 mM tris-HCl, pH 7.5, 100 mM NaCl, 2 mM $MgCl_2$, 25% glycerol, 80 μg/mL BSA, and 0.1 mM DTE. The reaction temperature was 5 °C. Different salt concentrations and temperatures were occasionally employed as indicated in the text. The reaction volume for dissociation experiments was typically 100 μL. Samples with a volume of 10 μL were applied to the gel or to the filter. Association reactions were carried out in a volume of 10 μL.

Gel-Retardation Assay. Electrophoresis was carried out on a 5% polyacrylamide gel at 4 °C with 20 V/cm. The electrophoresis buffer contained 25 mM tris base and 200 mM glycine. From the gels, autoradiograms (in the case of ^{32}P-labeled RNA) or fluorograms (with ^{35}S-labeled RNA) were obtained, which were quantified by scanning with an LKB Ultroscan XL laser densitometer. The LKB 2400 Gelscan-XL software was used for integrating the peaks. From the peak area of the bands of free and bound RNA compared to the total sum of the peak areas, the amount of these species in the solution was determined.

Filter-Binding Assay. Nitrocellulose membrane filters with a pore size of 0.45 μm, previously soaked in 50 mM tris-HCl, pH 7.5, 4 °C, were used. Immediately after application of the sample, the filter was washed with 1 mL of the same buffer. The filtrate of each sample was collected in a scintillation vial. All data points were determined in duplicate. The radioactivity of filter and filtrate was measured in a liquid scintillation analyzer, Tri Carb 1900 CA, Packard. The amount of free and bound RNA was determined from the ratio of radioactivity retained on the filter to the sum of the radioactivity of filter and filtrate. The background of unspecifically bound RNA of a sample without enzyme was always subtracted.

Results

The binding of Qβ-replicase to RNA templates was determined by using gel-retardation and filter-binding assays,

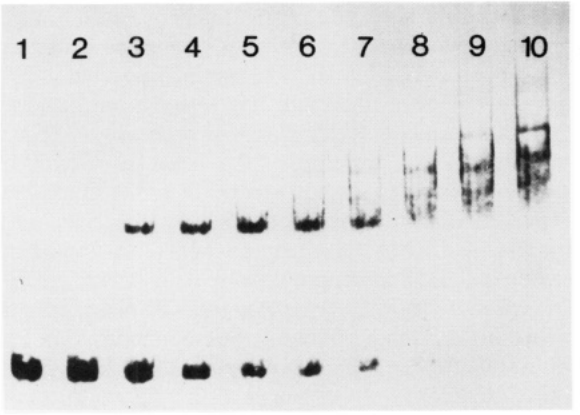

FIGURE 1: Gel retardation of single-stranded MDV, complexed with Qβ-replicase. The binding assay was performed under standard reaction conditions. The RNA concentration was 1 nM. The concentration of replicase from lane 1 to lane 10 was 0, 0.4, 0.8, 1.2, 1.6, 2.0, 3.0, 4.0, 6.0, and 10 nM.

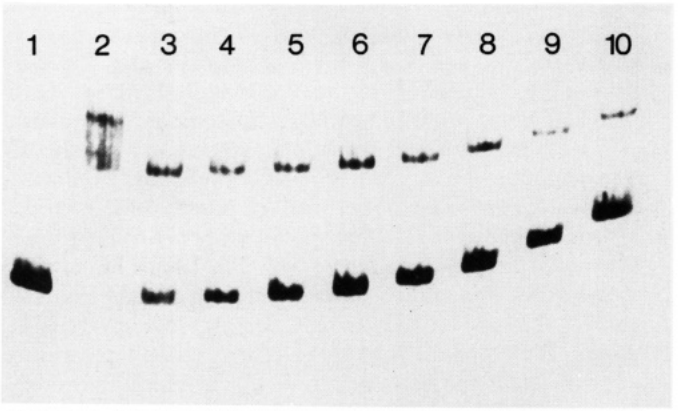

FIGURE 2: Dissociation kinetics of the Qβ-replicase–MDV complex. The reaction was carried out under standard conditions. The concentration of complex was 2 nM. (Lane 1) As a reference, RNA without replicase was loaded. (Lane 2) Replicase–MDV–complex before addition of an excess of nonlabeled RNA. (Lanes 3–10) Samples loaded to the gel at different time intervals after the addition of nonlabeled RNA. The intervals were 1, 2, 3, 4, 5, 6, 8, and 10 min.

predominantly the former method. The binding characteristics were measured under various external conditions. Single-stranded midivariant (MDV) RNA complexed with Qβ-replicase forms a sharp band on a nondenaturing gel, clearly separated from the free RNA (Figure 1). Quantitative comparison with reference lanes containing only MDV shows that 80–90% of the radioactivity is concentrated in the main band and not smeared in the lane. The general observation of low background supports this finding. If the molar ratio of enzyme to RNA is larger than a factor of two or if the concentration exceeds $5 \cdot 10^{-9}$ M, additional bands appear in the gel (Figure 1), which most likely contain complexes with a stoichiometry larger than one molecule of replicase per RNA molecule. These complex bands are less stable than the main band and cannot be quantified easily. All experiments were performed under conditions which guaranteed that predominantly the main complex band was present.

Control experiments carried out with the filter-binding method, yielded results similar to those obtained in the gel-retardation experiments. The filter-binding efficiency of all RNA samples used for experiments with nitrocellulose filters was determined quantitatively by titration of Qβ-replicase with RNA as described by Woodbury and von Hippel (1983).

Dissociation Kinetics. To determine the dissociation rate constant, radioactive RNA and replicase were mixed in nearly equimolar amounts and equilibrated. At the beginning of the reaction a 30-fold excess of nonlabeled MDV in a small volume was added to the solution. The dissociation of the radioactive RNA from the complex was monitored by taking aliquots from the reaction solution every 30 s and loading them immediately on to a gel to which voltage already was applied (Figure 2). The plot of the logarithm of the complex concentration as a function of time is linear, demonstrating that the dissociation of the replicase–RNA complex follows first-order kinetics.

Under most conditions, differences in the kinetic behavior of the minus (−) and the plus (+) strands of MDV are observed. The dissociation of Qβ-replicase from MDV− proceeds approximately twice as fast as the dissociation from MDV+.

The dissociation rate is very sensitive to the salt concentration (Table I), indicating a strong contribution of ionic contacts to the protein–RNA interaction. The dissociation becomes faster at higher ion concentrations. This effect is due to the recondensation of cations to the RNA when it dissociates from the protein and probably also to the binding of anions to the protein (Lohman, 1985). A quantitative interpretation of ionic effects on protein–nucleic acid interactions, based on the polyelectrolyte theory of Manning (1969, 1978), was described by Record et al. (1976) and Lohman (1985). The dissociation data have been evaluated according to these models. The possibility of anion binding to the protein, as well as the effect of the small concentration of Mg^{2+} ions, could be neglected in the quantitative interpretation. A value of 0.71 was used for ψ, the fraction of counterion bound per polyanionic charge. This value was given by Record et al. (1976) for single-stranded DNA. The double-logarithmic plot of the dissociation rate constant as a function of the sodium concentration is linear (Figure 3). The slope indicates that there exist approximately three ionic contacts between the nucleic acid and the protein.

Table I: Dissociation Rate Constants

$[Na^+]$ (mM)	$[Mg^{2+}]$ (mM)	T (°C)	MDV− k_d (s^{-1})	MDV+ k_d (s^{-1})
25	2	5	$(1.8 \pm 0.4) \cdot 10^{-4}$	$(1.0 \pm 0.1) \cdot 10^{-4}$
50	2	5	$(1.5 \pm 0.3) \cdot 10^{-3}$	$(5.6 \pm 2.5) \cdot 10^{-4}$
100	2	5	$(2.2 \pm 0.1) \cdot 10^{-3}$	$(1.4 \pm 0.2) \cdot 10^{-3}$
200	2	5	$(1.0 \pm 0.1) \cdot 10^{-2}$	$(7.1 \pm 0.6) \cdot 10^{-3}$
100	10	5	$\geq 2 \cdot 10^{-2}$	$(2.6 \pm 0.2) \cdot 10^{-3}$
100	2	20	$(5.9 \pm 1.0) \cdot 10^{-3}$	$(4.0 \pm 1.5) \cdot 10^{-3}$
100	2	30	$(1.1 \pm 0.2) \cdot 10^{-2}$	$(7.5 \pm 0.1) \cdot 10^{-3}$
100	2	40	$(1.5 \pm 0.1) \cdot 10^{-2}$	$(1.1 \pm 0.3) \cdot 10^{-2}$

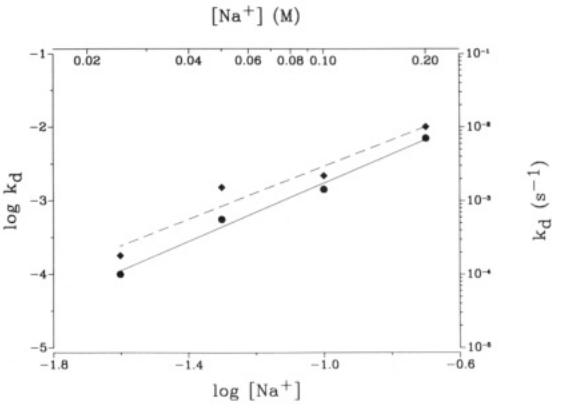

FIGURE 3: Double-logarithmic plot of the dissociation rate constant of the Qβ-replicase–MDV complex vs the sodium ion concentration for the plus (●) and minus (◆) strands. The slope of the fitted line is $m\psi = 1.8$ for MDV− and $m\psi = 2.0$ for MDV+, where m is the number of ion pairs between nucleic acid and protein and ψ is the fraction of a counterion bound per polyanion charge; a value of 0.71 was assumed (Record et al., 1976).

Table II: Association Rate Constants

$[Na^+]$ (mM)	$[Mg^{2+}]$ (mM)	T (°C)	MDV− k_a (L mol^{-1} s^{-1})	MDV+ k_a (L mol^{-1} s^{-1})
25	2	5	$(6.2 \pm 1.4)\cdot10^7$	$(4.2 \pm 0.2)\cdot10^7$
50	2	5	$(8.5 \pm 0.1)\cdot10^7$	$(6.4 \pm 0.2)\cdot10^7$
100	2	5	$(7.5 \pm 2.5)\cdot10^6$	$(3.5 \pm 0.6)\cdot10^7$
200	2	5	$(4.6 \pm 1.8)\cdot10^5$	$(4.1 \pm 1.8)\cdot10^6$
100	10	5	$(1.9 \pm 0.1)\cdot10^6$	$(6.9 \pm 0.1)\cdot10^6$
100	2	20	$(4.0 \pm 1.6)\cdot10^7$	$(7.9 \pm 1.0)\cdot10^7$
100	2	30	$(4.7 \pm 0.7)\cdot10^7$	$(9.5 \pm 1.7)\cdot10^7$
100	2	40	$(3.7 \pm 0.4)\cdot10^7$	$(6.4 \pm 0.5)\cdot10^7$

An increase of the magnesium ion concentration at a constant sodium concentration of 0.1 M leads to an increase of the dissociation rate (Table I). This effect is large in the case of MDV−; a structural change of the RNA molecule might be involved.

With increasing reaction temperature, the velocity of the dissociation grows (Table I). The dependence of the dissociation rate constant on the temperature can be fitted to the equation of Arrhenius (Figure 4). The linearity of this plot indicates that the reaction proceeds via a transition state. From the slope, the energy of activation can be determined. The activation energy for the dissociation of the enzyme–MDV− complex has a value of 35 kJ/mol and for the enzyme –MDV+ complex a value of 43 kJ/mol.

Association Kinetics. To determine the association rate, two solutions, one containing radioactivity labeled MDV, the other one containing Qβ-replicase, were mixed rapidly. After a short time interval (between 10 and 120 s), the association of the radioactive RNA was stopped by adding a 30-fold excess of nonlabeled MDV to the solution. Immediately afterward the sample was loaded onto an acrylamide gel to which voltage was already applied (Figure 5). In general, nine time points were measured in one association experiment. Under reaction conditions favoring a large dissociation rate, some of the complex decomposed before entering the gel matrix. In these cases, the apparent association rate was corrected by using the known dissociation rate constant. The data were interpreted in terms of a second-order kinetic expression. The plot of the integrated second-order rate equation versus time yields a linear function (data not shown). In the case of second-order kinetic behavior, the velocity of the reaction depends on the concentration of both reactants. Therefore, high association rate constants can be determined with biochemical methods by working with low reactant concentrations in the range 10^{-10}–10^{-9} M.

At higher salt concentrations, the association rate of replicase with MDV+ is significantly larger than with MDV− (Table II). With both strands of MDV, the association rate decreases with increasing concentration of sodium ions. Raising the concentration of magnesium ions results in a further decrease of the association rate (Table II). The double-logarithmic plot of the rate constant as a function of the monovalent ion concentration is not linear, excluding the possibility of any simple quantitative analysis according to polyelectrolyte models.

With increasing temperature (up to 30 °C), the bimolecular association reaction proceeds faster. At even higher temperatures (40 °C), the reaction rate decreases (Table II). The data cannot be fitted to the Arrhenius equation, and therefore the energy of activation could not be evaluated.

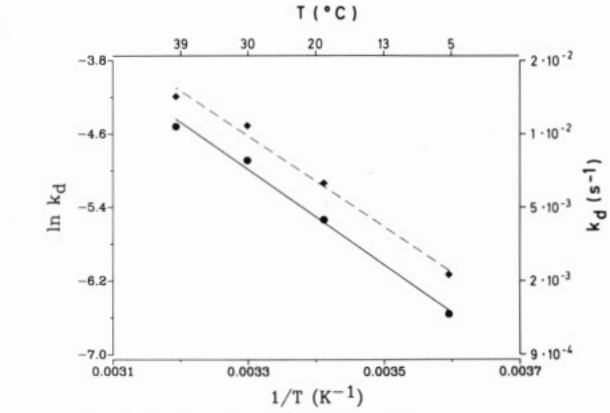

FIGURE 4: Arrhenius plot of the dissociation rate constants of the complexes with MDV− (◆) and MDV+ (●) as a function of the reaction temperature. The line has the slope E_a/RT. For MDV−, E_a = 35 kJ/mol; for MDV+, E_a = 43 kJ/mol.

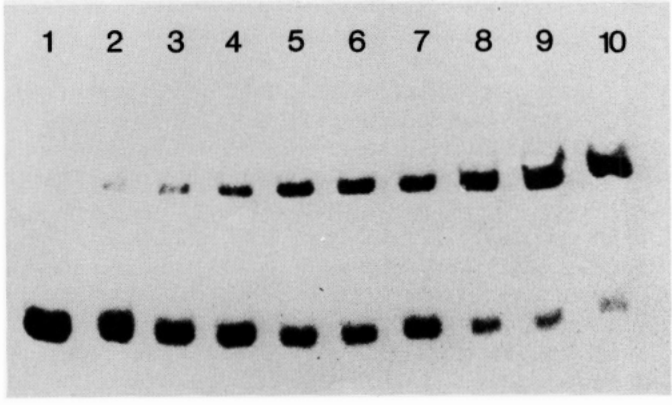

FIGURE 5: Association kinetics of Qβ-replicase and MDV−. The reaction buffer contained 25 mM NaCl. The concentration of replicase was $4\cdot10^{-10}$ M; the RNA concentration was $2\cdot10^{-10}$ M. (Lane 1) As a reference, only MDV was loaded. (Lanes 2–10) The reaction times were 10, 15, 20, 30, 40, 60, 80, 90, 100, and 120 s.

Binding of MDV to α-less Replicase. Three of the four subunits of Qβ-replicase are proteins of the host cell, the β-subunit being coded for by the phage RNA. The α-subunit of Qβ-replicase, which is the ribosomal protein S1 from the host cell, can be separated from the holoenzyme by ion-exchange chromatography (Kamen et al., 1972; Sumper & Luce, 1975). The remaining α-less replicase multiplies RNA more slowly than the holoenzyme (Biebricher et al., 1981). In order to examine the function of the α-subunit, the association and dissociation rates of α-less replicase to single-stranded MDV were measured and compared to the corresponding rates obtained for Qβ-replicase holoenzyme. The association rate constants for the binding of either minus or plus MDV strands to the replicase do not depend on whether the α-less replicase or the holoenzyme is used (Table III). In contrast, the dissociation rate of the α-less replicase RNA complex exceeds the dissociation of the holoenzyme complex by a factor of two (Table III). These results suggest that the S1 protein has a function in stabilizing the active complex.

Determination of the Equilibrium Constant. The equilibrium constants for the binding of Qβ-replicase to MDV− and

Table III: Comparison between the α-less Replicase and the Replicase Holoenzyme

	k_d (s^{-1})		k_a (L mol^{-1} s^{-1})	
	α-less	holo	α-less	holo
MDV−	$(5.8 \pm 0.6)\cdot10^{-3}$	$(2.2 \pm 0.1)\cdot10^{-3}$	$(7.8 \pm 0.4)\cdot10^6$	$(7.5 \pm 2.5)\cdot10^6$
MDV+	$(2.5 \pm 0.4)\cdot10^{-3}$	$(1.4 \pm 0.2)\cdot10^{-3}$	$(2.8 \pm 1.3)\cdot10^7$	$(3.5 \pm 0.6)\cdot10^7$

Table IV: Equilibrium Constants

$[Na^+]$ (mM)	$[Mg^{2+}]$ (mM)	T (°C)	MDV- K_a (M^{-1})	MDV+ K_a (M^{-1})
25	2	5	$(3.5 \pm 1.1)\cdot10^{11}$	$(4.2 \pm 0.5)\cdot10^{11}$
50	2	5	$(5.6 \pm 1.1)\cdot10^{10}$	$(1.1 \pm 0.5)\cdot10^{11}$
100	2	5	$(3.5 \pm 1.2)\cdot10^{9}$	$(2.5 \pm 0.6)\cdot10^{10}$
200	2	5	$(4.5 \pm 1.8)\cdot10^{7}$	$(5.8 \pm 2.6)\cdot10^{8}$
100	2	20	$(6.8 \pm 2.9)\cdot10^{9}$	$(2.0 \pm 0.8)\cdot10^{10}$
100	2	30	$(4.3 \pm 1.0)\cdot10^{9}$	$(1.3 \pm 0.2)\cdot10^{10}$
100	2	40	$(2.4 \pm 0.3)\cdot10^{9}$	$(5.8 \pm 2.2)\cdot10^{9}$

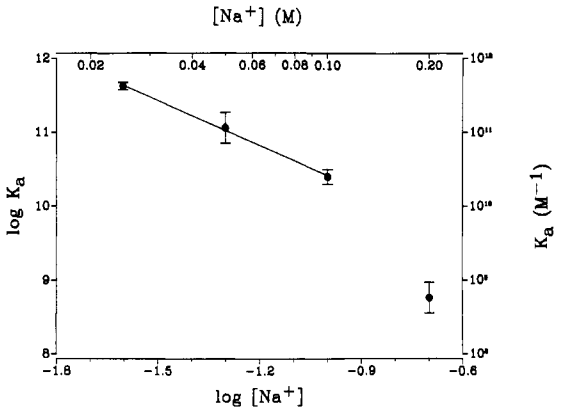

FIGURE 6: Double-logarithmic plot of the equlibrium constant of the replicase–MDV+ complex vs the sodium ion concentration. A line with a slope of $m\psi = -2.0$ is fitted through the data points between 25 and 100 mM NaCl.

MDV+ under different experimental conditions were calculated from the kinetic rate constants. The binding constants at different salt concentrations (Table IV) were compared and interpreted in terms of the polyelectrolyte model (Record et al., 1976). In a limited sodium concentration range up to 0.1 M, the data can be interpreted with this model (Figure 6). A number of ~3 ionic contacts between protein and nucleic acid is estimated, in agreement with the value determined from the kinetic data.

The temperature dependence of the binding constant (Table IV) can be interpreted according to the equation of van't Hoff, which describes the dependence of the equilibrium constant on the temperature. In the temperature range 20–40 °C, a linear function is obtained (Figure 7). Values of 39 and 47 kJ/mol are obtained for the enthalpy ΔH of binding of Qβ-replicase to MDV- and MDV+, respectively.

Under standard reaction conditions the equilibrium constant was determined in a separate titration experiment. The RNA concentration was kept constant, while the enzyme concentration was varied. The data were evaluated in terms of a double-reciprocal plot of the degree of binding as a function of the concentration of free enzyme (data not shown). An equilibrium constant $(2.8 \pm 2)\cdot10^9$ M^{-1} is determined from the slope and intercept of several plots, in good agreement with the value of $(3.5 \pm 1.2)\cdot10^9$ M^{-1} calculated from the kinetic rate constants.

Binding of Qβ-Replicase and Qβ-RNA. Because of Qβ-RNA is too large to enter a polyacrylamide gel, the binding of the phage RNA and Qβ-replicase was measured with a filter-binding assay under standard reaction conditions. To measure the dissociation rate, radioactive Qβ-RNA and Qβ-replicase were mixed and equilibrated. The equilibrium was perturbed by adding a 30-fold excess of nonlabeled phage RNA or a 1000-fold excess of aurintricarboxylic acid [a competitive binding inhibitor (Blumenthal & Landers, 1973)] to the solution. Samples were taken every 20 s and applied immediately to a nitrocellulose filter. When the first sample

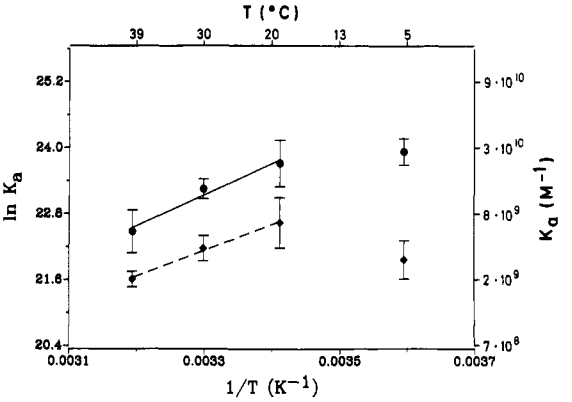

FIGURE 7: Van't Hoff plot of the equilibrium constant of the MDV–replicase complex as a function of the temperature for the plus (●) and the minus (◆) strand. A line is fitted through the data points between 20 and 40 °C. The slopes gives $\Delta H/R$; for MDV-, ΔH = −39 kJ/mol; for MDV+, ΔH = −47 kJ/mol.

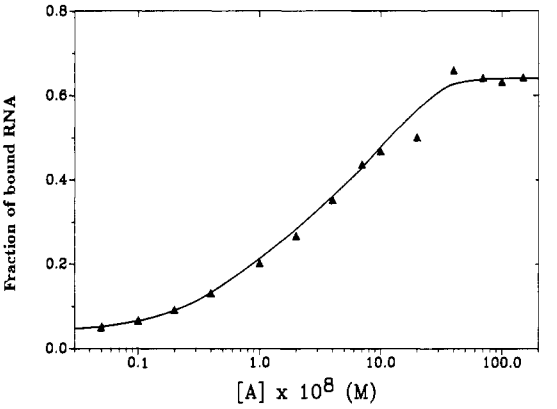

FIGURE 8: Protein excess binding curve with Qβ-RNA. The RNA concentration was $5\cdot10^{-11}$ M. [A] is the concentration of free enzyme.

was taken after 20 s, at least half of the complex had already dissociated. The reaction of the complex proceeds so fast that the rate cannot be determined exactly by this method. Assuming that the half-life of the complex is at most 20 s, a lower bound of $3.5\cdot10^{-2}$ s^{-1} is obtained for k_d.

The association rate constant was determined by rapidly mixing Qβ-replicase with radioactive Qβ-RNA and loading the sample after a short time interval on a nitrocellulose filter. Ten time points were measured in each association experiment. An association rate constant of $(4 \pm 2)\cdot10^5$ L mol^{-1} s^{-1} was obtained. This value is three orders of magnitude lower than the k_a of Qβ-replicase and single-stranded MDV under the same conditions.

Beside the kinetic determination, the equilibrium constant was also determined by two different types of titration. From a double-reciprocal plot, it was found that there is one binding site with a K_a of $(1.2 \pm 0.8)\cdot10^7$ M^{-1} (data not shown). A protein excess binding curve gives a similar value, $(3 \pm 1)\cdot10^7$ M^{-1}, for K_a (Figure 8).

Discussion

Several properties of the interaction between Qβ-replicase and template RNA molecules were investigated. The gel-retardation method, also used in this paper, was developed by Garner and Revzin (1981) and Fried and Crothers (1981) to study the binding of the lac repressor protein to the operator DNA. Up until now, this method was not applied to quantitative studies of protein–RNA interactions.

A quantitative characterization of an RNA–protein interaction was performed by Carey, Uhlenbeck, and co-workers (Carey et al., 1983; Carey & Uhlenbeck, 1983; Lowary &

Uhlenbeck, 1987), who studied the binding of the R17 coat protein to its binding site on the phage RNA, using a filter retention assay. They found a smaller association rate and a larger dissociation rate, compared to the kinetic rates of the binding of single-stranded MDV (particularly MDV+) to Qβ-replicase under similar conditions (low temperature, 80–100 mM monovalent salt, 10 mM Mg^{2+} ions). In the Qβ system, a high association rate of replicase and template leads to an efficient multiplication of the template RNA.

The reaction temperature has a smaller influence on the equilibrium constant of Qβ-replicase and MDV than on the binding of R17 coat protein and its binding site. This corresponds to a smaller enthalpy of binding. The dependence of the binding constant on the salt concentration is larger for the Qβ-replicase–MDV interaction than for the binding of the R17 coat protein to RNA, suggesting a larger contribution of electrostatic effects on the binding free energy. The thermodynamic parameters of the Qβ-replicase MDV interaction differ from the corresponding values of the R17 system. For the binding of replicase to MDV- and MDV+ at 25 °C in standard reaction buffer, ΔH is −39 and −47 kJ/mol, ΔG is −56 and −58 kJ/mol, and ΔS is +317 and +353 J mol^{-1} deg^{-1} K, respectively. The values for the binding of the R17 coat protein to its binding site are: ΔH = −80 kJ/mol, ΔG = −41 kJ/mol, and ΔS = −126 J mol^{-1} deg^{-1} K (Carey & Uhlenbeck, 1983). In the R17 system the contribution of ΔH to ΔG is nearly twice as large as with the Qβ system, whereas the contribution of ΔS is negative and therefore unfavorable. A large ΔH indicates the presence of weak contacts such as van der Waals interactions and hydrogen bonds in a nucleic acid–protein complex. Ionic bonds and hydrophobic interactions lead to an increase in ΔS (Beaudette & Langerman, 1980). In the case of the Qβ system, this would be in agreement with the large influence of the ion concentration on the binding behavior.

In interpreting the salt dependence of the kinetic rates, one has to take into account that the polyelectrolyte model is a simplified description of the charge distribution around DNA molecules. These molecules are pictured as linear rods of infinite length, where no end effects occur. The application of this model to short single-stranded RNA molecules, which are highly structured in solution, is not straightforward. The results obtained can only be regarded as an approximation.

The dependence of the dissociation rate constant on salt concentration and temperature permits an interpretation according to simple kinetic models. The number of ion pairs between the replicase and MDV RNA in the complex, and also the energy of activation of the dissociation reaction, can thus be determined. In contrast to the dissociation rates, the association rates do not show the temperature and salt concentration dependencies implied by these simple models. The association of the two macromolecules is a complex process in which the spatial orientation of the molecules and probably also structural changes are involved. Most likely, the reaction proceeds in several steps. The first would be an association due to electrostatic interactions, followed by structural changes which permit the formation of the tight complex.

In both the association and dissociation kinetics, a distinct difference between the plus and the minus strand of MDV exists. Because the number of ionic contacts between the RNA and the enzyme is the same, the difference is caused most likely by weaker interactions like hydrogen or van der Waals bonds. The larger difference in ΔH compared to the difference in ΔG also suggests this interpretation. The asymmetric binding behavior of the complementary strands should lead to dissimilar replication rates, resulting in different concentrations of plus and minus strand. Indeed, Biebricher et al. (1984) reported a faster growth of RNA with plus strand as template than with minus strands.

The binding of the plus strand of the Qβ-phage RNA to Qβ-replicase is several orders of magnitude weaker than the binding of the small variant MDV under the same conditions. In vivo, the host factor, a protein originating from the bacterial cell that binds to the RNA (DeHaseth & Uhlenbeck, 1980), is involved in the replication of Qβ-replicase (August et al., 1968; Spiegelman et al., 1968). The detailed role of this host factor in template binding is not yet elucidated.

Since the rates found differed from values assumed by Biebricher et al. (1983), computer simulations with the measured rate constants were performed and found to be in agreement with the experimental growth curves of RNA. By varying the parameters in the simulations, the association rate value was found to be important for the replication behavior, whereas the dissociation rate values may differ over a wide range without impact on the replication kinetics.

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Registry No. Qβ-Replicase, 9026-28-2.

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Induction of Mammalian DNA Topoisomerase I and II Mediated DNA Cleavage by Saintopin, a New Antitumor Agent from Fungus

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ABSTRACT: Saintopin is an antitumor antibiotic recently discovered in mechanistically oriented screening using purified calf thymus DNA topoisomerases. Saintopin induced topoisomerase I mediated DNA cleavage comparable to that of camptothecin, and topoisomerase II mediated DNA cleavage equipotent to those of 4′-(9-acridinylamino)methanesulfon-*m*-anisidide (*m*-AMSA) or 4′-demethylepipodophyllotoxin 9-(4,6-*O*-ethylidene-β-D-glucopyranoside) (VP-16). Treatment of a reaction mixture containing saintopin and topoisomerase I or II with either elevated temperature (65 °C) or higher salt concentration (0.5 M NaCl) resulted in a substantial reduction in DNA cleavage, suggesting that the topoisomerase I and II mediated DNA cleavage induced by saintopin is through the mechanism of stabilizing the reversible enzyme–DNA "cleavable complex". Consistent with the cleavable complex formation with both topoisomerases, saintopin inhibited catalytic activities of both topoisomerase I and topoisomerase II. The DNA cleavage intensity pattern induced by saintopin with topoisomerase I was different from that by camptothecin. A difference in cleavage pattern was also detected between saintopin and *m*-AMSA or VP-16 in topoisomerase II mediated DNA cleavage. DNA unwinding assay using T4 DNA ligase showed that saintopin is a weak DNA intercalator like *m*-AMSA. Thus, saintopin represents a new class of antitumor agent that can induce both mammalian DNA topoisomerase I and mammalian DNA topisomerase II mediated DNA cleavage.

DNA topoisomerases are nuclear enzymes that catalyze the concerted breaking and rejoining of DNA strands, thereby controlling the topological states of DNA. Two major topoisomerases, topoisomerase I and topoisomerase II, have been detected in all eukaryotic cells. Topoisomerase I catalyzes the passage of the DNA strand through a transient single-strand break, while topoisomerase II catalyzes the passage of DNA double strands through a transient double-strand break. These topoisomerases have been known to be involved in many important DNA metabolism reactions including replication, recombination, transcription, and chromosome segregation at mitosis (Wang, 1985). In addition, both topoisomerase I and topoisomerase II have generated extensive clinical interest in chemotherapy. There is now good evidence showing that topoisomerase II is the principal intracellular target for a number of clinically important antitumor drugs (Liu, 1989; D'Arpa & Liu, 1989). Despite their apparent structural diversity, these drugs have the common property of stabilizing a key covalent reaction intermediate of topoisomerase II,

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