dUTPase from the retrovirus equine infectious anemia virus: specificity, turnover and inhibition

Johan Nord[a,*], Gunilla Larsson[a], Jan-Olov Kvassman[b], Anna Maria Rosengren[a], Per Olof Nyman[a]

[a]*Department of Biochemistry, Center for Chemistry and Chemical Engineering, University of Lund, P.O. Box 124, S-221 00 Lund, Sweden*
[b]*Henry Ford Health Sciences System, Division of Biochemical Research, Detroit, MI 48202-3450, USA*

Received 27 June 1997

Abstract The kinetic properties of dUTPase from equine infectious anemia virus (EIAV) were investigated. K_M (1.1 ± 0.1 μM) and k_{cat} (25 s^{-1}) were found to be independent of pH in the neutral pH range. Above pH 8.0, K_M increases slightly. Below pH 6.0, the enzyme is rapidly deactivated. Detergent was found to enhance activity, leaving K_M and k_{cat} unaffected. Compared to the *Escherichia coli* dUTPase, the EIAV enzyme is equally potent in hydrolyzing dUTP, but less specific. Inhibition of the viral enzyme by the nucleotides dTTP, dUMP and a synthetic analogue, 2′-deoxyuridine 5′-(α,β-imido)triphosphate, is stronger by one order of magnitude.

Key words: dUTPase; Equine infectious anemia virus; Kinetic constant; Inhibition; Deoxyuridine; Analogue

1. Introduction

dUTP pyrophosphatase (dUTPase, EC 3.6.1.23) catalyzes the hydrolysis of dUTP to dUMP and pyrophosphate [1–3], thereby preventing hazardous incorporation of uracil into DNA. Mutants with reduced dUTPase activity exhibit DNA fragmentation with concomitant increase in mutation and recombination [4]. dUTPase has been reported from a variety of cellular and viral systems. It has been shown to be essential for the viability of *E. coli* [5] and *Saccharomyces cerevisiae* [6] and to be a target for a cytotoxic drug in human cancer cells [7]. The crystal structures of dUTPase from *E. coli*, man, and the retrovirus feline immunodeficiency virus have been solved [8–10].

The genomes of retroviruses are small and contain only a few genes, and the occurrence of an encoded dUTPase in several of these viruses is therefore notable. dUTPase null-mutants of lentiviruses, a subgroup of retroviruses, replicate to wild-type levels in dividing cells, but are attenuated for replication in resting cells, in which the wild-type virus is able to replicate [11–13]. Apparently, these viruses cannot depend on their host dUTPase, which makes their dUTPases possible targets for antiviral drugs. Kinetic and structural characterizations of viral and host dUTPases would be requisites for design of such virus-specific inhibitors.

*Corresponding author. Fax: (46) 46-222-4534.
E-mail: Johan.Nord@biokem.lu.se

Abbreviations: α,β-Imido-dUTP, 2′-deoxyuridine 5′-(α,β-imido)triphosphate; $C_{12}E_8$, octa ethylene glycol mono *n*-dodecylether; dUrd, deoxyuridine; dUTPase, dUTP pyrophosphatase; *E. coli*, *Escherichia coli*; EIAV, equine infectious anemia virus

To enable detailed studies of the EIAV dUTPase, a method for high-level overproduction of recombinant enzyme has been developed in our laboratory [14]. The enzyme has recently been crystallized [15] and X-ray studies are under way. Here, we describe the kinetic properties of the enzyme, assessed mainly by using a stopped-flow spectrophotometric method [16]. For the first time, inhibition constants and turnover rates for various nucleotides are presented for a viral dUTPase. So far, the only dUTPase thoroughly characterized kinetically is the one from *E. coli* [16]. Here, the kinetic constants for the bacterial enzyme are compared with those obtained in the present study.

2. Materials and methods

2.1. Chemicals

2′-Deoxyuridine 5′-(α,β-imido)triphosphate (α,β-imido-dUTP) was synthesized as described [17]. Other nucleotides and pH indicators were from Sigma, USA. All other chemicals were of highest available quality. Nucleotides were purified on a Mono Q anion-exchange column from Pharmacia, using 10 mM KCl in a 10–200 mM HCl gradient. Recombinant EIAV dUTPase, carrying an extra Met at the amino terminus not encoded in the native sequence [18], was prepared according to Bergman et al. [14], and stored at −20°C. The concentrations of enzyme [14,19] and nucleotides were determined spectrophotometrically (Shimadzu UV-160, Japan).

2.2. Spectroscopic monitoring of nucleotide hydrolysis

The kinetic properties of the EIAV dUTPase were studied by mixing enzyme and substrate in a stopped-flow spectrophotometer (Durrum D130, USA), using a pH indicator as reporting agent [16]. The reaction was followed to completion under multiple ($C_E \ll [S]_0$) and single ($C_E \gg [S]_0$) turnover conditions. Experiments were carried out at 25°C with deaerated solutions of dUTPase, nucleotides, buffer (50 μM to 1.3 mM), indicator (5–25 μM), and $MgCl_2$ (5 mM, unless otherwise stated), keeping the ionic strength at 0.10 M with KCl. When used, the monodisperse detergent, octa ethylene glycol mono *n*-dodecylether ($C_{12}E_8$, Nikkol Chemicals, Japan), was kept at a concentration of 0.01% (w/v). The indicator/buffer systems were bromothymol blue/MOPS (pH 6–8) and cresol red/BICINE (pH 7.4–9). The apparent pK_a values of the indicators were determined by titration, in the stopped-flow apparatus, of solutions identical to those used in the kinetic experiments, but without enzyme and substrate.

Multiple turnover was accomplished by reacting 0.04–0.50 μM dUTPase with 6–25 μM dUTP. The inhibition by nucleotides other than dUTP was studied by including inhibitor at 5–10 different concentrations ranging from zero to twice the K_i of the inhibitor. V_{max}, K_M and K_i were evaluated by fitting the integrated Michaelis-Menten equation to the resulting absorbance traces [16,20]. Data points in the region of equilibrium and the immediate start of the trace were omitted (Fig. 1A). The reproducibility of the method was tested by determining the K_M of *E. coli* dUTPase at pH 7.8 [16].

Single turnover of the dUTPase-dUTP system was effectuated by mixing 5–35 μM dUTPase with 0.5–1 μM dUTP. The rate constant observed for the exponential absorbance traces is given by $k_{obs} \approx k_{cat}/(1+K_M/C_E)$ [16]. The measurements were conducted with no buffer

PII S0014-5793(97)00935-6

added to the system, and with saturating enzyme concentration (due to the low signal to noise ratio obtained at low enzyme and substrate concentration) giving $k_{obs} \approx k_{cat}$.

The single-turnover approach was not applicable when determining k_{cat} for dCTP, due to the high K_i for this nucleotide. Instead, the constant was determined at multiple turnover conditions by mixing enzyme with dCTP at a subsaturating concentration. dUTP at a saturating concentration was included as internal standard. This resulted in a biphasic curve (Fig. 1B), the first phase reflecting the complete hydrolysis of dUTP at a rate given by $k_{cat,dUTP} \times$[dUTPase], and the second one the slow hydrolysis of dCTP at the rate $k_{cat,dCTP} \times$[dUTPase]$\times$[dCTP]$_0$/([dCTP]$_0$+$K_{i,dCTP}$). $k_{cat,dCTP}$ was obtained by dividing the slope of dCTP hydrolysis with the slope of dUTP hydrolysis and substituting with the values for $K_{i,dCTP}$ and $k_{cat,dUTP}$ determined as above.

The effect of detergent was analyzed by combining the results from single- and multiple-turnover measurements. The fraction of available active sites in the presence and absence of detergent was estimated as $V_{max}/k_{cat,dUTP}$ divided by the spectrophotometrically determined enzyme concentration.

2.3. Chromatographic analysis of nucleotide hydrolysis

Reaction mixtures (200 µl) with cresol red (15 µM), BICINE (2–10 mM) pH 8.0, $MgCl_2$ (5 mM), KCl (85 mM), dUTPase (10 µM) and nucleotide (380 µM) were incubated over night at 25°C. Each tube had a reference treated in the same way but without enzyme. To calculate k_{cat}/K_M for dCTP, the exponential rate constant for dCTP hydrolysis was determined at known concentration of active enzyme by taking samples at time intervals and quenching by the addition of EDTA (final concentration, 50 mM). After chromatography on a Mono Q column as described in section 2.1, hydrolysis was estimated as the change in the absorbance-peak area on the chromatogram.

3. Results

3.1. Measurements at multiple turnover conditions

As demonstrated by Fig. 1A, the integrated Michaelis-Menten equation could be nicely fitted to the data obtained by continuous spectroscopic monitoring of multiple-turnover hydrolysis of dUTP. There was no second-order dependence on substrate concentration (which could indicate cooperativity between the subunits). K_M and V_{max} were insensitive to variations in the composition of the reaction mixture, e.g., variations in buffer concentration, increase in the Mg^{2+} concentration up to 20 mM, and the interchange of the two indicator/buffer systems in an overlapping pH interval (pH 7–8). As judged by V_{max}, k_{cat} is relatively constant over the pH range investigated. However, at low pH, the stability of the enzyme is poor: at pH $\leq$ 6, the activity decreases rapidly and irreversibly. K_M is a constant 1.1 ± 0.1 µM between pH 6.2 and 8.0. It raises slowly above pH 8.0, to reach 1.4 µM at pH 9.0. The hydrolysis product, dUMP, as well as the other nucleotides investigated caused competitive inhibition (Table 1 and Fig. 1B). The concerted inhibitory effect of dUMP and PP_i was not significantly different from the inhibition by dUMP alone.

Chromatographic experiments showed that the EIAV dUTPase hydrolyzes UTP, dTTP and dCTP to their corresponding monophosphates, with no nucleotide diphosphate formed. dUDP and α,β-imido-dUTP are not hydrolyzed by the viral enzyme.

3.2. Turnover-rate constants

The V_{max} values obtained indicate a k_{cat} for dUTP greater than 10 s^{-1} and approximately constant in the neutral pH range. When the concentration of enzyme was increased, the reaction curve obtained at multiple-turnover conditions (Fig. 1) gradually changed into an exponential course of reaction. At saturating enzyme concentration (i.e., at single-turnover conditions) and pH 7.4, a first-order rate constant of around 25 s^{-1} was obtained (Fig. 2). Similar exponential rates were obtained from single measurements using a saturating enzyme concentration at pH 7.0 and pH 8.0, which confirmed that k_{cat} is approximately constant in the neutral pH range. k_{cat} for dCTP was estimated by measuring relative rates of dCTP and dUTP hydrolysis (Fig. 1B). This value together with the K_i determined for dCTP is in accordance with the apparent second-order rate constant, k_{cat}/K_M, determined chromatographically for dCTP (Table 1). dTTP is hydrolyzed at a significantly lower rate, i.e. $k_{cat,dTTP} < 0.5$ s^{-1}, and was not investigated further (Fig. 1B).

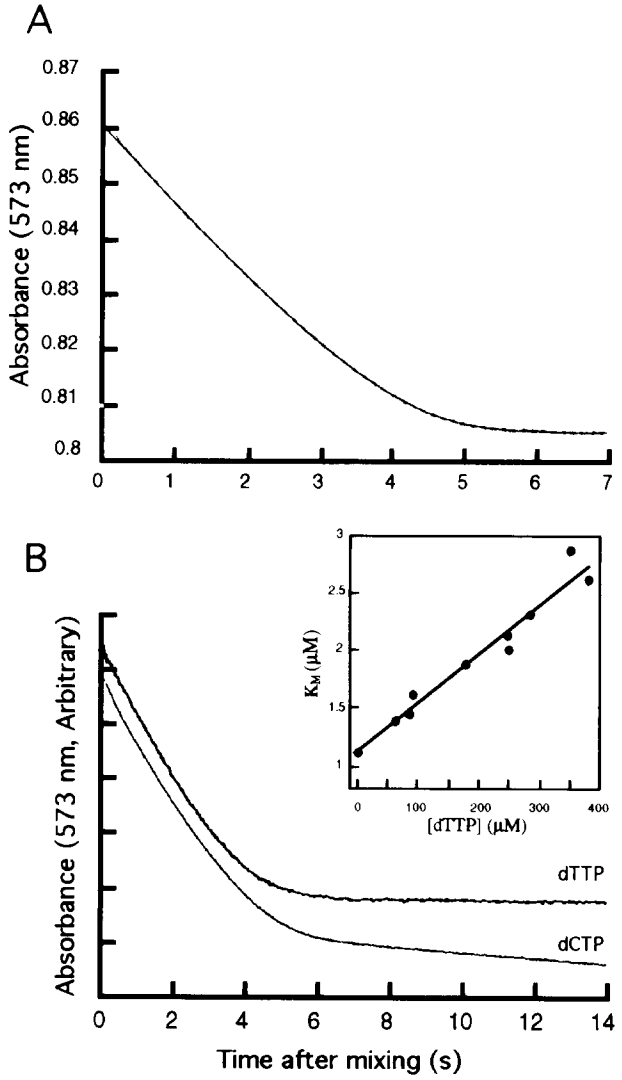

Fig. 1. (A) dUTP (8.9 µM) hydrolyzed by EIAV dUTPase (0.2 µM active sites) at pH 7.8 and multiple-turnover conditions. The best fit of the integrated Michaelis-Menten equation is superimposed (K_M = 1.1 µM, and V_{max} = 2.5 µM/s). (B) Hydrolysis of 10.9 µM dUTP with dCTP (1.9 mM) or dTTP (250 µM) present. After the hydrolysis of dUTP has reached completion, hydrolysis of dCTP and dTTP continues at a slower rate. The hydrolysis of dCTP is clearly visible, while that of dTTP is too slow. Inset: Inhibition of dUTP hydrolysis by dTTP (apparent K_M for dUTP as a function of the concentration of dTTP).

3.3. Effect of detergent

Upon the addition of detergent, the activity of the EIAV dUTPase increases, as noted previously [14]. V_{max} was found to increase by 70–90%, while K_M remained unaffected. The integrated Michaelis-Menten equation could be nicely fitted to the multiple-turnover data. Also k_{cat} was found to be unaffected, according to single-turnover experiments carried out as described in Fig. 2 but with detergent present (Table 1). This result indicates that the increase in activity upon addition of detergent is due to an increase in the fraction of available active sites. Without detergent, this fraction was estimated to 30–50%, occasionally as low as 10%. In the presence of detergent, the fraction increased to 70–90% of the total amount of sites present.

4. Discussion

Earlier investigations of cellular dUTPases in general report a low K_M (0.1–12 μM) and a dependence upon divalent metal ions such as Mg^{2+} [3,9,16,21–24]. Similar properties have been reported for viral dUTPases, with $K_M < 1$ μM [12,25–27]. However, up to now, the only dUTPase for which turnover numbers and inhibition constants for nucleotides have been investigated is the *E. coli* dUTPase [16]. Here, we present those constants for the EIAV dUTPase and compare them with those of the bacterial enzyme (Table 1).

Among the properties of the EIAV dUTPase are a low K_M, a broad pH optimum (in contrast to the narrow one reported for the dUTPase of the feline immunodeficiency virus), and first order Michaelis-Menten kinetics (contradicting the proposal that the intertwined quaternary structure of trimeric dUTPase should render its active sites cooperative [9]). The EIAV dUTPase is a potent catalyst of dUTP hydrolysis. Its specificity constant (k_{cat}/K_M) for dUTP is close to the diffusion controlled rate limit, as found for the *E. coli* dUTPase [16]. However, it is significantly less specific and exhibits a specificity constant for dCTP ten times that of the *E. coli* dUTPase. Also, despite a five times higher K_M for dUTP, the binding of the analogous nucleotides dTTP, α,β-imido-dUTP and dUMP, is at least one order of magnitude stronger in the viral dUTPase (Table 1). If K_M for dUTP is used to normalize inhibitor binding by the two enzymes (which is allowed if the association rate constant of substrate and enzyme is assumed to be equal in the two systems), the binding discrimination between dUTP and the other nucleotides would be more than 40 times better by the *E. coli* dUTPase than in the case of the EIAV enzyme. For dUDP, the factor would be greater than 20. These observations are interesting, since viral dUTPases have earlier been reported to be highly specific [12,27].

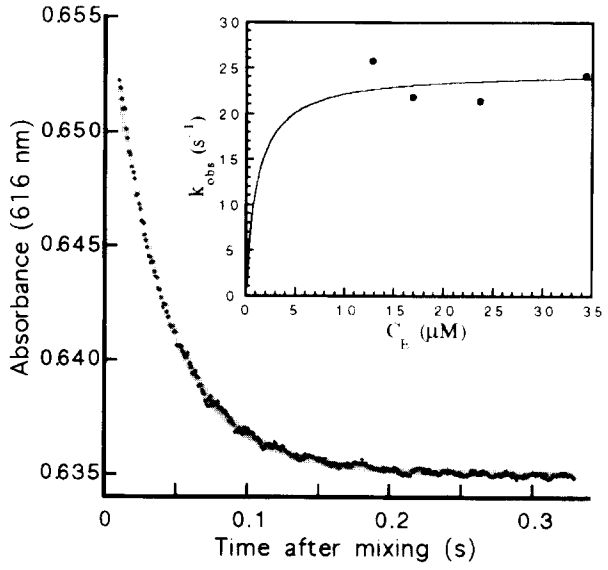

Fig. 2. dUTP hydrolysis catalyzed by EIAV dUTPase at single-turnover conditions at pH 7.4 and exponential regression of the data (grey). Inset: the rate constants (k_{obs}) obtained at different concentrations of dUTPase (C_E, active sites). The equation $k_{obs} \approx k_{cat}/(1+K_M/C_E)$ [16] is fitted to the data, using a K_M of 1.1 μM, to give a k_{cat} of 25 s^{-1}.

Table 1
Kinetic properties of the EIAV dUTPase at pH 8 (unless otherwise stated)

Nucleotide species	EIAV dUTPase	*E. coli* dUTPase
dUTP		
K_M (μM, pH 6.2–8.0)	1.1 ± 0.1[a]	0.2
k_{cat} (s^{-1}, pH 7.4)	25[a]	7
k_{cat}/K_M ($M^{-1}s^{-1}$)[b]	2×10^7	3.5×10^7
dCTP		
K_M (μM)[c]	3000	4000
k_{cat} (s^{-1})	2	0.4 s^{-1}[b]
k_{cat}/K_M ($M^{-1}s^{-1}$)	1000	100
dTTP		
K_M (μM)[c]	260 ± 20	> 20000[b]
k_{cat} (s^{-1})	< 0.5	–
k_{cat}/K_M ($M^{-1}s^{-1}$)	< 2000[b]	–
K_i of non-substrate species		
α,β-imido-dUTP (μM)	0.60 ± 0.03	5[d]
dUDP (μM)	3.6 ± 0.1	15
dUMP (μM)	130 ± 10	1500
	150 ± 10 (pH 7)	150 (pH 5)
deoxyuridine (μM)	1500 ± 50	1000

The kinetic constants (with standard deviations) were assessed as described in Section 2. As a comparison, the properties of the *E. coli* dUTPase are also included [16], see Section 4.
[a]The same values were also obtained with 0.01% (w/v) of the detergent $C_{12}E_8$ added. [b]Calculated value. [c]$K_M \approx K_i$. [d][17].

The data reported here should facilitate an understanding of the molecular interactions between enzyme and substrate. In the absence of the crystal structure, the following observations can be made. In the *E. coli* dUTPase, dUMP is bound more weakly than dUrd, which suggests a repelling force acting on the α-phosphate of a nucleotide when bound to the active site. In the EIAV dUTPase, dUMP is instead bound more strongly than dUrd. Comparison of $K_{M,dUTP}$, $K_{i,dUDP}$, $K_{i,dUMP}$ and $K_{i,dUrd}$ of the two enzymes at pH 8 suggests that, in the viral dUTPase-dUTP complex, the triphosphate moiety is bound mainly via the α- and β-phosphates, whereas in the complex with the bacterial enzyme, it is bound preferentially via the β- and γ-phosphates. This notion is consistent with the weaker binding of magnesium pyrophosphate to the viral dUTPase than to the bacterial enzyme [16].

dTTP is bound more strongly by the EIAV dUTPase compared to the bacterial dUTPase, with a K_i for dTTP lower by 2 orders of magnitude. Predicted from structural data for the *E. coli* and human dUTPases, the binding of dTTP is prevented by a steric clash of the thymine methyl group with

residues in the wall of the nucleotide-binding pocket [8,9]. According to our kinetic data, the pocket of the EIAV dUTPase would be more prone to accept the methyl group. However, the k_{cat} for dTTP is low, suggesting that the enzyme dTTP complex is distorted and non-optimal for hydrolysis. dCTP is weakly bound by both enzymes. However, for the EIAV dUTPase, $k_{cat,dCTP}$ is fairly high. The data obtained here will be useful in a mechanistic interpretation of a future X-ray structure of the EIAV dUTPase, and in understanding the role of dUTPase in the life cycle of lentiviruses.

Acknowledgements: We gratefully thank Gösta Pettersson for discussions on kinetic theory, and Rebecca Persson for laboratory assistance. α,β-imido-dUTP was kindly provided by Tina Persson. This work was supported by grants from the Swedish Cancer Society, Craafords Stiftelse, Magnus Bergwalls Stiftelse and Kungliga Fysiografiska Sällskapet in Lund.

References

[1] Bertani, L.E., Häggmark, A. and Reichard, P. (1961) J. Biol. Chem. 236, PC67–PC68.
[2] Bertani, L.E., Häggmark, A. and Reichard, P. (1963) J. Biol. Chem. 238, 3407–3413.
[3] Hoffmann, I. (1987) Doctoral thesis, University of Saarbrücken, Germany.
[4] Tye, B.-K., Nyman, P.O., Lehman, I.R., Hochhauser, S. and Weiss, B. (1977) Proc. Natl. Acad. Sci. USA 74, 154–157.
[5] El-Hajj, H.H., Zhang, H. and Weiss, B. (1988) J. Bacteriol. 170, 1069–1075.
[6] Gadsden, M.H., McIntosh, E.M., Game, J.C., Wilson, P.J. and Haynes, R.H. (1993) EMBO J. 12, 4425–4431.
[7] Zalud, P., Wachs, W.O., Nyman, P.O. and Zeppezauer, M.M. (1995) in: Purine and pyrimidine metabolism in man (Sahota, A. and Taylor, M., Eds.) VIII pp. 135–138. Plenum publishing corp., New York.
[8] Larsson, G., Svensson, L.A. and Nyman, P.O. (1996) Nature Struct. Biol. 3, 532–538.
[9] Mol, C.D., Harris, J.M., McIntosh, E.M. and Tainer, J.A. (1996) Structure 4, 1077–1092.
[10] Prasad, G.S., Stura, E.A., McRee, D.E., Laco, G.S., Hasselkus-Light, C., Elder, J.H. and Stout, C.D. (1996) Protein Science 5, 2429–2437.
[11] Threadgill, D.S., Steagall, W.K., Flaherty, M.T., Fuller, F.J., Perry, S.T., Rushlow, K.E., Le Grice, S.F.J. and Payne, S.L. (1993) J. Virol. 67, 2592–2600.
[12] Wagaman, P.C., Hasselkus-Light, C.S., Henson, M., Lerner, D.L., Phillips, T.R. and Elder, J.H. (1993) Virology 196, 451–457.
[13] Turelli, P., Pétursson, G., Guiguen, F., Mornex, J.-F., Vigne, R. and Quérat, G. (1996) J. Virol. 70, 1213–1217.
[14] Bergman, A.-C., Björnberg, O., Nord, J., Rosengren, A.M. and Nyman, P.O. (1995) Protein Express. Purif. 6, 379–387.
[15] Persson, R., Rosengren, A.M., Nyman, P.O., Dauter, Z. and Cedergren-Zeppezauer, E. (1997) Protein and Peptide Letts 4, 145–148.
[16] Larsson, G., Nyman, P.O. and Kvassman, J.-O. (1996) J. Biol. Chem. 271, 24010–24016.
[17] Persson, T., Larsson, G. and Nyman, P.O. (1996) Bioorg. Med. Chem. 4, 553–556.
[18] Yaniv, A., Dahlberg, J., Gazit, A., Sherman, L., Chiu, I.M., Tronick, S.R. and Aaronson, S.A. (1986) Virology 154, 1–8.
[19] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[20] Walker, A.C. and Schmidt, C.L.A. (1944) Arch. Biochem. 5, 445–467.
[21] Hokari, S. and Sakagishi, Y. (1987) Arch. Biochem. Biophys. 253, 350–356.
[22] Pardo, E.G. and Gutiérrez, C. (1990) Exp. Cell Res. 186, 90–98.
[23] Wist, E. (1979) Biochim. Biophys. Acta 565, 98–106.
[24] Ingraham, H.A., Dickey, L. and Goulian, M. (1986) Biochemistry 25, 3225–3230.
[25] Björnberg, O. and Nyman, P.O. (1996) J. Gen. Virol. 77, 3107–3111.
[26] Sommer, P., Kremmer, E., Bier, S., König, S., Zalud, P., Zeppezauer, M., Jones, J.F., Mueller-Lantzsch, N. and Grässer, F. (1996) J. Gen. Virol. 77, 2795–2805.
[27] Roseman, N.A., Evans, R.K., Mayer, E.L., Rossi, M.A. and Slabaugh, M.B. (1996) J. Biol. Chem. 271, 23506–23511.