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QUARTERLY

Inhibition of the helicase activity of HCV NTPase/helicase by 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide-5′-triphosphate (ribavirin-TP)

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In the presented study the ribavirin-TP — an established inhibitor of the NTPase activity of the superfamily NTPase/helicases II — was investigated as an inhibitor of the unwinding activity of the hepatitis C virus (HCV) NTPase/helicase. The kinetics of the reaction revealed that ribavirin-TP reduces the turnover number of the helicase reaction by a mechanism that does not correspond to that of the inhibition of the NTPase activity. Our results suggest that derivatives of ribavirin-TP with enhanced stability towards hydrolytic attack may be effective inhibitors of the enzyme.

NTPase/helicases unwind duplex RNA or DNA structures by disrupting the hydrogen bonds that keep the two strands together [1–3]. This reaction is coupled with the hydrolysis of γ-phosphate of ATP. All NTPase/helicases known to date display ATPase activity [2, 3]. Recently, it was found that, in the presence of a nonhydrolyzable ATP analogue, adenosine-5′-(3-thiotriphosphate) (ATP-γ-S), only a low level of unwinding of HCV dsRNA

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Abbreviations: ATP-γ-S, adenosine- 5′-(3-thiotriphosphate); HCV, hepatitis C virus; NDP, nucleoside-5′-diphosphate(s); NMP, nucleoside-5′-monophosphate(s); NS, nonstructural proteins; NTP, nucleoside-5′-triphosphate(s); NTPase, nucleoside triphosphatase, ribavirin-TP, 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide-5′-triphosphate; TBE, Tris/borate/EDTA buffer.

could be detected [4]. The NS3 – like proteins of flaviviruses, pestiviruses and of HCV belong to superfamily II (SFII) [2]. The ATP-binding site of the NTPase/helicase contains conserved amino-acid sequences called Walker motifs A and B that are located on the surface of domain I of the three-domain enzymes [5]. These motifs are responsible for the binding of the terminal phosphate groups of ATP and the Mg^{2+} of the Mg-ATP complex respectively [5–7]. Mutations in these motifs eliminate the ATP hydrolytic activity of the enzymes [6, 8].

On the basis of these results one could speculate that a reduction of the accessibility of the ATP-binding site for ATP may lead to inhibition of the ATPase and, consequently, of the helicase activity of the NTPase/helicases. Indeed, in our previous studies we have demonstrated that compounds that reduced the binding of ATP to proteolytically isolated domain I of the HCV NTPase/helicase are capable of inhibiting the ATPase activity of the enzyme [9]. Nevertheless, there is no simple correlation between the turnover number of the NTPase reaction and the number of helicase unwinding cycles of the enzymes. Further analysis using a West Nile (WN) virus NTPase/helicase, a further SFII enzyme, revealed that O^6-benzylguanine and N-chloroethylguanine derivatives modulated, i.e. enhanced or reduced, the ATPase activity of the enzyme without influencing the helicase activity. Moreover, the helicase activity may be up- or down-regulated without corresponding changes of the requirement for ATP [10].

Therefore we performed a study, presented here, in which the inhibitory potential of 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide-5′-triphosphate (ribavirin-TP) towards the helicase activity of HCV NTPase/helicase was tested. This compound was established to be a competitive (with regard to ATP) inhibitor of the NTPase activity of HCV and WN virus NTPase/helicases [10, 11].

MATERIALS AND METHODS

Production and purification of the HCV NTPase/helicase. HCV NTPase/helicase was expressed in *Escherichia coli* and purified as previously described [12, 13]. The purity of the obtained enzyme was around 85%. The purification procedure was completed by gel exclusion chromatography on Superdex 200 (Amersham-Pharmacia) [11]. The final preparation contained homogeneous HCV NTPase/helicase.

ATPase and helicase assays. A standard ATPase assay was performed according to the charcoal adsorption method described previously [9, 11]. The helicase activity of the enzyme was determined using a DNA substrate that was obtained by annealing two partly complementary DNA oligonucleotides. The synthesised oligonucleotides had sequences corresponding to the deoxynucleotide version of the RNA strands described previously [4, 10].

Synthesis of 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide-5′-triphosphate (ribavirin-TP). Ribavirin-TP was synthesised according to the modified Yoshikawa-Ludwig-Mishra-Broom procedure [11, 14–17].

Other methods. Kinetic parameters of the reactions were determined by non-linear-regression analysis using ENZFITTER (BioSoft) and SIGMA PLOT (Jandel Corp.). Protein concentration was measured by the method of Lowry *et al.* [18]. The purity of the proteins was determined by densitometric analysis of the SDS/polyacrylamide gels stained with Coomassie Blue [19].

RESULTS AND DISCUSSION

Purity of the enzyme

It is known that the NTPase and helicase activities of different preparations of HCV en-

zymes expressed in *E. coli* differ in their specific activity, the response to polynucleotides or their affinity to substrate [11]. Since the discrepancies between the data result often from differences in the purity of the final preparations or various reaction conditions, the HCV NTPase/helicase used in this study was purified so as to obtain a homogeneous enzyme preparation and optimised conditions of the reaction had to be established.

Substrates and kinetic parameters of HCV NTPase/helicase

The NTPase activity of the enzyme was assayed by a previously described charcoal adsorption method [9, 11]. Direct comparison of the kinetic parameters obtained under optimised conditions showed no significant differences between the enzyme preparation used here and that used in previous studies [9, 11]. The two enzymes displayed similar temperature optima, time course of the reaction and dependency on divalent ions. However, the K_m value for ATP estimated for the enzyme used here was 143.5 μM, in contrast to 11.0 μM for the enzyme described by us previously [9, 11].

The helicase activity of the enzyme was investigated with a partly double-stranded DNA substrate well characterised in previous studies. The substrate was applied at non-saturating concentrations [4, 10]. When the kinetic parameters of the unwinding and ATPase activities of the enzyme were compared, some discrepancies between the optimum conditions were observed. The most prominent difference, important for the further study, probably due to the strongly limited concentrations of the DNA–substrate, is that the half-optimum ATP concentration for the helicase turnover was 5 fM (Borowski, P., Niebuhr, A. & Schmitz, H., unpublished data).

An important feature of the unwinding reaction is that at the ATP concentration corresponding to the K_m value for the ATPase reaction (143.5 μM) and with 4.7 pM of the DNA-substrate (nucleotide base), the plateau of the unwinding reaction was reached after 20 min (Fig. 1). There were no significant differences when the helicase and ATPase activities were compared in respect of their dependency on divalent ions and temperature optimum (not shown).

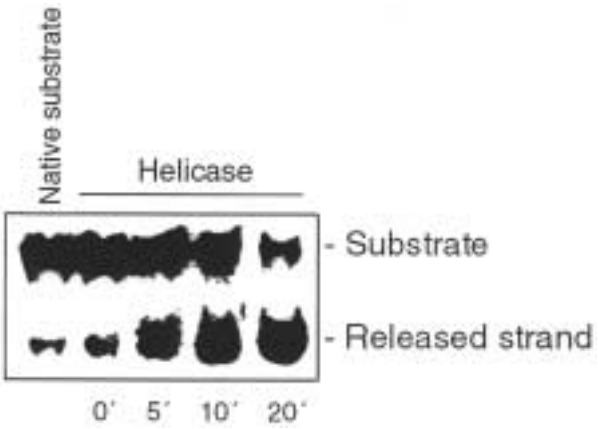

Figure 1. Time course of the unwinding reaction mediated by HCV NTPase/helicase.

The helicase assays were performed with 10 pmol of the enzyme and the unwinding reaction was terminated at the times indicated in the figure as described under Materials and Methods. The samples were separated on a 15% polyacrylamide TBE gel containing 0.1% SDS and the dried gel was exposed to X-ray film at –80°C for 14 h. The autoradiography shown is representative of three independent experiments.

Response of the HCV NTPase/helicase to ribavirin and ribavirin-TP

Previous studies indicated that, at a very low ATP concentration, i.e. <1/1000 of K_m values, ribavirin added at a concentration range of 50–750 μM could act as a competitive ATPase inhibitor [11]. Ribavirin *versus* ATP titration curves did not reveal, however, any inhibitory potential of the compound towards the helicase activity of the enzyme. Similar results were obtained previously with the WN virus NTPase/helicase [10] and with the Japan encephalitis virus (JEV) enzyme, belonging to the SFII NTPase/helicases too (not shown).

It was found previously that ribavirin-TP exhibited a higher inhibitory effect on the

NTPase activity of the HCV and WN virus enzymes than did ribavirin [11]. Thus, one could speculate that an effective inhibition of the NTPase activity can reduce the energy supply and therefore inhibit the unwinding activity of the enzyme. The helicase activity of the enzyme was investigated as a function of increasing concentrations of ribavirin-TP and the reaction was terminated after 20 min, i.e. at a time at which >95% of the DNA–substrate was unwound. As expected, when the ATP concentration was adjusted to a concentration equal to the K_m value, only a moderate inhibition with an IC_{50} of 150–200 μM was seen. The inhibition was not complete and reached a maximum of 35% of the control at ribavirin-TP concentrations higher than 300 μM (±10). Surprisingly, a similar extent of inhibition was also observed when ATP concentration was reduced to 0.01 μM or 5 fM, i.e. to concentrations at which ribavirin-TP acts as a potent inhibitor of the ATPase activity of the enzyme (ref. [11] and unpublished data). Interestingly, the inhibitory potential of the compound was higher when the reaction was terminated earlier, i.e. at times at which the DNA–substrate was unwound only to some extent. Thus, a reduction of the reaction time to 10 min at which about 60% of DNA–substrate was unwound resulted in IC_{50} of 90 μM (±10). When the reaction time was limited to 5 min (35% of substrate unwound) the IC_{50} was 30 μM (±13). Nevertheless, the inhibition was not complete and reached 35% of the control at ribavirin-TP concentrations corresponding to a values 3–5 times as high as the IC_{50} i.e. 200 μM (± 8.5) (Fig. 2).

These data are obviously contrary to the previous results obtained with the WN virus NTPase/helicase [10]. In the case of the WN virus enzyme no apparent correlation between the duration of the reaction and inhibitory potential of the ribavirin-TP was seen (see ref. [10] and unpublished data). One possible explanation for this kinetic behaviour is the relatively low specificity of the HCV enzyme for NTP [20, 21]. Thus it could not be ruled out that the enzyme hydrolysed the ribavirin-TP to less potent derivatives. Indeed, our preliminary data indicate that the HCV enzyme produces not only NDP, but also substantial amounts of NMP in the course of hydrolysis of ATP and other NTPs. Moreover, the products of the hydrolysis were not capa-

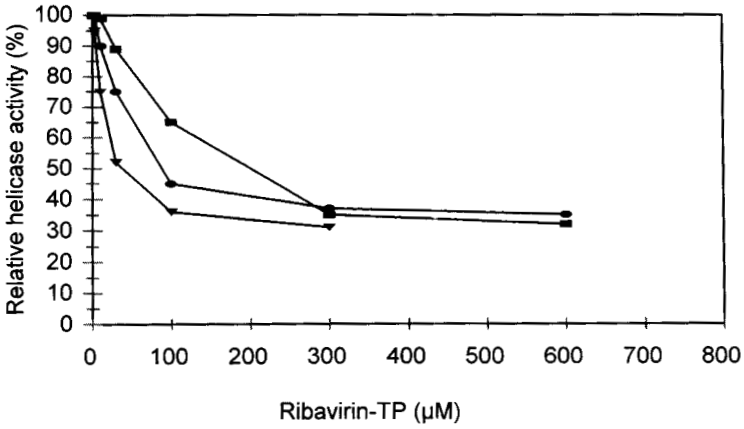

Figure 2. Inhibition of the unwinding reaction mediated by HCV NTPase/helicase and its dependency on the duration of the reaction.

The helicase assays were performed as described above in the presence of increasing concentrations of ribavirin-TP. The reaction was terminated after 5 min (▼), 10 min (●) and 20 min (■) duration of the reaction. The samples were proceeded as described in the legend to Fig. 1 and the helicase activity was quantified according to the procedure reported previously [10]. The obtained values are presented as percentages of controls (no inhibitor added) and are averages of three independent assays.

ble inhibiting the helicase activity of the enzyme (Borowski, P., Niebuhr, A., Schmitz, H., unpublished data)

In this context, it is apparent that introduction of β- and γ-phosphate groups into the 5′-position of ribavirin molecule could enhance the inhibitory potential of the drug. On the other hand, in the light of the low specificity of the enzyme for phosphate groups of NTPs only the compounds with enhanced resistance to hydrolysis of the terminal phosphate group(s) seem to be suitable as inhibitors of the helicase activity. Investigations on nonhydrolysable analogues of ribavirin-TP are currently under way.

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