Guanosine Triphosphatase Activity of the Human Coronavirus Helicase

ANJA SEYBERT AND JOHN ZIEBUHR
Institute of Virology, University of Würzburg, 97078 Würzburg, Germany

1. INTRODUCTION

RNA helicases are a diverse class of enzymes that use the energy of nucleoside triphosphate (NTP) hydrolysis to unwind duplex RNA structures. They are involved in virtually every aspect of RNA metabolism, including transcription, RNA splicing, translation, RNA export, ribosome biogenesis, mitochondrial gene expression and regulation of mRNA stability (Schmid and Linder 1992, Lohman and Bjornson 1996, Kadaré and Haenni 1997). On the basis of conserved sequence motifs, helicases have been divided into 3 large superfamilies (SF), SF1 to SF3, and two smaller families (Gorbalenya *et al.* 1989, Gorbalenya and Koonin 1993b), which contain both RNA and DNA helicases.

Apart from the RNA-dependent RNA polymerases (RdRp), helicases are the most conserved subunits of the replication machinery of (+)RNA viruses (Gorbalenya *et al.* 1988, Gorbalenya and Koonin 1993a, Koonin and Dolja, 1993). Thus, based on sequence analyses, putative helicase domains have been identified in most (+)RNA virus genomes and there is a large body of genetic and reverse-genetic information to suggest a key function of helicases in the life-cycle of (+)RNA viruses (Kadare and Haenni 1997). Remarkably, (–)RNA viruses and retroviruses do not encode helicases (Gorbalenya *et al.* 1988). While a number of (+)RNA virus helicases of SF2 have been characterized in considerable detail, there is nearly no information on the biochemical properties of RNA virus SF1 helicases. The latter superfamily includes putative helicases from more than 15 (+)RNA virus

families but, to date, no convincing evidence for duplex-unwinding activity has been obtained for most of these proteins.

The human coronavirus 229E (HCoV) replicase gene encodes two large polyproteins that are extensively processed by virus-encoded proteinases (Ziebuhr *et al.* 2000). One of the mature processing products, p66^{HEL}, has previously been predicted to contain an SF1 helicase domain (Gorbalenya *et al.* 1988). Biochemical data to support these predictions have recently been reported (Seybert *et al.* 2000). Specifically, clear evidence was presented to show nucleic acid duplex-unwinding activity associated with this viral protein. Interestingly, the functional analysis of p66^{HEL} revealed a 5'-to-3' polarity of the unwinding reaction, whereas SF2 RNA virus helicases have been shown to operate in 3'-to-5' direction.

2.   MATERIALS AND METHODS

2.1   Protein Expression and Purification Using Baculovirus Recombinants

The construction of baculovirus recombinants expressing recombinant forms of p66^{HEL} has been described previously (Seybert *et al.* 2000). Briefly, the coding sequence of the HCoV pp1ab amino acids 4998 to 5592 was inserted into pBlueBacHis2B DNA (Invitrogen; Groningen, Netherlands). The resultant plasmid, pBlueBacHis2B-Hel, essentially encodes the complete HCoV helicase domain fused to an amino-terminal histidine tag.

A recombination-PCR method was used to introduce a point mutation into the helicase-coding sequence of pBlueBacHis2B-Hel. In the resultant plasmid, pBlueBacHis2B-Hel-KA, the codon for the HCoV pp1ab amino acid Lys-5284, AAA, was substituted by GCA, which encodes Ala.

The plasmids pBlueBacHis2B-Hel and pBlueBacHis2B-Hel-KA were used to derive two recombinant baculoviruses, designated vBac-Hel and vBac–Hel–KA, respectively. Cell culture, transfections, isolation of recombinant baculoviruses, plaque purifications, protein expression in High Five™ insect cells (Invitrogen) and nickel-affinity chromatography purification were done as previously described (Seybert *et al.* 2000). The recombinant proteins purified from vBac–Hel- or vBac–Hel–KA-infected insect cells were designated HEL and HEL–KA, respectively.

2.2   Nucleoside Triphosphatase Assay

In the adenosine triphosphatase (ATPase) assay, HEL (3, 15 and 450 fmol, respectively) or HEL–KA (10 pmol) were incubated in a volume of

40µl containing 20 mM HEPES-KOH pH 7.4, 300 µM ATP, 5 mM magnesium acetate, 2 mM dithiothreitol, 25 µg/ml bovine serum albumin and 250 nCi of [γ-^{32}P] ATP (3000 Ci/mmol). In the guanosine triphosphatase (GTPase) assay, ATP and [γ-^{32}P] ATP were replaced by 300 µM GTP and 250 nCi [γ-^{32}P] GTP (3000 Ci/mmol), respectively. When included, poly(U) was at a concentration of 150 µg/ml. The reactions were incubated at 30°C for 30 min and stopped by adding EDTA to a final concentration of 100 mM. The samples were analyzed by polyethyleneimine-cellulose thin layer chromatography with 0.15 M formic acid-0.15 M LiCl (pH 3.0) as the liquid phase. The reaction products were analyzed by phosphorimaging of the dried chromatographic plates (ImageQuant software; Molecular Dynamics, Sunnyvale, CA).

2.3   Preparation of the Partial-Duplex DNA Substrate

The synthetic oligonucleotide 5'–GGTGCAGCCGCAGCGGTGCTCG– d(pT)$_{30}$–3' was labelled with [γ-^{32}P] ATP (3000 Ci/mmol) using T4 polynucleotide kinase and purified by phenol/chloroform extraction and gel filtration chromatography using Micro Bio-Spin 6 columns (Bio-Rad Laboratories, Munich, Germany). The DNA duplex was produced by annealing the labelled oligonucleotide to the oligonucleotide 5'–d(pT)$_{30}$– CGAGCACCGCTGCGGCTGCACC–3' in buffer E (25 mM HEPES-KOH pH 7.4, 500 mM NaCl, 1 mM EDTA, 0.1% (w/v) SDS). The annealing reaction, which contained a tenfold excess of unlabelled DNA over [γ–^{32}P] ATP-labelled DNA, was denatured for 5 min at 95°C and slowly cooled to room temperature. The resultant substrate was a twin-tailed ("forked") DNA duplex; i.e., it contained both 5' and 3' single-stranded regions on one end of the partial-duplex DNA.

2.4   Duplex-Unwinding Assay

HEL (30 fmol) or HEL–KA (210 fmol) were incubated in a volume of 40 µl with 15 fmol of the partial-duplex DNA substrate for 30 min at 30°C in a buffer containing 20 mM HEPES-KOH pH 7.4, 37.5 mM NaCl, 5 mM ATP or GTP, respectively, 10% glycerol, 5 mM magnesium acetate, 2 mM dithiothreitol and 0.1 mg/ml bovine serum albumin. The reactions were stopped by the addition of 10 µl of 5% (v/v) SDS, 15% (w/v) Ficoll and 100 mM EDTA. The reaction products were separated on 12% polyacrylamide-1×TBE gels (acrylamide/bis-acrylamide ratio of 19 to 1) at 4 W until the bromphenol blue dye approached the bottom of the gel. The gels were exposed to X-ray film at –70°C.

3. RESULTS AND DISCUSSION

A recombinant form of the HCoV helicase, HEL, has recently been shown to have ATPase activity *in vitro* (Seybert *et al.* 2000). To examine if HEL is also able to hydrolyze GTP, we have incubated [γ-^{32}P] GTP with HEL and the ATPase-deficient control protein HEL–KA. As Figure 1 (lane 2) shows, HEL effectively hydrolyzes GTP. We also found that the substrate conversion by HEL can be significantly stimulated by poly(U). Thus, in the presence of 150 µg/ml poly(U), 15 fmol of HEL gave a similar rate of hydrolysis as 450 fmol HEL without the polynucleotide cofactor. Even very low amounts of enzyme gave significant GTPase activity if poly(U) was included (lane 6). The rate of GTP hydrolysis was found to be similar to that of ATP hydrolysis (Fig.1, cf. lanes 3, 5 and 4, 6). As expected, no significant substrate hydrolysis was observed in the control reactions using buffer or HEL–KA (Figure 1, lanes 7 to 10). We conclude from these data that the HCoV helicase is able to effectively hydrolyze GTP and, in agreement with the structural model for the nucleic acid-induced activation of the *Bacillus stearothermophilus* PcrA helicase-associated ATPase activity (Soultanas *et al.* 1999), we propose that the binding to single-stranded RNA induces a

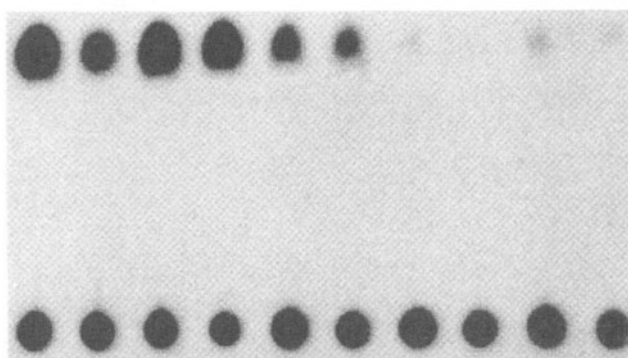|	1	2	3	4	5	6	7	8	9	10
ATP (300 µM)	+	−	+	−	+	−	+	−	+	−
GTP (300 µM)	−	+	−	+	−	+	−	+	−	+
HEL (fmol)	450	450	15	15	3	3	−	−	−	−
HEL–KA (pmol)	−	−	−	−	−	−	−	−	10	10
poly(U) (150 µg/ml)	−	−	+	+	+	+	+	+	+	+

Figure 1. ATPase and GTPase activities of HEL. Reactions containing either 300 µM ATP (lanes 1, 3, 5, 7, and 9) or 300 µM GTP (lanes 2, 4, 6, 8, and 10) were incubated with (i) different amounts of HEL (lanes 1 to 6), (ii) buffer alone (lanes 7 and 8), or (iii) ATPase-deficient control protein HEL–KA (lanes 9 and 10). The reaction products were analyzed by thin-layer chromatography and phosphorimaging (see Materials and Methods for details). The composition of the individual reactions is indicated above.

conformational change in the NTPase active site of HEL, which stabilizes the bound NTP molecule in a conformation that is required for rapid substrate hydrolysis.

It is generally accepted that the strand separation of nucleic acid duplexes by helicases is an energy-dependent process. Thus, NTP is a necessary cofactor for duplex-unwinding activity. In agreement with this model, we recently found that HEL requires ATP for duplex-unwinding activity. Also, the substitution of a lysine residue (Lys-5284 to Ala), which is part of the Walker A motif (Walker *et al.* 1982) and is predicted to be involved in binding and/or hydrolysis of NTP, resulted in an ATPase-deficient and, hence, helicase-deficient protein (HEL–KA).
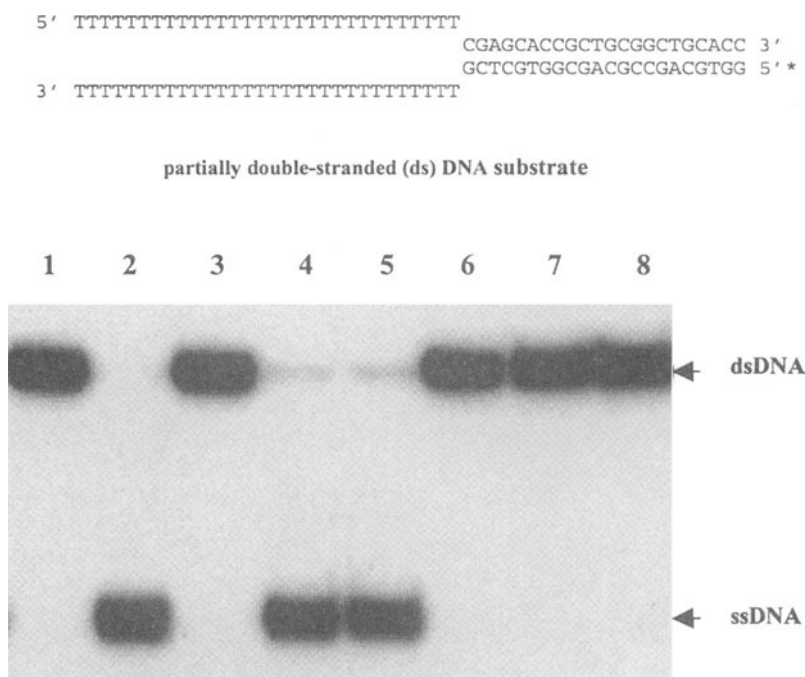

Figure 2. NTP-dependent DNA duplex-unwinding activity of HEL. Reaction conditions were as described in Materials and Methods with approximately 15 fmol of DNA substrate per reaction. The structure of the partial-duplex DNA substrate is shown schematically with the radiolabelled strand marked by an asterisk. The reaction products were separated on a non-denaturing 12% polyacrylamide gel and visualized by autoradiography. The positions of the partially double-stranded substrate (dsDNA) and the displaced, monomeric product (ssDNA) are indicated. Lanes: 1, reaction without protein; 2, heat-denatured DNA substrate; 3, reaction containing 30 fmol HEL in the absence of NTP; 4, reaction containing 30 fmol HEL in the presence of 5 mM ATP; 5, reaction containing 30 fmol HEL in the presence of 5 mM GTP; 6, reaction containing 210 fmol HEL–KA in the absence of NTP; 7, reaction containing 210 fmol HEL–KA in the presence of 5 mM ATP; 8, reaction containing 210 fmol HEL–KA in the presence of 5 mM GTP.

ACKNOWLEDGMENTS

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