4 Discussion

4.1 RepA enzymatic activity

4.1.1 Optimal activity conditions

RepA shows optimal dsDNA unwinding activity at pH 5.6, while at pH 7.6, only 20% of the unwinding activity remains (Scherzinger et al., 1997). In the present study, we found ssDNA to stimulate the ATPase activity of RepA much more at acidic pH (5.3-6.0) than at pH 7.6. This might be the reason why RepA unwinds dsDNA optimally only in the narrow acidic pH range 5.5-6.0. Fluorescence correlation spectroscopy and analytic ultracentrifugation have further shown that between pH 5.5-6.0 in the presence of ATPγS the binding of ssDNA to RepA is optimal while at pH 7.6, the binding affinity is so weak that it is difficult to monitor formation of the helicase-DNA complex. There is a strict correlation between optimal ssDNA binding at acidic pH to RepA and stimulation of ATPase activity which drives efficient unwinding of dsDNA. The low optimal pH of RepA may lead to reduced enzymatic activity at physiological pH, similar as reported for yeast Saccharomyces cerevisiae RAD3 helicase (Sung, 1988).

4.1.2 Mutation studies

Substitution of conserved Lys43 in the conserved sequence SXGGXGKS (Walker A motif) of RepA with alanine abolished the ATPase activity and reduced the binding affinity of ATP analog TNP-ATP. Therefore, Lys43 is important for both NTP binding and hydrolysis, indicating that lysine is essential in the Walker A motif in DnaB-like helicase family.

4.2 Interactions with ssDNA

4.2.1 NTP binding is essential for ssDNA interaction with RepA

The NTPs can function as switches that induce conformational changes necessary to promote DNA binding and release-steps required for translocation and dsDNA unwinding of helicase. In general, the hexameric helicases appear to interact tightly with ssDNA in the presence of NTP and weakly in the presence of NDP (Patel et al., 2000). From our studies, we found that

ssDNA binding to RepA is optimal only in the presence of nucleoside-5'-triphosphate analogs. Without NTP analogs or in the presence of NDP analogs, binding of ssDNA to RepA is so weak that it was not detectable under our experimental conditions. Similar results were also found for T7 gp4 (Hingorani et al., 1993; Yong et al., 1995), *E. coli* DnaB (Jezewska et al., 1996), and *E. coli* RuvB helicases (Muller et al., 1993).

4.2.2 A certain length of ssDNA required for optimal binding to RepA

The finding that RepA requires at least a decanucleotide as cofactor to stimulate ATPase activity provides clues for the importance of the interaction between RepA and ssDNA and suggests that it is the minimum length of single-stranded DNA in the template strand that will enable RepA helicase to bind and initiate unwinding of duplex DNA. In the presence of ATPγS at pH 5.8 the FCS measured affinity of RepA for a 12 mer ssDNA is the same as for a 30 mer. This indicates that even for a long oligonucleotide only a limited length (10 to 12) of the respective oligonucleotde interacts with hexameric RepA; the optimal length required for RepA/ssDNA interaction is between 10~12 nucleotides.

In the presence of the ATP nonhydrolyzable analog AMP-PNP, the DnaB helicase binds polymer DNA with a site-size of 20±3 nucleotides per protein hexamer. This site-size is independent of the type of nucleic acid base as well as type and concentration of salt (Bujalowski et al., 1995).

4.2.3 Only one ssDNA binding site on RepA

Jezewska et al. have proposed strong and weak ssDNA binding sites present on helicase DnaB which may be involved in the unwinding reaction. It was demonstrated that during the unwinding reaction only one ssDNA strand passes through the central channel and the other ssDNA strand moves along the outsite of the DnaB helicase.(Jezewska et al., 1998). According to this model the 5´ end of ssDNA binds strongly to a subsite within the channel, while the ssDNA near the entry site of duplex DNA is only weakly bound. The 3´ ssDNA leaves helicase DnaB at the outsite during the unwinding reaction.

In analytic ultracentrifugation experiments we observed only one single ssDNA binding site for both, RepA hexamer at pH 7.6 and dimeric form of RepA hexamer at pH 5.8. We can exclude additional weak binding sites present on RepA in the concentration range studied

here. From the X-ray structure of RepA, we learned that only ssDNA but not dsDNA can thread into the central hole (Niedenzu et al., 2001), therefore the other ssDNA (3'-tail) must move along the outsite of the helicase. Additionally, from FCS experiments we found forked DNA substrate with 14 nucleotides in the 5'-tail and 3 nucleotides in the 3'-tail (Fig. 2.2) has lower binding affinity than the normal 12mer or 30 mer ssDNA. Scherzinger et al (1997) demonstrated that substrates with a 3' - tail 4 nt were not efficiently displaced and optimal length of the 3'- tail required for helicase unwinding activity is near 12 nt.

4.2.4 Only one subunit of the RepA hexamer is predominantly engaged in interactions with ssDNA

Insight in the number of subunits involved in the ssDNA binding can be obtained by studying photo-cross-linking of the ssDNA oligomers of different lengths to the RepA hexamer. We have shown that only a single radioactive band appears on the SDS polyacrylamide gel around the molecular weight of 30,000, corresponding with the expected molecular weight of the $d(T)_n$-RepA monomer complex. Even in the case of oligomer $d(T)_{30}$, which is considerably longer than the optimal 10~12 mer for RepA-ssDNA interaction, only one subunit of the hexamer is primarily engaged in interactions with the oligonucleotide, suggesting that only one oligonucleotide is accommodated in the cavity of RepA where it photo-crosslinks to one subunit. This experiment excludes binding of ssDNA at the periphery of RepA and excludes the possibility of extensive wrapping of the ssDNA around the hexamer and formation of a complex in which all six protomers are simultaneously bound to ssDNA.

UV photo-cross-linking experiments of DnaB helicase with oligonucleotides of different lengths, $d(T)_{19}$, $d(T)_{55}$, and $d(T)_{69}$ also suggest that primarily a single subunit of the DnaB helicase hexamer is in contact with the DNA. The presence of a single, strong binding site on the hexamer, built of six chemically identical subunits, the site-size of the large helicase-DNA complex, and the involvement of a single subunit in contact with the nucleic acid indicate the presence of long-range allosteric interactions in the DnaB helicase which encompass the entire DnaB hexamer (Jezewska et al., 1996).

4.2.5 RepA hexamers associate to dimeric hexamers at acidic pH conditions

Analytic ultracentrifugation experiments show that in solution at pH 7.6, RepA exists as a homohexamer of 180 kDa but forms a dimeric hexamer of 360 kDa at pH 5.8. X-ray studies also show that at acidic pH 5.6-6.0, two RepA hexamers are stacked bottom-to-bottom in an asymmetric unit while at neutral pH, no such associated hexamers are found in crystal structures.

In the presence of ATP, the hexameric RuvB protein forms a dimer of hexamer on dsDNA in which two stacked hexameric rings encircle the DNA and are orientated in opposite directions (back-to-back) with D_6 symmetry (Stasiak et al., 1994). The *E. coli* Rho helicase forms hexameric rings (Gogol et al., 1991) as well as dodecamers and the dodecameric organization D_3 occurs only in the presence of an oligonucleotide cofactor that binds to three of the six RNA-binding sites (Geiselmann et al., 1992a; Geiselmann et al., 1992b). The docecameric form of Rho might be similar to that of RuvB, in that it would consist of two rings of oppositely oriented polarity (Stasiak et al., 1994), similar as observed for RepA.

Although we found that dimeric form of hexamer RepA binds to ssDNA at pH 5.8 in the presence of the non-hydrolysable ATP analog ATPγS by both, fluorescence correlation and photo correlation spectroscopy, further FCS experiments show that in the presence of ATP and ssDNA at pH 5.8, dimeric forms of hexamer RepA dissociate to hexamers dependent on RepA concentrations and incubation time (data not shown), suggesting that in the process of ssDNA translocation and DNA unwinding the dimeric forms of hexamer dissociate into hexamers.

4.2.6 High salt concentration inhibits ssDNA binding to RepA helicase

Changes in solution conditions (salt concentration and type, pH, temperature, etc) can influence the energetics (stability) and kinetics, as well as the specificity, of protein-DNA complexes (Lohman et al., 1996). Due to the polyelectrolyte nature of DNA, these properties are influenced most dramatically by salt concentration (Record et al., 1978; Record et al., 1991; Lohman, 1986; Lohman et al., 1992) as a result of differential cation or anion binding to the complex or the free protein and DNA. Since the DNA binding sites of most proteins are positively charged, protein binding to DNA generally results in partial neutralization of DNA phosphates with concomitant release of cations from the DNA, although differential ion

binding to the protein can also occur (Overman et al., 1994). In fact, the increase in entropy accompanying cation release from the DNA provides a major favorable contribution to the stability of most protein-DNA complexes (Record et al., 1976; Record et al., 1978; Record et al., 1991; Lohman et al., 1992). As a result of the release of cations from the DNA (and potentially ions from the protein), an increase in salt concentration will generally lower the observed association equilibrium constant (Record et al., 1991; Lohman, 1986) and also influence the kinetic rate constants for protein-DNA complex formation.

Analytical ultracentrifugation experiments showed the dissociation constant for the $d(A)_{30}$/RepA dimeric form of hexamer-complexes at pH 5.8 in the presence of 60 mM NaCl to be 0.94 µM. By contrast, in the absence of NaCl the dissociation constant was determined to be 0.52 µM by FCS at the same pH. These findings show that high concentration of salt increases the apparent dissociation constant of the ssDNA/RepA helicase-complex, in agreement with the inhibitory effect of NaCl on the helicase reaction (Scherzinger et al., 1997).

4.3 Nucleotide binding

4.3.1 Negative cooperativity for nucleotide binding to hexameric RepA

Previous studies (Xu et al., 2000) and the present results show that the six potential nucleotide binding sites of hexameric RepA are not identical. RepA either shows three high-affinity and three low-affinity nucleotide binding sites for the ADP analog εADP, or may bind three TNP-ATP, suggesting a negative cooperativity for nucleotide binding to RepA, similar as observed for most of the hexameric helicases. Nucleotide binding to T7 gp4 showed that only three or four nucleotides were bound per hexamer (Patel et al., 1995; Hingorani et al., 1996). In DnaB, three high-affinity NTP binding sites and three low-affinity sites were observed at 10°C, and only three high-affinity sites were observed at higher temperatures (Biswas et al., 1986; Bujalowski et al., 1993). *E. coli* rho protein also binds NTP with a negative cooperativity, i.e, three ATP molecules bind with a K_d in the range of 0.3-2 µM, and three or four AMP-PNPs bind with a K_d of 4-6 µM, both with and without RNA (Stitt, 1988).

As the nucleotide binding sites in RepA are located at the interfaces between adjacent monomers, this configuration may facilitate conformational changes transferred to

neighboring subunits upon NTP binding and hydrolysis. It is possible that the binding of an NTP molecule to the interface between two monomers causes conformational changes that are communicated to the adjacent subunit, resulting in a decrease of the binding affinity for NTP to the second subunit interface. This could give rise to high affinity and low affinity binding sites adjacent to each other in the RepA hexamer.

The best model that describes this negative cooperativity is a hexameric helicase with C_3 symmetry, where alternating sites on the hexamer bind NTP with high and low affinity. EM studies at pH 7.0 show that C_3 and C_6 symmetry architectures of the RepA hexamer coexist in solutions and that there is an equilibrium between them. The percentages of these populations are about the same, 55% 6-fold, and 45% 3-fold. At pH 6.0, only 6-fold symmetry structures exist (Bárcena, 2000). From our crystal structures, only 6-fold symmetrical structures have been observed at both acidic and neutral pH. It could be that it is only the C_6 symmetry molecules that can form crystals so that the equilibrium is shifted.

4.3.2 Conformational changes upon NTP binding

In near UV CD spectra (250 nm – 320 nm) a negative spectral change is observed for RepA upon binding of ATPγS. Since the CD signal in this region is due to electronic transitions of the aromatic amino acids of the protein or/and of the adenine base of the cofactor, the negative CD spectral change indicates a variation in enviroment of aromatic groups of RepA upon binding of ATPγS, suggesting tertiary structure changes. This is consistent with the X-ray structure of RepA showing that the modeled adenine-ribose moiety of ATP is located in a cleft formed between adjacent monomers, with the adenine base stacked between Arg86 of the same and Tyr243 of the adjacent monomer.

EM shows that upon binding of ATPγS to RepA, the diameter of the whole structure is larger than that of unliganded RepA. X-ray studies show that even the binding of hydrolysed product phosphate (sulfate) not only changes the conformations at the active site but also induces conformational changes of the whole structure.

Thermal unfolding studies show that binding of ATPγS and ADP to RepA stabilizes intersubunit contacts of the two monomers. In presence of ATPγS, RepA is more stable (+ 2.3°C) than in the presence of ADP (+ 0.7°C), compared to free RepA. This finding suggests

that the γ phosphate of ATP contributes additional contacts that stabilize the structure of RepA, in contrast to ADP where this phosphate is missing.

Similar results were also observed for other hexameric helicases. In studies reported by Jezewska et al (1996) using analytical sedimentation equilibrium, sedimentation velocity provided evidence for dramatic global conformational changes of the DnaB helicase hexamer, induced by NTP binding, and the existence of multiple structural states of the enzyme. Upon binding the ATP analog AMP-PNP, the DnaB hexamer transforms into a "tense" state while in the presence of ADP, the DnaB hexamer assumes a "relaxed" conformation. The functional difference between these two conformations is reflected in the much weaker allosteric effect of ADP on ssDNA binding with an affinity constant about three orders of magnitude lower than in the presence of AMP-PNP.

In electron microscopy studies, a small difference does exist between the DnaB hexamer C_6 rings formed in the presence of ADP and those formed in the presence of AMP-PNP. When these two averages are superimposed, it appears that the hole in the center of the C_6 ring does not change, but that the peak density in each subunit in the presence of AMP-PNP ring is radially extended by about 5 Å, leading to a ring that is about 10 Å larger in diameter than the ring formed by the DnaB-ADP complex (Yu et al., 1996).

4.3.3 Dynamic segment movements caused by NTP binding and hydrolysis

The fluorescent dye Lucifer Yellow attached to the unique Cys172 between helix F and β-strand 5 of RepA serves as a reporter group for distinct changes in diffusional dynamics of the protein segment it is attached to (Fig. 4.1). In this region of the protein changes of motion and conformation were detected upon interaction with ATP and ssDNA. His 179 at the end of β-strand 5 is involved in ATP hydrolysis. Therefore, upon addition of ATP, changes in the lable flexibility in this protein region are expected. The decrease of the second correlation time ϕ_2 upon addition of ATP to RepA probably is due to the motion of either helix F or β-strand 5 or of both. The interaction with ssDNA increases the final anisotropy, suggesting that significant changes in the dynamics of that segment and in the degree of steric hindrance upon binding of ssDNA do occur.

However, the experiments reported here do not distinguish between sterical hindrance caused by the protein itself or by ssDNA. It remains an open question whether the increase of r_∞ in

the RepA/ssDNA complex is due to a conformational change of the protein or due to direct interactions with the ssDNA.

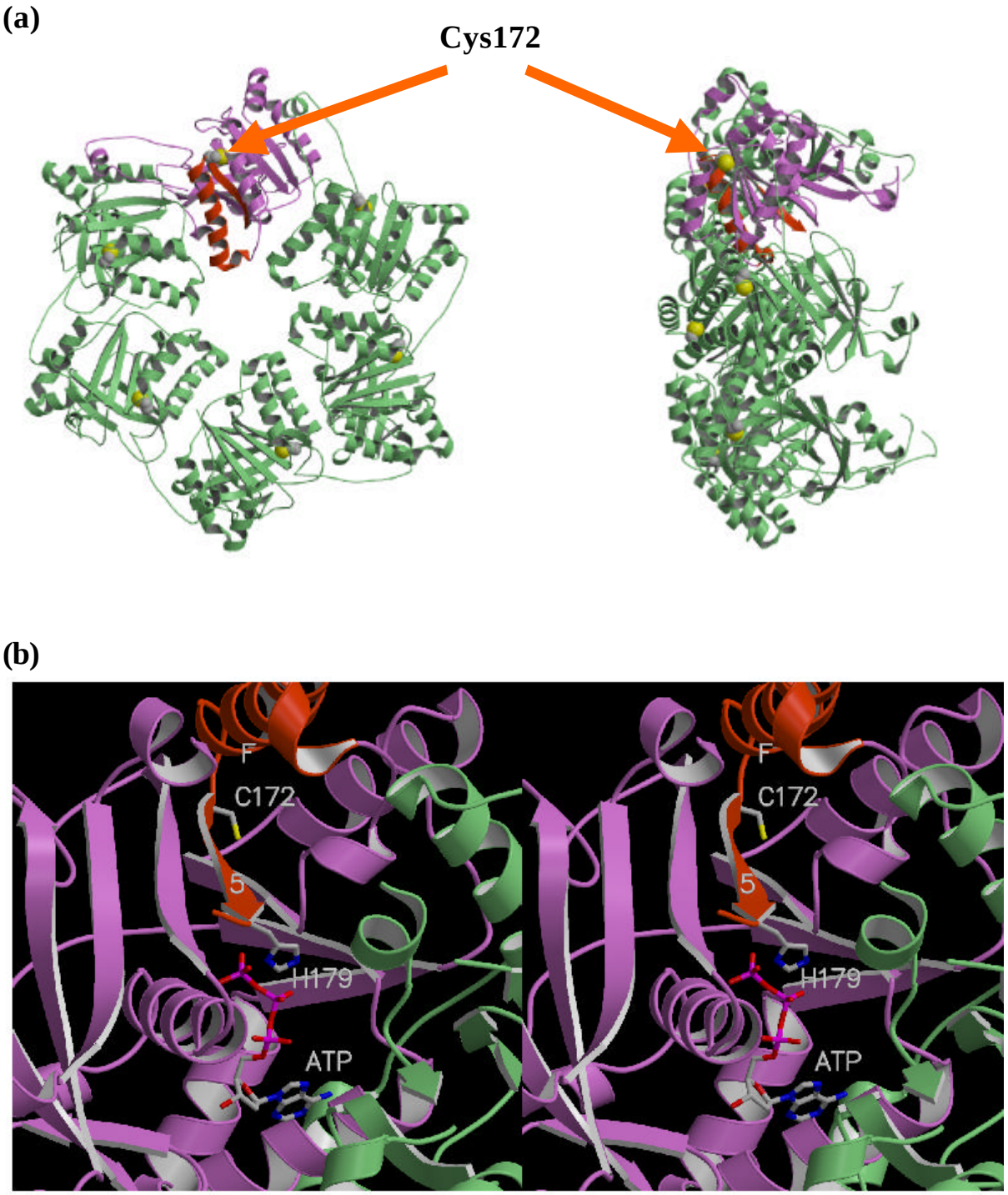

Figure 4.1: (a) Overview of the labelling site for Cys172 (indicated by yellow spheres) of RepA hexamer. (b) Segment of the structure of RepA which shows the location of the LY labeled Cys172 residue and ATP. The monomer which the labeled Cys172 belongs to is in pink; the next adjacent monomer is in green and red segment is the β-strand 5 linking the labeled Cys172 and His179 which is involved in the ATP binding and hydrolysis.

4.3.4 Possible subunit movements upon nucleotide binding and hydrolysis

X-ray structure of RepA shows that except for the interactions between the N-terminal amino acid residues (2-18) of one monomer and the adjacent monomer, there are additional intersubunit interactions formed between Ser153 O^{γ} ··· Glu149' $O^{\varepsilon 2}$, Tyr243 OH ··· Asp78' $O^{\ddot{a}2}$, Glu164 $O^{\varepsilon 1}$ ··· Arg144' N^{ε} and Glu164 $O^{\varepsilon 2}$ ··· Arg144' $N^{\eta 2}$ (Fig. 4.2). Additionally, there are several van der Waals contacts between the intersubunits (data not shown).

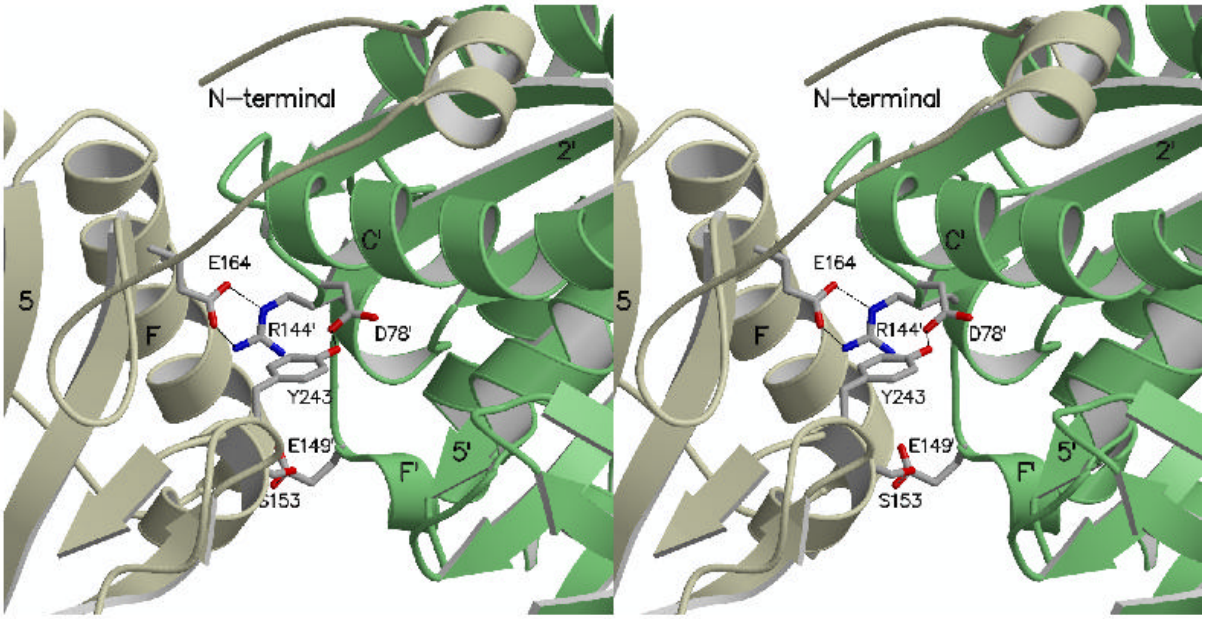

Figure 4.2: Stereoview of the interactions at the interface between two subunits of hexameric RepA.

Arg144' is on the loop C-terminal of β-strand 4 which contains Asp140 (H2 motif) that is involved in ATP binding and hydrolysis. Asp78' is just located on the same loop with Glu77 which is in the H1A motif, and Tyr243 is directly involved in the interaction with the adenine base of bound ATP (Niedenzu et al., 2001). Additionally, Glu164, Ser153 and Glu149' are all located at or near the α-helices F and F' of each subunit at the interface (Fig. 4.2). The hydrogen bonding within the interface cleft formed between the adjacent subunits provides a mechanism whereby nucleotide-induced conformational changes between these domains can directly affect the subunit-subunit interactions and suggest that subunit may change positions (rotate) during the ATPase and DNA unwinding reaction cycle.

It is anticipated that such relative subunit motions occur upon binding of ligands to many other oligomeric proteins, particularly allosteric proteins (Ladner et al., 1977; Steitz et al., 1982).

4.4 Two-site sequential NTPase model

ATP hydrolysis by RepA shows positive cooperativity both in the absence and presence of ssDNA. Here we assume that the ATP binding sites of RepA can exist in either of two conformational states, R (relaxed), when the sites are filled by ADP and inorganic phosphate Pi, and T (tense), when they are filled by ATP. We also assume that the transition from R to T forms occurs sequentially. During hydrolysis, ATP molecules bound to three strong affinity sites (form T) of RepA are hydrolysed and the three strong ATP binding sites are now occupied by ADP and inorganic phosphate Pi; RepA undergoes a conformational change, with the former ATP binding sites changing from T to R form and vice versa. Since ATPase active site and adenine binding site are formed by adjacent subunits, conformational changes may be easily transmitted to neighboring subunits such that the weak binding sites become strong ATP binding sites (from R to T form). They bind and hydrolyse ATP again and the cycle continues (Xu et al., 2000).

In the presence of ssDNA, this scheme becomes more complicated. From the above studies of RepA, we already learned that ssDNA passes through the 17 Å diameter central hole of the hexamer and interacts only with one monomer of the hexameric helicase. It has also been suggested that ssDNA passes through the central hole of the hexameric ring formed by *E. coli* RuvB helicase (Stasiak et al., 1994), bacteriophage T7 gp4 helicase (Egelman et al., 1995), SV40 large T antigen (Dean et al., 1992) and E. coli DnaB helicase (Bujalowski et al., 1995). ssDNA binds tighly to only one or two subunits of the hexameric helicases (Egelman et al., 1995; Bujalowski et al., 1995; Yu et al., 1996). This may be required for dsDNA unwinding where each subunit or pair of subunits of RepA alternately participates in ssDNA binding and release reactions. Such an asymmetric pattern can also generate nonequivalent NTP hydrolysis active sites in a multisubunit enzyme so that only one or two of the six subunits are engaged in NTP hydrolysis at each unwinding cycle, and they may sequentially bind and hydrolyse NTPs at the catalytic sites with positive cooperativity (Hingorani et al., 1997; Marrione et al., 1995; Marrione et al., 1996). This is similar to F_1 - ATPase (Washington et

al., 1996; Abrahams et al., 1994), because the γ - subunit of F_1 - ATPase is positioned within the central cavity of the hexameric ring of α and β subunits, analogous to the way in which the hexameric helicases bind ssDNA (West, 1996).

Combining the above cases we propose here a general two-site sequence NTPase mechanism for RepA. In this two-site sequential mechanism, catalytic sites are located between adjacent subunits and each of two neighbouring catalytic sites cycles between the T-and R-states in a sequential manner. When the T-site hydrolyzes NTP, the adjacent R-site releases the hydrolysis products, ADP and Pi. The R-site then quickly rebinds an NTP and convert to the T-site. Thus the E-state (empty site) does not accumulate. Sequential catalysis is ensured by the interdependence of reactions at each site. Hydrolysis of ATP does not occur unless product is released from the alternative site and vice versa (Fig. 4.3).

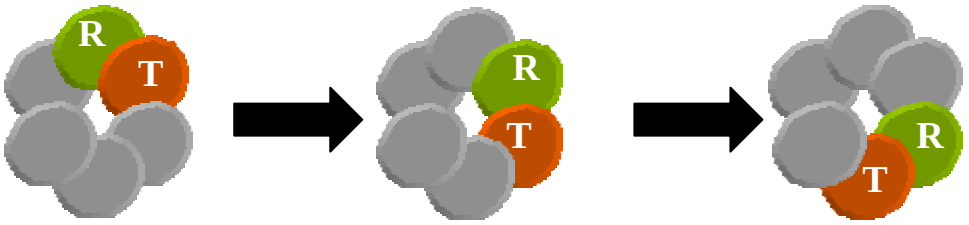

Figure 4.3: Proposed models for NTP hydrolysis in RepA helicase. In the two-site sequential model, each of the two catalytic sites cycles between the T- and R-states, in a sequential manner.

4.5 Model for RepA helicase coupled translocation on ssDNA and unwinding reaction

Based on the model for NTPase, we propose models for helicase coupled translocation on ssDNA and dsDNA unwinding reaction.

The two-site sequential translocation model is based on the two-site sequential NTPase model described above (Fig. 4.3). In this model, it is proposed that the sites cycle between the T and R states and the ssDNA binds in the central hole and interacts tightly with the T-state, weakly with the R-state. As shown in Fig. 4.4, when the subunit in the T-state (which is tightly bound to ssDNA) undergoes a conformational change that results in movement, the subunit in the R-state (which is weakly bound to ssDNA) releases the DNA and also starts to rebind at a

different position on ssDNA. The two-site sequential mechanism also allows processive unidirectional movement.

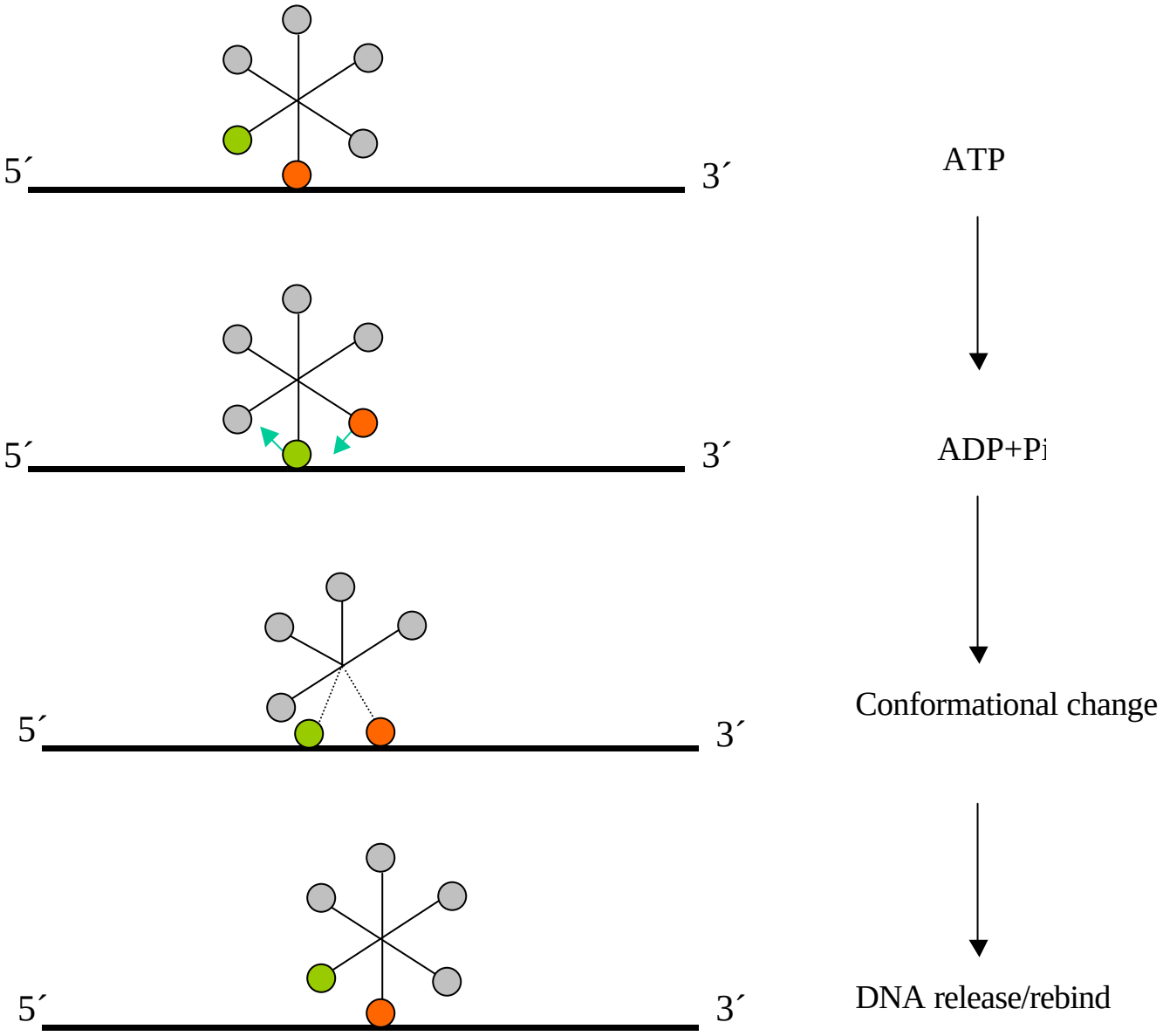

Figure 4.4: Two-site sequential translocation model. In this model, the sites cycle between the T (shown in red) and R states (shown in green) and the ssDNA interacts tightly with the T-state, weakly with the R-state.

The dsDNA unwinding reaction involves unidirectional translocation and base-pair separation processes, i.e. the latter reaction involves disruption of the Watson-Crick hydrogen bonding.

To understand how DNA can be unwound in a processive manner, we need to know how the above processes are coupled to the NTPase and DNA translocation reaction.

Here we propose a dsDNA unwinding model where the hexamer helicase interacts with only one of the separated strands (the 5'-strand) in its central channel at the unwinding junction and excludes the other strand (the 3'-strand) from the channel. The excluded strand does not have tight or specific interactions with the helicase. The helicase moves unidirectionally along the 5'-strand bound in the central channel using the two-site sequential translocation model, and the NTPase-coupled movement provides enough force to enable the helicase to destabilize the base pairs at the junction by a process resembling the action of a bulldozer (Fig. 4.5).

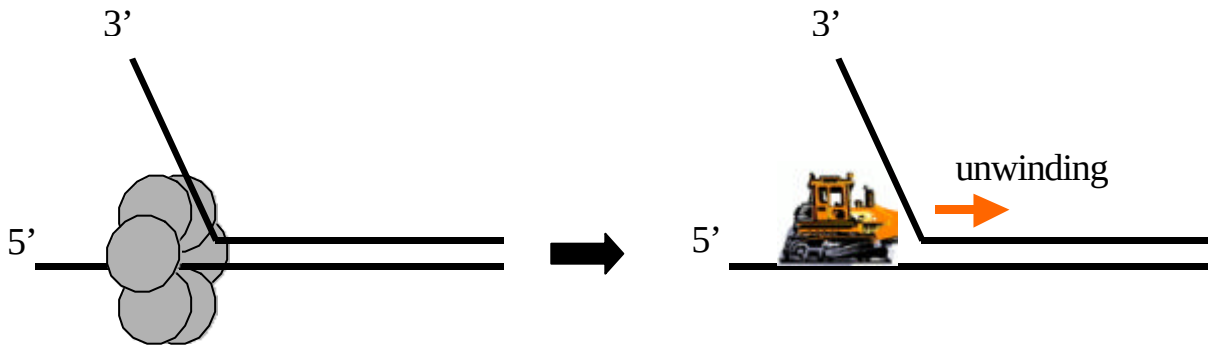

Figure 4.5: Proposed DNA unwinding model.

4.6 Helicase inhibitors as new antibiotics for cell growth

In contrast to a variety of enzymes involved in the metabolism of nucleic acids, e.g. RNA polymerase or DNA gyrase, no easily available inhibitors for helicases are known. Such inhibitors would be helpful in studying the mechanisms of unwinding and they could also be useful as antibiotics to control pathogenic bacteria. Flavones are naturally occurring polyphenolic compounds ubiquitously found in plants. The cellular mechanisms underlying known anticarcinogenic effects associated with the flavones are still unclear but are thought to be linked to inhibition of enzymes involved in transduction of mitogenic signals (Middleton et al., 1993).

The kinetic inhibition study of ssDNA stimulated ATPase activity of RepA shows that the flavones inhibit RepA non-competitively with respect to ATP. Since myricetin did neither disrupt RepA and other hexameric helicases tested, nor prevent hexamerisation, we hypothesise that certain flavones may block binding of ssDNA to RepA rather than the binding of ATP, thereby preventing RepA from translocation on ssDNA. This hypothesis is supported by our observation that myricetin caused only little inhibition of the intrinsic RepA ATPase activity in the absence of ssDNA effector. The fact that flavones are known to bind weakly to single-stranded DNA might also add to the inhibitory effect. Additionally it is known that these compounds bind to double-stranded DNA by intercalation due to the planar structure; such intercalation, has been associated with cytotoxicity (Austin et al., 1992).

Walker *et al*. recently published structural information on the inhibition of phosphoinositide 3-kinase (PI3K) by flavones and other inhibitors (Walker et al., 2000). In PI3K, myricetin and quercetin were found to be located in the ATP binding pocket. There is a salient difference, however, in the ATPase active site of PI3K and RepA. In the ATPase active site of PI3K, the kβ3-kβ4 loop ^{804}MASKKKP^{810} (P loop) contains no glycine, the side chain of Ser806 interacts with the β-phosphate of ATP and Lys833 at the end of β-strand kβ5 interacts with the α-phosphate of bound ATP. The loop between strands kβ7 and kβ8 forms the bottom of the ATP binding pocket and provides two hydrophobic contacts with the adenine moiety of ATP (Walker et al., 1999).

In the complex formed between myricetin and PI3K, the phenyl ring of myricetin is partially overlapping and coplanar with the space occupied by the adenine moiety, and other flavones occupy the ATP binding site in a comparable fashion. By superimposing the ATPase active sites of PI3K and RepA, we learned that the structure of the ATP binding motif of PI3K is very different compared to that of RepA helicase. The ATP binding site in RepA is much smaller than that in PI3K (Fig. 4.6) and would be unable to accommodate flavone compounds if no major structural rearrangements would occur. The flavones studied here appear to select a different way to regulate the RepA helicase function. This is further supported by the finding that wortmannin, an inhibitor which is covalently bound with high affinity at the ATP active site of PI3K (Walker et al., 2000), has nearly no inhibitory effect on RepA ATPase activity (data not shown).

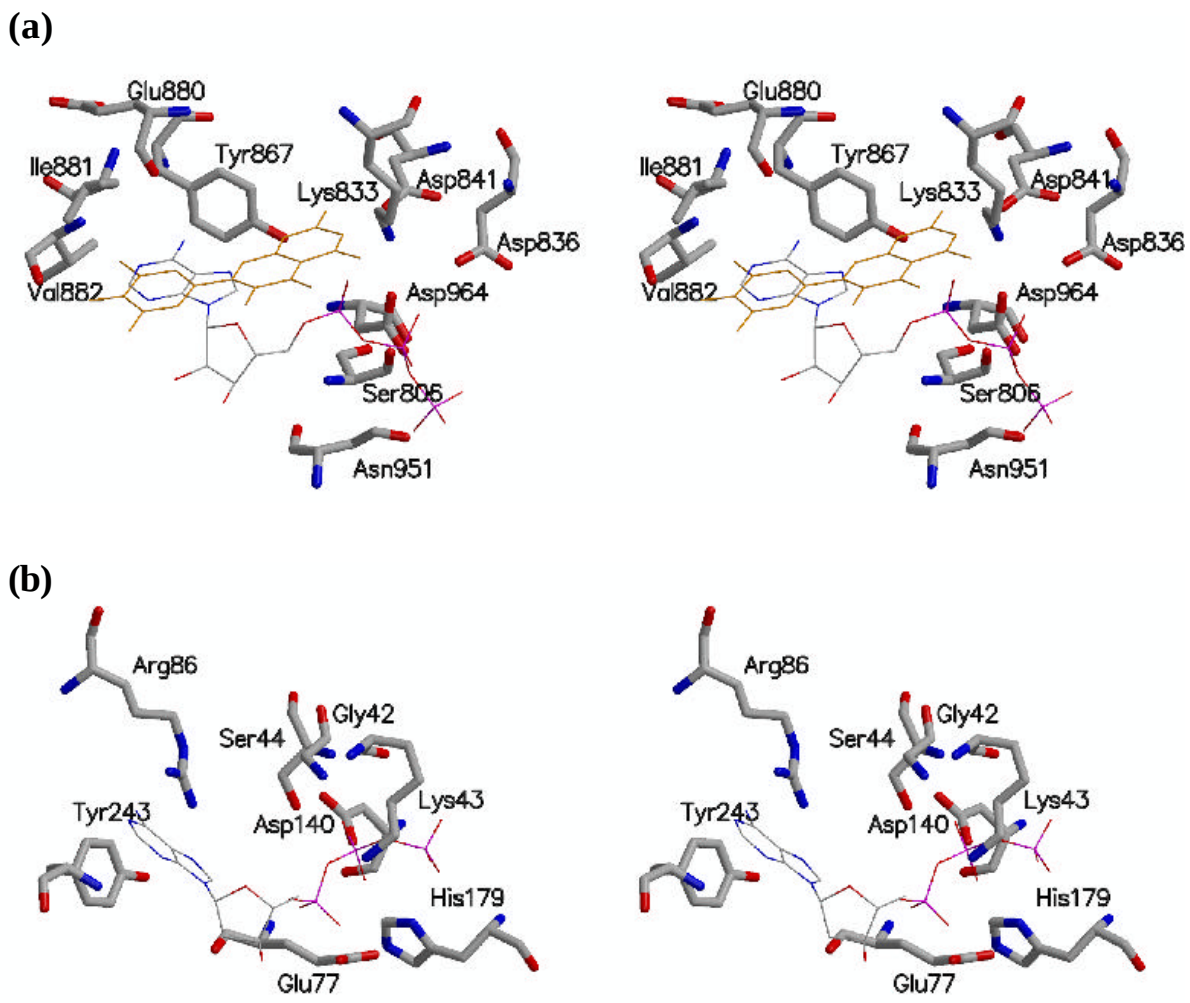

Figure 4.6: Comparison of the ATPase active sites of PI3K and RepA. (a) The catalytic domain of PI3K with bound ATP and myricetin (Walker et al., 2000). (b) ATPase active site of RepA hexamer showing the position of ATP in the cleft between two neighbouring RepA monomers. ATP was modeled into the RepA hexamer as described (Niedenzu et al., 2001).

The target(s) of myricetin, the only one of the here investigated flavones that inhibited cell growth, may be localized within the cytoplasm or within the bacterial membrane. We suggest that myricetin inhibits at least one cytoplasmic enzyme essential for bacterial viability. One target may be a replicative helicase, since *in vitro* myricetin not only inhibited RepA helicase, but also a variety of helicases of different function and origin, including the phage P1 Ban protein which can functionally replace the *E. coli* DnaB replicative helicase (Lanka et al., 1978). The specific sensitivity of each of these helicases towards myricetin suggests to design molecules by molecular modelling with enhanced specificity for a given helicase once the site of binding is known by crystallographic studies. Of the compounds studied *in vitro*,

dimyricetin was shown to be the most active molecule against RepA, but it did not inhibit bacterial growth, probably because dimyricetin does not cross the cell membrane.

Although the inhibition of RepA by luteolin, morin, myricetin and dimyricetin is only in the µM range, these compounds are advantageous as they are commercially available and nontoxic. The discovery of naturally occurring helicase inhibitors provides information regarding the type of small molecules that may be used to inhibit helicase action, and our results suggest that these compounds may provide lead compounds for the design of novel drugs. Since the flavone compounds are achiral, their binding affinity to RepA can certainly be enhanced by introducing chiral centers that augment their interaction with RepA. These studies will be guided by crystal structure analyses of complexes between RepA and the flavone compounds with the aim to synthesize better inhibitors with higher specificity.