The P4 protein of bacteriophage φ12 is a hexameric molecular motor closely related to superfamily 4 helicases. P4 converts chemical energy from ATP hydrolysis into mechanical work, to translocate single-stranded RNA into a viral capsid. The molecular basis of mechanochemical coupling, i.e. how small ~1 Å changes in the ATP-binding site are amplified into nanometer scale motion along the nucleic acid, is not understood at the atomic level. Here we study in atomic detail the mechanochemical coupling using structural and biochemical analyses of P4 mutants. We show that a conserved region, consisting of superfamily 4 helicase motifs H3 and H4 and loop L2, constitutes the moving lever of the motor. The lever tip encompasses an RNA-binding site that moves along the mechanical reaction coordinate. The lever is flanked by γ-phosphate sensors (Asn-234 and Ser-252) that report the nucleotide state of neighboring subunits and control the lever position. Insertion of an arginine finger (Arg-279) into the neighboring catalytic site is concomitant with lever movement and commences ATP hydrolysis. This ensures cooperative sequential hydrolysis that is tightly coupled to mechanical motion. Given the structural conservation, the mutated residues may play similar roles in other hexameric helicases and related molecular motors.

Helicases are molecular motors that unwind double-stranded nucleic acids using the energy of NTP hydrolysis. Helicases have been divided into four superfamilies (SF1–SF4) based on the sequence identity among the conserved helicase motifs (1). Helicases belonging to SF1 and SF2 (e.g. RecBCD and RecQ) function as monomers and provide simple model systems for studying DNA translocation and strand separation. An inchworming mechanism of translocation and unwinding was proposed on the basis of extensive structural and biochemical data (2, 3).

Members of the SF3 and SF4 function as hexameric rings that encircle their polynucleotide substrates (DNA or RNA) and translocate along one strand. A recent landmark structural study on human papilloma virus helicase E1 (SF3 helicase) demonstrated that DNA is bound to conserved β-hairpins on multiple E1 subunits (4). A correlation between the positions of E1 β-hairpin and the conformations of the corresponding ATP-binding site provided evidence for sequential hydrolysis and translocation by an “escort” mechanism (4). Another important study on Rho transcription terminator (SF5 helicase (5)) has shown that RNA binds to multiple sites (Q loop, R loop, and P loop) in a cleft between two neighboring Rho protomers (6). Rho helicase also utilizes a sequential hydrolysis scheme (7); however, the translocation along RNA was proposed to proceed by a directed hand-off mechanism mediated by local conformational changes within the Q loops. A well known SF4 helicase, gp4 from bacteriophage T7 (T7 gp4), also employs a sequential ATP hydrolysis (8–12). A set of gp4 x-ray structures suggested that ATP hydrolysis triggers sequential subunit rotations, which were proposed to effect nucleic acid translocation (13). However, in this case the structural details of DNA binding are not known, and the interplay between observed subunit rotation and translocation is under debate.

On the basis of the five helicase motifs (H1, H1a, H2, H3, and H4) and the structural homology, bacteriophage φ12 protein P4 was found to be closely related to the SF4 family of helicases.
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(14) and more recently to the DNA packaging ATPase from bacteriophage T4 (15). The primary function of the P4 ATPase is packaging genomic ssRNA precursors into preformed capsids (16). Hexameric P4 loads onto ssRNA and translocates in the 5’ to 3’ direction (17) while using its helicase activity to destabilize the ubiquitous secondary structure elements of the viral genomic precursors (18). Crystallographic analysis revealed structures of key intermediates along the catalytic pathway (14). The nucleotide-binding sites, which are located at the perimeter of the hexamer (Fig. 1, A and B), are connected via α-helices (helix α6, motif H4; Fig. 1C) to the central channel (L2 loops), through which RNA is thought to pass. The structures showed that the α6 helix-L2 loop region may act as a lever, which is in the “up” position in the presence of ATP and moves downward following hydrolysis and release of the phosphate. Each lever was proposed to effect an ~6 Å translocation of the RNA backbone bound to residue Lys-241 (motif H4). This movement constitutes the mechanical reaction coordinate. Six levers would act sequentially to pull the RNA strand through the channel. Moreover, the movement of the lever in one subunit may stimulate hydrolysis at the adjacent subunit via insertion of Arg-279 into the neighboring catalytic site. This arginine residue would act as an “arginine finger” in analogy to GAP-G protein system and F1-ATPase (19, 20). The P4 hexamer exhibits enzymatic properties compatible with sequential hydrolysis of ATP (21). P4, unlike gp4, Rho, and F1-ATPase, achieves catalytic cooperativity without substrate binding cooperativity, i.e. all nucleotide sites possess identical affinity for ATP irrespective of RNA binding. In summary, the sequential hydrolysis mechanism seems well established and conserved among remotely related hexameric motors, whereas mechanisms of nucleic acid binding and translocation differ considerably.

Mutation analysis of the related SF4 helicases highlighted the importance of specific residues within the H3 and H4 motifs for nucleic acid binding, translocation, and duplex unwinding (10, 22–27). This suggests that these residues play important roles in coordinating the chemical state of the ATP-binding site (i.e. the chemical reaction coordinate) with nucleic acid translocation (i.e. the mechanical reaction coordinate). However, in the absence of direct correlation between these mutations and mechanical motion, their exact roles in mechnochemical coupling remained unclear.

Here we test mutations of selected conserved residues within the H3 and H4 motifs in the context of the P4 hexamer with a well defined mechanical reaction coordinate. We demonstrate that motion of the RNA-binding site is essential for ATP hydrolysis, i.e. mechanical motion of the lever triggers hydrolysis. The structure of an intermediate state shows that the trigger is accomplished by insertion of the arginine finger into the neighboring active site. Finally, we show that two conserved residues within H3 (Asn-234) and H4 (Ser-252) motifs, respectively, act as γ-phosphate (γP) sensors and control the position of the lever. Structural comparison with SF3 and SF4 helicases suggests that the conserved residues may play similar roles in mechnochemical coupling in other hexameric molecular motors and perhaps in viral double-stranded DNA packaging motors (15).

EXPERIMENTAL PROCEDURES

Mutation and Protein Preparation—Mutants of φ12 P4 (N234G, K241A, K241C, S252Q, S252A, R272A, and R279A) were generated using site-directed mutagenesis strategy (Stratagene) using plasmid pPG27 as a template (28). All mutants were sequenced to confirm the mutations. P4 mutants were expressed in Escherichia coli BL21 (DE3) cells and purified to homogeneity as described (28). K241C mutant was purified in the presence of 1 mM dithiothreitol in the reduced form and then oxidized to produce the inter-subunit cross-links. Cross-linking of the K241C subunits was assisted by 0.1% H2O2 oxidation on ice in a buffer A containing 20 mM Tris, 50 mM NaCl, 5 mM MgCl2, pH 8.0, for 1 h. The presence of the cross-link was assayed by nonreducing SDS-PAGE (supplemental Fig. 1), size exclusion chromatography, and matrix-assisted laser desorption ionization time-of-flight mass spectrometry. The cross-linked subunits were only associated with the hexameric form.

Analytical Gel Filtration—The oligomeric state of P4 proteins was determined by analytical gel filtration at 20 °C on a Superdex-200 10/30 column (GE Biosciences) in buffer A. Molecular masses were determined by light scattering as described previously (17).

ATP Hydrolysis—ATP hydrolysis and subsequent phosphate release were measured using the EnzChek phosphate assay kit (Molecular Probes, Inc.) in the standard reaction buffer (20 mM Tris–HCl, 7.5 mM MgCl2, 75 mM NaCl, pH 7.5) at 28 °C, as described (29). ATP and ADP were purchased from Amersham Biosciences; poly(rC) was from Sigma (concentration is expressed as moles of bases). The dependence of the hydrolysis rate, v, on molar NTP concentration, [S], was described by the Hill Equation 1,

$$v = \frac{[P] \times k_{cat} \times [S]^n}{(K_{m} + [S]^n)}$$  

where [P] is molar protein concentration; $K_{m}$ is the apparent Michaelis constant; $k_{cat}$ is the turnover number, and $n$ is the Hill coefficient. The RNA-induced cooperativity and stimulation of ATPase activity were measured in the presence of 1 mM poly(C).

RNA Binding—In φ12 P4 RNA binding cannot be directly examined by gel mobility shift or filter assays because the affinity of φ12 P4 for RNA is low (30). RNA binding can be indirectly detected by monitoring the stimulation of ATPase by RNA (increased Hill coefficient and $k_{cat}$, and decreased $K_{m}$). This assay has been proved to be reliable for the packaging motor from a related cystovirus φ8 (31), for which binding to RNA can be detected by the standard gel mobility shift assay.

FIGURE 1. Key mutated residues mapped onto the P4 structure and sequence. A, ribbon diagram of a P4 monomer viewed from the bottom. B, ribbon diagram of P4 monomer. C, detailed view (stereo) of the nucleotide binding cleft: P loop (red), helix α6 (crimson), and L2 loop (cyan). Mutated residues are shown as ball-and-stick models and are colored according to their classification in Table 1 (green, sequential hydrolysis; blue, RNA binding; magenta, coupling of movement to hydrolysis). The bound AMP-PNP is shown in yellow. D, sequence alignment of cystoviral P4 proteins, where the positions of the mutations are indicated by arrows and color-coded according to the same scheme as in A–C. Identical residues are highlighted in red, and similar residues are in red type; red boxes indicate the positions of the conserved helicase motifs of the RecA-like family (H1, H1a, H2, H3, and H4).
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TABLE 1
Enzymatic properties of mutant P4 hexamers

| Mutants grouped by class | No RNA | With 1 m M poly(C) |
|-------------------------|--------|--------------------|
|                         | $k_{cat}$ | $K_{m}$ | $n$ | $k_{cat}$ | $K_{m}$ | $n$ |
| WT*                     | 0.84 ± 0.12 | 1.5 ± 0.4 | 1.4 ± 0.2 | 2.52 ± 0.07 | 0.49 ± 0.02 | 2.7 ± 0.3 |
| RNA binding             | 0.46 ± 0.02 | 1.0 ± 0.1 | 2.0 ± 0.2 | 0.75 ± 0.02 | 1.5 ± 0.1 | 1.9 ± 0.1 |
| K241A                   | 0.10 ± 0.01 | 1.3 ± 0.2 | 2.3 ± 0.4 | 0.12 ± 0.02 | 1.2 ± 0.3 | 1.7 ± 0.3 |
| Coupling of movement to nucleotide state | 0         | 0         | 0 | 0         | 0         | 0 |
| K241C                   | 0         | 0         | 0 | 0         | 0         | 0 |
| K241C + dithiothreitol  | 0         | 0         | 0 | 0         | 0         | 0 |
| N234G                   | 0         | 0         | 0 | 0         | 0         | 0 |
| S252A                   | 0         | 0         | 0 | 0         | 0         | 0 |

*Activity of the WT protein is shown for comparison (21). All symbols are defined in the text and under “Experimental Procedures.”

**Mutant protein names are based on one-letter abbreviations, e.g. K241A harbors Lys-241 to Ala2–41 substitution.

RESULTS

Mutagenesis Strategy and Mutant Classes—Several conserved residues are found within motifs H3 and H4 in P4 sequences from different bacteriophages (Fig. 1D). Structurally equivalent residues are also conserved among SF4 helicases and other RecA-like proteins (14). Structural studies on P4 suggest that these conserved residues coordinate ATP binding and hydrolysis with the lever motion (14). Thus residues belonging to the H3 (Asn-234) and H4 (Ser-252) motifs and loop L2 (Lys-241) were mutated to investigate the basis of mechanochemical coupling. We also probed the role of the proposed arginine finger candidates (Arg-279 and Arg-272) that are contributed in trans to the catalytic site in the adjacent subunit. The arginine finger is believed to stabilize the transition state (TS) (14, 36).

We describe the results for seven mutants, all hexameric in solution as judged by analytical gel filtration (data not shown) with enzymatic activities summarized in Table 1. All except R272A yielded diffraction quality crystals and interpretable electron density maps. Crystallographic and refinement statistics are given in Table 2. All diffraction data were obtained from crystals belonging to the same space group and having the same crystal packing, minimizing the possibility that the observed differences were crystallization artifacts. Based on the biochemical and structural properties, the mutants were classified into three groups (Table 1), which are mapped onto the structure in Fig. 1 and discussed in detail below.

Mobility of the Lever Is Essential for ATP Hydrolysis—The central channel of hexameric P4 is lined by the tip of helix α6 (residues 241–252, motif H4) and two loops, L1 (residues 196–206) and L2 (233–238). Motif H4 has been shown to encompass the DNA-binding sites of several related helicases (12, 27). In P4 the residue Lys-241, which is conserved among P4s from different phages, is located at the tip of the moving lever (Fig. 1A). We have proposed that this residue interacts with the RNA backbone (14).

Replacing Lys-241 with alanine reduced RNA stimulation of ATP hydrolysis by a factor of 2 compared with WT (1.6-fold, see Table 1) without alteration to the structure (Table 2). Substitution of this lysine with cysteine (K241C) blocked the
RNA stimulation under reducing conditions. This suggests that Lys-241 may act as the principal RNA-binding site. The residual stimulation by RNA observed in the K241A mutant could be due to weak RNA binding to a secondary site.

We have previously proposed that Lys-241 tracks the RNA during the catalytic cycle (14). The lever should undergo swinging motion along the mechanical reaction coordinate. If this motion is required for ATP hydrolysis, then immobilization of the lever should severely affect the basal activity (in the absence of RNA). To test this hypothesis we oxidized the cysteine residues in the K241C mutant and reversibly cross-linked the levers from the neighboring subunits (supplemental Fig. 1). The oxidized K241C P4 had no ATPase activity (Table 1). Addition of 10 mM dithiothreitol reduced the disulfide bonds and restored the basal ATPase activity. Hence, the mobility of the RNA-binding site and the lever is essential for the hydrolysis step, presumably by aiding the formation of the transition state.

The Reverse Stroke of the Lever Is Coupled to ATP Binding—A plausible sensor motif (Gln-194), which detects the presence of the γP of ATP and relays the information to other sites, was first identified in E. coli RecA (37). In mycobacterial RecA, the corresponding residue (Gln-195) interacts with the γP of ATP γS (Fig. 5B) and triggers ordering of the DNA-binding loop L2, possibly modulating DNA affinity (38). In P4, the structurally equivalent Asn-234 is fully conserved among members of the Cystoviridae family (Fig. 1D) and contacts the γP of AMPcPP in the phi2 structure (14).

Replacement of Asn-234 with glycine rendered P4 completely inactive (Table 1). Structural analysis of N234G complex with AMPcPP and Mn$^{2+}$ revealed that the mutant adopts the conformation of WT protein with ADP and Mg$^{2+}$ (or Mn$^{2+}$) bound, i.e. the product state (α6 helix in the “down” conformation, P loop folded inside the binding pocket) (Fig. 2, A and B). It appears that this mutation disrupts the communication between the nucleotide state and the conformation of the moving lever so that the enzyme is locked in a nonfunctional, product-like state. We conclude that binding of γP to Asn-234 in the WT P4 drives the recoil (upwards) stroke of the motor.

In addition to Asn-234 the hydroxyl of the conserved residue Ser-252 is in the vicinity of γP in the active site of the neighboring subunit. This residue has a structural counterpart in T7 gp4 helicase Ser-496 (13) (Fig. 6), which is essential for gp4 dTTPase and DNA binding activities (27). As Ser-252 flanks α6 helix, it may either control the lever position (i.e. sensor function) or respond to the mechanical motion in the neighboring subunit changing the chemical environment of the γP (i.e. effector function). Mutation to an alanine rendered the hexamer inactive (Table 1). The overall structure of the S252A in complex with either ADP or ATP was identical to WT. Only a minor rearrangement of Arg-251 was observed in the presence of ADP. Significant departures from the corresponding WT:AMPcPP structure, however, were seen for conformations of L2 loop, arginine finger (Arg-279), and the P loop in the presence of ATP (Fig. 3A). The position of P loop was well defined but slightly displaced from the WT. The L2 loop and tip of the α6 helix were significantly disordered and clearly displaced from the WT:AMPcPP configuration. This suggests that...
interactions of the Ser-252 hydroxyl control the $\alpha_6$ helix mobility. The role of Ser-252 in $\gamma_P$ interaction is further confirmed by the altered ATP binding kinetics (Fig. 4A), which exhibited an apparent first-order behavior. A much faster second-order phase ($k_{app} > 80 \text{ s}^{-1}$) could not be resolved in the present experimental setup suggesting much faster binding and release of ATP by this mutant. The ATP affinity of S252A decreased about 2-fold with respect to the wild type as judged by equilibrium amplitudes (Fig. 4A, inset). ADP binding to S252A was not significantly different from the WT (Fig. 4B).

The arginine finger was inserted into the active site of the neighboring subunit but in a different position from that seen in the ADP:Mg state. The side chain formed a hydrogen bond with the $\gamma_P$ oxygen (P-O=H-N distance 2.5Å).
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Arginine fingers are thought to stabilize the transition state (TS) during hydrolysis (36) and may trigger conformational changes following hydrolysis (39–41). In all known cases, only one arginine finger per subunit is needed for coordination of NTP hydrolyses within the hexameric ring (e.g. Arg-522 in T7 gp4 (9), Arg-207 in RSF1010 RepA (42), Arg-376 in the α-subunit of E. coli F1-ATPase (39), and Arg-373 in the α-subunit of bovine heart F1-ATPase (43)) (Fig. 6C). φ12 P4 contains two arginines contributing to the active site from the adjacent monomer, Arg-272 and Arg-279, which are highly conserved among the cystoviral packaging NTases (Fig. 1D). Based on their proximity to the triphosphate moiety in the three-dimensional structure, both arginines were implicated in ATP hydrolysis (14). Indeed, replacement of either arginine with alanine (R272A and R279A) abolished ATPase activity (Table 1).

The conformation of Arg-279 depends on the nucleotide-binding state of the protein. In the substrate-bound state (P4:AMPcPP:Mg2⁺), Arg-279 is locked away from the nucleotide pocket by the hydrogen bonding of Arg-251 (within the α6 helix). After hydrolysis the α6 helix swings down, and Arg-279 is inserted into the nucleotide-binding site of the next subunit to contact γP, in the transition state (see S252A:ATP structure discussed above, see Fig. 3A), and αP, in the product P4:ADP state (14). Such insertion effectively couples ATP hydrolysis in one subunit with the adjacent active site. The R279A mutant had nondetectable ATPase activity, suggesting that stimulation of hydrolysis around the ring plays an essential role in the ATPase reaction. The R279A substitution did not affect the overall structure of the mutant in neither the ATP-bound (Fig. 3B) nor the ADP-bound complex (data not shown). The mutation had no significant effect on ATP and ADP binding kinetics (Fig. 4). We conclude that the interaction of Arg-279 with the αP does not affect the lever position, i.e. Arg-279 does not have a sensor function. Furthermore, the S252A:ATP:Mg2⁺ structure revealed an intermediate state in which the arginine finger interacts with γP (Fig. 3A). This structure may represent a configuration close to the transition state in which the arginine finger stabilizes the buildup of charge on the terminal phosphate (19, 44). Thus, Arg-279 most likely plays a catalytic role rather than acting as a sensor.

**DISCUSSION**

**Structural Basis of Cooperativity and Sequential Hydrolysis**

The enzymology of φ12 P4 is compatible with a cooperativity model involving three consecutive subunits, two of which bind ATP and one binds ADP (21). Previous structural studies demonstrated that the moving lever can adopt either the up or down configuration depending on the bound nucleotide (14). The former configuration is preferred in the ATP-bound state, whereas the latter was only observed for the product (ADP: Mg2⁺) state. Here we demonstrate that the up position of the lever is stabilized by concerted interactions of Asn-234 and Ser-252 with the γP of the bound nucleotides. Both Asn-234 and Ser-252 act as γP sensors albeit in two neighboring binding sites. Simultaneous interaction with two γP is necessary for stabilizing the up conformation of the lever. Thus, we show that the concurrent ATP binding to two consecutive sub-
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FIGURE 5. Schematic description of the sequential coordination of hydrolysis. A, top panel shows a schematic representation of the conserved motifs in the context of one P4 subunit together with a bound ATP (yellow). B, initial state before hydrolysis. Only three consecutive subunits of the unraveled hexamer are shown for clarity (as perceived from the central channel, i.e. from the bound RNA "perspective"). C, hydrolysis and Pi release from subunit i - 1 allows the downward movement of helix α6 and insertion of the arginine finger Arg-279 into subunit i active site (ADP-P* designates the transition state). The L2 loop drags down the bound RNA (cyan). D, next round of sequential hydrolysis. The stretched RNA (a stress loop, magenta) links L2 loops on i and i - 1. RNA is brought to the vicinity and binds to L2 at subunit i + 1.

units is necessary for the recoil stroke, i.e. the return of the lever to its initial state.

The S252A:ATP:Mg2+ structure sheds light on the role of the third subunit, the ADP state, in the catalytic mechanism. This structure mimics the transition state configuration (see detailed justification below) in which the presence of ADP at the preceding subunit triggers the lever motion and arginine finger insertion at the catalytic subunit, resulting in sequential hydrolysis (Fig. 5C). Upon hydrolysis and Pi release from i - 1 subunit (ADP state) Asn-234-γP interaction is lost, and the lever moves from the up to the down position. The movement triggers the transition state at subunit i. Pi release further stabilizes the down position of lever in i - 1 because of the loss of the Ser-252-γP interaction. Pi release also allows the lever of i to move down and trigger the catalytic event at i + 1, beginning another cycle (Fig. 5D).

In the structures of WT P4, the conformation of the P loop correlates with the nucleotide-binding state, i.e. P loop is relaxed in the ATP:Mg2+ state and is strained in the P4:ADP: Mg2+ structure (14). Interestingly, in the S252A:ATP structure the P loop is in a configuration distinct from the two mentioned above. Thus, P loop may adopt multiple conformations, one of which is essential for transition state formation. This is consistent with the conservation of the pivotal glycine residue in P loop sequences, which presumably ensures the necessary flexibility.

Tight Coupling between Chemical and Mechanical Reaction Coordinates

The K241C cross-linking experiment indicates that motion of the α6 helix is necessary for productive hydrolysis, suggesting that the mechanical and chemical reactions are tightly coupled. In fact, the movement along the mechanical reaction coordinate is a prerequisite for the hydrolytic step in the neighboring subunit. The biochemical and structural phenomenology of the R279A and S252A mutants suggest that the coupling relies on the controlled, stepwise insertion of the arginine finger into the neighboring catalytic site, ensuring that ATP hydrolysis proceeds in a sequential manner, consistent with enzymatic and kinetic results (21).

The structure of the S252A mutant with ATP captures the arginine finger interacting with γP. The position is distinct from both the initial (observed in the P4:AMPcPP:Mg2+) and the final (P4:ADP:Mg2+) state. We propose that inability of S252A to hydrolyze ATP, even when the arginine finger is interacting with γP, is because of the lack of the Ser-252 hydroxyl group that coordinates the catalytic water molecule (45). In fact, a water molecule that in WT P4 is in the vicinity of γP (P=O...O distance 2.97 Å) and is hydrogen bonded to the hydroxyl group, is much further from its target (P=O...O distance 3.64 Å) in the mutant (Fig. 3A). This presumably blocks the reaction just before the chemical step takes place. Hence, the conformation of Arg-279 found in the S252A mutant may be very close to that assumed in the transition state. In this transient state the positive charge of Arg-279 would help to neutralize the charge on the γP and steer the electronic distribution on the β-γP linkage to an unstable configuration (36, 46, 47). After the dissipation of the transition state into product state a rotation of Ser-252 hydroxyl group most likely assists inorganic phosphate release, whereas Arg-279 retires to the stable position observed in the ADP state. In summary, a slight motion of the lever in the preceding subunit (Fig. 5B, i-1) is essential for the insertion of the Arg-279 side chain into the active site of the immediate neighbor (i), which in turn enables hydrolysis.

RNA-induced Cooperativity

To explain the RNA-induced cooperativity in the absence of structural data for a P4-RNA complex, we combine the biochemical data with the new structural information. Binding of nucleic acids stimulates the ATPase activity of P4 and other helicases (11). Two recent structures of hexameric helicases with bound nucleic acids, E1 (4) and Rho (6), show that β-hair-
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...pins or loops protruding into the central channel interact with the sugar-phosphate backbone. However, the number of subunits interacting with the bound nucleic acid differs between the two systems. The RNA-binding loops in Rho (Q loop and R loop) have direct structural equivalents in P4 (L1 and L2 loops, respectively) (Fig. 6f), whereas the β-hairpins in E1 and large T antigen (LTag) of SV40 virus (48) are not emanating from the central β-sheet of the catalytic domain (Fig. 6, E and F). The structural and sequence similarity of the RNA-binding loops between P4 and Rho suggests that binding of P4 to RNA might share similar features. The shortest ssRNA to stimulate P4 activity, a pentaoligonucleotide (21), is of similar length as that the four-nucleotide-long RNA bound between neighboring Rho subunits (6).

We propose that two neighboring subunits interact simultaneously with RNA via their L2 loops (Lys-241, Fig. 5C). At the stage depicted in Fig. 5C, the lever on subunit \( i - 1 \) is in an intermediate position and promotes formation of the transition state in the active site of subunit \( i \). After hydrolysis and \( P_i \) release at subunit \( i \) the lever at \( i - 1 \) is stabilized in the down position and in turn promotes formation of the intermediate lever configuration at subunit \( i \) via dragging on the bound RNA (Fig. 5D). RNA is then handed off to the neighboring subunit \( i + 1 \) and the cycle repeats. In effect, RNA coordinates motion of L2 loops along the mechanical reaction coordinate and acts as a “timing belt” by augmenting sequential firing of active sites (4). This timing effect results in the observed increased \( k_{cat} \), because at high ATP concentrations sites \( i \) and \( i + 1 \) are likely to contain ATP. Conversely, at low ATP concentrations the sequential chain of hydrolysis will be frequently interrupted and the enzyme would have to reinitiate, decreasing the overall rate and increasing the apparent cooperativity in the presence of RNA (21).

In our model, RNA also acts as a stress loop between the two subunits and may play a role akin to the deformation of the central β-sheet in F₁-ATPase (49) and the related hexameric helicases (7, 8). In contrast to F₁-ATPase, strain on P4-bound RNA develops as a result of ATP hydrolysis This stabilizes the down position on one lever, whereas the neighboring L2 loop is still in the up position. When the L2 loop moves down (after ATP hydrolysis), RNA may act as a “bungee cord,” biasing the motion of the attached lever (Fig. 5D). An ATP-induced change in the backbone conformational of P4-bound RNA was detected for the related P4 bacteriophage φ8 (29). This change may be related to the elastic stretching and relaxation of the bound ssRNA backbone.

The proposed RNA-driven coordination of L2 loop movement is compatible with a version of the staircase model for nucleic acid binding as seen in the E1 helicase (4). However, a maximum of three subunits would bind to RNA at a time (Fig.

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**FIGURE 6.** Structural similarity and evolutionary relationship among hexameric molecular motors. A–I, ribbon diagrams of the ATPase core from P4 and other hexameric motors. A, P4 (PDB code 1w49 chain B); B, E. coli RecA (PDB code 1XMS chain A); C, bovine mitochondrial F₁-ATPase subunit α (PDB code 1H8H chain B); D, subunit β (PDB code 1H8H chain F); E, SV40 LTag (PDB code 2GXA chain E); F, papillomavirus E1 (PDB code 2GXA chain E), His-507 and Lys-506 constitute the DNA-binding site; G, T7 gp4 (PDB code 1EOJ chain B); H, plasmid RSF1010 RepA (PDB code 1G8Y chain A); I, Rho transcription terminator factor (PDB code 2HT1 chain A); The conserved RecA-motifs are highlighted (H1, red; H1a, yellow; H2, green; H3, blue; H4, magenta; L2 loop, cyan). The conserved side chains are shown as ball-and-stick models (Asn-234 equivalents in magenta, Ser-252 equivalents and arginine fingers in light green, Lys-241 equivalents in blue). Percentage of sequence identity (1st line) and root mean square differences (rms) in C-α positions with number of aligned residues (2nd line) between structure (A) and structures (B–I) are shown at the bottom of B–I. Superimposition of coordinates and calculation of root mean square was conducted using SHP (53).
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5D). Simultaneous binding of five to six subunits to the nucleic acid is unlikely given the low affinity of \( \Phi 12 \) P4 for RNA (30) and is inconsistent with the structural and biochemical models (14, 21) that involve cooperation of three neighboring subunits.

Implications for Other Helicases

Structural Comparison—The P4 fold belongs to the RecA-like ATPase family, and the motor domain structure is closely related to other members within this group (Fig. 6, A–I). Quantitative structural comparison of the Rec-A-like domains reveals that the closest P4 relative is the T7 gp4 (Fig. 6, A and G). This may not be surprising given that both motors are hexameric and translocate along single-stranded nucleic acids (12). However, it is surprising that the structure and location of the helicase motifs are more or less conserved even for the distantly related subunits of the rotary motor F1-ATPase. For example, the Asp-333 in the \( \alpha \)-subunit and Asp-316 and Asp-319 in the \( \beta \)-subunit of bovine mitochondrial F1-ATPase (Fig. 6, C and D) are in contact with the moving \( \gamma \)-subunit and might play a similar function as the residues within the L2 loop of helicases. The structural conservation goes beyond the helicase motifs and encompasses also the arginine fingers (except for RecA in which there is a lysine in the equivalent position, Lys-250; see Fig. 6B). Taken together, certain mechanistic features may be common to different hexameric motors.

H3 and H4 Motifs Encompass \( \gamma \)-Phosphate Sensors—Sensors of the \( \gamma P \) that are structurally equivalent to Asn-234 in P4 have been identified in hexameric (H3 motif) (26, 42, 50) as well as SF1 and SF2 helicases (motif III) (2, 22, 51). A substitution of histidine within the H3 motif of SF4 helicase RepA (His-179) disturbed the coupling between ATP hydrolysis and the concerted binding/release of ssDNA (42). In the monomeric helicase PcrA a mutation of the structurally homologous glutamine decoupled the ATPase activity from DNA unwinding (24). It was concluded that this residue acts as a conformational switch, a \( \gamma P \) sensor, which modulates the affinity for nucleic acid. Our results suggest that the affinity for nucleic acids could be modulated by physical motion (including domain motions) or mechanical stress applied to the H3/III motif through the interaction with ATP. Upon hydrolysis and P1 release, the contact between Asn-234 and the nucleotide is lost allowing the lever to relax into a different conformation. Unlike for other helicases, the P4 Asn-234 sensor is essential for the basal ATPase activity. This reflects the tight coupling in P4 between the lever movement and catalysis.

The sensor residue Ser-252 belongs to the H4 motif and is structurally equivalent to Ser-496 in T7 gp4 helicase. Mutation of Ser-496 led to a complete loss of both dTTPase activity and DNA binding (27). Our present results suggest that the hydroxyl group of Ser-252 is essential for ATP hydrolysis because it coordinates the catalytic water molecule for an “in-line” attack on \( \gamma P \). A similar role was found for the structurally equivalent Gln-254 in the Pcr domain 2A using a theoretical approach (45).

Interestingly, Asn-234 and Ser-252 are structurally equivalent to Asn-523 and Ser-537 of the distantly related AAA+ E1 helicase (Fig. 6, A and F). According to our results, Ser-537 is most likely involved in the chemical step by coordinating the network catalytic water molecules. Both Asn-523 and Ser-537 may also act as sensors because their distance to the bound ADP depends on the position of the DNA binding hairpin (4). However, the mechanism by which these residues would control the hairpin positions may not be as straightforward as in P4 because these residues are not directly connected to the moving lever (\( \beta \)-hairpin).

Arginine Fingers—Arginine fingers are structurally conserved features of hexameric motors. Insertion of an arginine finger correlates with subunit motion, and it was proposed that it mediates translocation in ‘T7 gp4 (13) and is essential for cooperativity (9). We have demonstrated that the insertion of the P4 arginine finger (Arg-279) correlates with the conformation of the moving lever. This ensures sequential hydrolysis of ATP that is triggered by mechanical motion. In addition, we show that there is an intermediate conformation of the arginine finger, which may be essential for reaching the transition state.

In contrast to Arg-279, Arg-272 occupied an identical position in all structures of the WT (14) and mutant proteins (Table 2), irrespective of the nucleotide state. The fixed position of its side chain suggests that it is not involved in relaying conformational changes between neighboring subunits, but it may simply provide a positive charge to neutralize the bound nucleotide di- and triphosphates. It may also stabilize the catalytically important water network. Therefore, it may act in a fashion similar to the so-called “position 3” arginine (52), which is common to many AAA+ and RecA-like proteins and is involved in ATP binding.

The arginine fingers in E1 and LTag helicases are not structurally equivalent to Arg-279 (Fig. 5, E and F) and seem to be inserted into the catalytic pocket upon ATP binding. It remains to be seen whether these residues are \( \gamma P \) sensor or serve as a trigger for transition state formation and hydrolysis.

Nucleic Acid Binding—It has been proposed that ssRNA binding in the central cavity promotes sequential hydrolysis and thereby stimulates ATPase activity (14, 21). Here we show that the RNA is very likely to interact with the L2 loop (Lys-241) within the central channel and that this interaction is responsible for the observed stimulation. The structurally equivalent L2 loop in T7 gp4 has recently been identified as the DNA-binding site (10). However, each L2 loop in gp4 contains three lysines, and consequently this motor exhibits a much higher affinity for ssDNA (Fig. 6G).

P4 from bacteriophage \( \Phi 8 \) does not possess an equivalent residue to Lys-241, and the L1 loop has been shown to bind RNA instead (31). The Q loop, which is the L1 loop equivalent in Rho transcription terminator, also binds RNA (6) (Fig. 6F). It is very likely that the L1 loop in \( \Phi 12 \) P4 also interacts with RNA. However, it is not clear at the present whether there is any communication between L1 and the active site.

The nucleic acid binding \( \beta \)-hairpins of AAA+ hexameric DNA helicases E1 and LTag do not emanate from the catalytic core and are not related to either L1 or L2 loop (Fig. 6, E and F). Thus, unlike the sensor residues and arginine fingers, the nucleic acid binding mode does not appear to be universally conserved among hexameric molecular motors.
Conclusions

We have demonstrated a tight coupling between the chemical and mechanical reaction coordinates for the viral packaging motor φ12 P4. Conserved residues within the H3 and H4 hexamer helicase motifs encompass the moving lever with RNA-binding site. The lever is flanked by γ-phosphate sensors that control its position. Sequential hydrolysis is assisted by a stepwise insertion of the arginine finger, which is coupled to lever motion in the neighboring subunit. Given the remarkable degree of structural conservation of the involved motifs, the proposed model may be applicable to other helicases and oligomeric motor proteins.

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