Cooperative Mechanism of RNA Packaging Motor*

Received for publication, March 10, 2005, and in revised form, April 12, 2005
Published, JBC Papers in Press, April 18, 2005, DOI 10.1074/jbc.M502655200

Jiří Lisal‡ and Roman Tumas§

From the Department of Biological and Environmental Sciences and Institute of Biotechnology, University of Helsinki, Helsinki FIN-00014, Finland

P4 is a hexameric ATPase that serves as the RNA packaging motor in double-stranded RNA bacteriophages from the Cystoviridae family. P4 shares sequence and structural similarities with hexameric helicases. A structure-based mechanism for mechano-chemical coupling has recently been proposed for P4 from bacteriophage φ12. However, coordination of ATP hydrolysis among the subunits and coupling with RNA translocation remains elusive. Here we present detailed kinetic study of nucleotide binding, hydrolysis, and product release by φ12 P4 in the presence of different RNA and DNA substrates. Whereas binding affinities for ATP and ADP are not affected by RNA binding, the hydrolysis step is accelerated and the apparent cooperativity is increased. No nucleotide binding cooperativity is observed. We propose a stochastic-sequential cooperativity model to describe the coordination of ATP hydrolysis within the hexamer. In this model the apparent cooperativity is a result of hydrolysis stimulation by ATP and RNA binding to neighboring subunits rather than cooperative nucleotide binding. The translocation step appears coupled to hydrolysis, which is coordinated among three neighboring subunits. Simultaneous interaction of neighboring subunits with RNA makes the otherwise random hydrolysis sequential and processive.

Genomes of many viruses are encapsulated through NTP-driven packaging motor into preformed capsids. This process requires a portal complex that operates as the molecular motor and converts chemical energy into mechanical work. Double-stranded RNA bacteriophages from the Cystoviridae family (φ6-φ14) package their ssRNAI genomic precursors using a hexameric portal complex, the packaging NTPase P4 (1). P4 proteins share sequence and structural similarities with hexameric helicases and some of them possess helicase activity (2, 3). The P4 hexamer is also used as a passive pore for the exit of nascent transcripts from the viral core (4).

A power stroke mechanism was proposed on the basis of P4 structures encompassing the key states of the catalytic cycle (3). Nucleotide exchange and hydrolysis was shown to induce concerted structural changes in two regions, namely the P-loop and the L2 loop/α6 helix segment. The P-loop (Walker A or H1a motif in hexameric helicases) interacts with α and β phosphates of the nucleotide bound within the catalytic site. The L2 loop (H3 motif) is directly connected to the α6-helix (H4 motif) and binds to RNA. In the pre-hydrolysis state (P4·MgAMP-CPP complex) the P-loop is in a “relaxed” configuration and the L2 loop/α6 helix is in an “up” configuration. After hydrolysis (P4·MgADP complex) the P-loop is in the “strained” configuration and the L2 loop/α6 helix is in the “down” configuration. In the absence of any nucleotide the P-loop is in the relaxed configuration and the L2 loop/α6 helix can swivel between the up and down configurations. Thus, it was proposed that the binding of ATP locks the L2 loop/α6 helix in the up configuration, where it engages the ssRNA. Upon ATP hydrolysis, the nucleotide binding P-loop strains, push against one end of the L2 loop, so that the rest of the L2 loop and the α6 helix are forced to pivot down, carrying along the engaged RNA (3).

The structure also revealed that nucleotides bind at the subunit-subunit interfaces (3). Half of the adenine binding pocket and so called “arginine fingers” are contributed by neighboring subunits, whereas the rest of the binding site is contributed by the catalytic subunit. This provides the structural basis for cooperativity. However, the mechanism of ATP hydrolysis coordination within the P4 hexamer and RNA-dependent stimulation of hydrolysis remains elusive (4).

Here we present detailed enzymatic study of nucleotide binding, hydrolysis, and product release by φ12 P4 in the presence of different nucleic acids, nucleotides, and inhibitors. Using these data we delineate the mechanism of ATP hydrolysis coordination and cooperativity during RNA translocation.

MATERIALS AND METHODS

Protein Purification—φ12 P4 protein was expressed in Escherichia coli and purified as previously described (5). P4 concentrations were determined by absorption at 280 nm using an extinction coefficient of 26,930 M⁻¹ cm⁻¹, which was calculated based on the amino acid composition.

Phosphate Release Measurements—Phosphate release assays were done using the EnzChek Phosphate Assay Kit (Molecular Probes, Inc.) in the standard reaction buffer (20 mM Tris-HCl, pH 7.5, containing 75 mM NaCl and 7.5 mM MgCl₂) as described (6). Absorbance values at 360 nm were converted to concentrations of inorganic phosphate (P₄) using calibration with KH₂PO₄ standards. AMP was used as a background control in all experiments. Steady-state kinetics of phosphate release were measured using a Victor plate reader (Wallac-PerkinElmer) with time resolution of 20 s at 28 °C. ATP, ADP, and UTP were purchased from Amersham Biosciences, AMP-PNP from Fluka, poly(C)₆ was from Sigma (concentration expressed as mole of base per liter), and oligoribocytidines of lengths 5, 7, 10, 15, 20, and 30 and 60 nucleotides were custom-made by Dharmacon (concentrations expressed as mole of oligonucleotide strand per liter).
Cooperativity of Hexameric Molecular Motor

Rapid Kinetics of Nucleotide Binding—Fluorescent nucleotide analogs 2′-O-(N-methylanthraniloyl)-3′-deoxyadenosine 5′-triphosphate (2′-MANT-3′-dATP) and 3′-O-(N-methylanthraniloyl)-2′-deoxyadenosine 5′-triphosphate (3′-MANT-2′-dATP) were obtained from Jena Bioscience. 2′- (or-3′)-O-(N-methylanthraniloyl) adenosine 5′-triphosphate (MANT-ATP) and 2′- (or-3′)-O-(N-methylanthraniloyl) adenosine 5′-diphosphate (MANT-ADP) were purchased from Molecular Probes. All experiments were done in 20 mM Tris-HCl buffer, pH 7.5, containing 75 mM NaCl and 7.5 mM MgCl₂ unless stated otherwise. Rapid mixing of fluorescent nucleotide analogs with P4 protein was achieved in a μSPM 20 stopped-flow apparatus (Bio-Logic) equipped with a P-15 flow cell (dead time approximately 6 ms). Fluorescence resonance energy transfer (FRET) between the three P4 tryptophans and the MANT-labeled nucleotides was detected by a MOS 250 fluorometer (Bio-Logic), using λ_ex = 290 nm (20 nm slit) and λ_det = 440 nm (20 nm slit) with a time step of 1 ms at 28 °C.

Equilibrium Competitive Binding Assay—1 μM P4 was mixed with 50 μM 3′-MANT-2′-dATP in 20 mM Tris-HCl buffer, pH 7.5, containing 75 mM NaCl and 7.5 mM MgCl₂ (final concentrations). The solution was aliquoted. Fluorescence spectra (310–550 nm, 10 nm slit) were recorded within seconds upon addition of appropriate concentrations of the competing nucleotide (ATP, ADP, AMP-PNP, or UTP) to the aliquots using a MOS 250 fluorometer (Bio-Logic), λ_ex = 290 nm (20 nm slit), at 28 °C.

Data Analysis—The steady-state rate of NTP hydrolysis (measured by phosphate release), \( v \), is in the simplest case described by the Michaelis-Menten equation,

\[
\frac{v}{[P]} = \frac{k_{\text{cat}}[S]}{K_m + [S]}
\]

where \([P]\) is the protein concentration, \([S]\) is the substrate concentration, and \(K_m\) is the Michaelis constant. In the case of apparent cooperativity, the steady-state kinetics can be phenomenologically described by the Hill equation,

\[
\frac{v}{[P]} = \frac{k_{\text{cat}}[S]^n}{K_m + [S]^n}
\]

where the parameter \( n \) is the Hill coefficient.

P4 translocates along the RNA strand of length \( L \) and falls off the RNA after an average of \( x \) bases because of limited processivity or limited substrate length. \( x \) is related to the processivity parameter \( M \) as follows: \( x = LM/(L + M) \). The processivity parameter \( M \) is an intrinsic property of the enzyme and reflects the average number of translocated bases at which half of the enzymes release RNA. Taking into account release and rebounding to RNA, the rate of phosphate release \( v \) during translocation along RNA is given by,

\[
1/v = 1/(k_{\text{RNA}}x) + 1/k_H
\]

where \( k_{\text{RNA}} \) is the rate of RNA binding (which happens once per \( x \) bases translocated) and \( k_H \) is the rate of ATP hydrolysis per one base translocated (i.e. rate of ATP hydrolysis divided by the number of bases translocated per one ATP molecule). Combining these two equations gives,

\[
\frac{v}{[P]} = \frac{ML_hk_{\text{RNA}}}{(M + L)k_H + ML_hk_{\text{RNA}}}
\]

if \( k_{\text{RNA}} \ll k_H/M \) (RNA binding is limiting) this equation simplifies into as follows.

\[
\frac{v}{[P]} = \frac{ML_hk_{\text{RNA}}}{M + L}
\]

Taking into account the footprint of the P4 on RNA, \( R \) (RNA longer than \( R \) bases must be bound to achieve translocation), and the basal ATPase activity, \( k_B \) (in the absence of RNA), the final equation for obtaining the processivity parameter \( M \) is as follows.

\[
\frac{v}{[P]} = \frac{[(L - R)ML_hk_{\text{RNA}}]}{M + L - R}
\]

Fluorescence intensity traces, \( f \), corresponding to binding of MANT-ADP, 3′-MANT-2′-dATP, and 2′-MANT-3′-dATP were approximated by a single exponential term,

\[
f = b - a \exp(-kt)
\]

where \( a \) is the amplitude of fluorescence change, \( b \) is the fluorescence plateau at infinite times, \( k \) is the apparent first-order rate, and \( t \) is time. The kinetic fluorescence traces corresponding to MANT-ATP binding were described by two exponential terms.

\[
f = b - a_1 \exp(-k_{t,1}t) - a_2 \exp(-k_{t,2}t)
\]

In the case of a simple second-order reaction, the linear dependence of the apparent rate on substrate concentration \([S]\) was described by,

\[
k = k_{\text{on}}[S] + k_{\text{off}}[S]
\]

\( k_{\text{on}} \) and \( k_{\text{off}} \) are the true second-order association and first-order dissociation rate constants.

Equilibrium dissociation constants were estimated from the fluorescence change amplitudes \( a \) (corrected for the inner filter effect as previously described (6)).

\[
a = C [P] + [S] + K_{\text{app}}\sqrt{[P][S] + K_{\text{app}}^2 - 4[P][S]}/2
\]

This equation describes stochiometric binding of the nucleotide analog (concentration \([S]\)) to P4 (protein concentration \([P]\)) with the apparent dissociation constant \( K_{\text{app}} \). Parameter \( C \) describes the maximum increase in quantum yield upon binding.

The equilibrium competition assays against the fluorescent nucleotide analog were performed at low protein concentration and the fluorescence intensity was approximated by,

\[
f = d - e[S]/K_{\text{app}} + [S]
\]

where \( d \) and \( e \) are constants. The correct dissociation constant \( K_d \) was calculated from the apparent dissociation constant \( K_{\text{app}} \) as follows,

\[
K_d = K_{\text{app}}/[1 + K_{\text{app}}]
\]

where \([I]\) is the concentration and \( K_i \) is the dissociation constant of the fluorescent analog, respectively (7).

RESULTS

\( \phi 12 P4 \) Is a RNA Specific Motor with Low Translocation Processivity—P4 \( \phi 12 \) slowly hydrolyzed ATP in the absence of any nucleic acid, whereas ssRNA but not ssDNA stimulated the activity (Fig. 1A). RNA affected both \( k_{\text{cat}} \) (3-fold increase) and \( K_m \) (2-fold decrease), and induced cooperativity in ATP hydrolysis with an apparent Hill coefficient \( n = 2.7 \pm 0.3 \) (Table I). ATP analogs AMP-PNP and AMP-CPN were not hydrolyzed by \( \phi 12 P4 \) (Fig. 1A, triangles). On the other hand, slow but detectable hydrolysis of AMP-PCP was observed (data not shown).

Dependence of \( K_{\text{on}} \) on ssRNA concentration provided an indirect measure of RNA-P4 complex formation (6). The apparent dissociation constant of the RNA-P4 complex was \( 0.40 \pm 0.06 \) mM in the presence of 1 mM ATP and 0.39 ± 0.04 mM in the presence of 1 mM ATP and 0.5 mM ADP, respectively (Fig. 1B). Thus, the low affinity for RNA remained unaffected by ADP. Unfortunately, this method does not permit quantitative assessment of RNA affinity in the absence of ATP. However, no stable complex was detected by gel mobility shift (4) and thus we conclude that the affinity in the nucleotide free state remained low.

Fig. 1C shows the dependence of \( k_{\text{off}} \) on the ssRNA length. RNA as short as a pentamer stimulated P4 activity. Extrapolation of the experimental data using Equation 6 suggested that (rC)4 would not stimulate and (rC)5 constitutes the minimum length of several thousand bases (6). Using Equation 6 a processivity parameter \( M \) of 2.1 ± 0.7 was obtained indicating frequent dissociation of the P4-RNA complex. This is in contrast
Cooperativity of Hexameric Molecular Motor

Table I

Parameters of hydrolysis of ATP and its analogs by \( \phi 12 \ P4 \)

|          | \( k_{\text{cat}} \) | \( K_m \) | \( n \) |
|----------|-------------------|--------|-------|
| ATP      | \( 0.84 \pm 0.12 \) | \( 1.5 \pm 0.4 \) | \( 1.4 \pm 0.2 \) |
| No nucleic acid | \( 2.52 \pm 0.07 \) | \( 0.49 \pm 0.02 \) | \( 2.7 \pm 0.3 \) |
| RNA      | \( 0.72 \pm 0.03 \) | \( 1.3 \pm 0.1 \) | \( 1.5 \pm 0.1 \) |
| AMP-PNP  | \( 0 \)            | \( 0 \)  | \( 0 \)  |
| No nucleic acid | \( 0.021 \pm 0.001 \) | \( 0.12 \pm 0.01 \) | \( 1.5 \pm 0.1 \) |
| RNA      | \( 0.189 \pm 0.008 \) | \( 0.10 \pm 0.01 \) | \( 2.0 \pm 0.2 \) |
| 3'-MANT-2'-dATP | \( 0 \)            | \( 0 \)  | \( 0 \)  |
| No nucleic acid | \( 0.071 \pm 0.006 \) | \( 0.42 \pm 0.04 \) | \( 1.9 \pm 0.1 \) |
| RNA      | \( 0.77 \pm 0.02 \) | \( 0.12 \pm 0.01 \) | \( 3.4 \pm 0.4 \) |

FIG. 1. A, plot of steady-state ATP hydrolysis rate as a function of substrate concentration without nucleic acid (open circles), in the presence of 1 mM poly(C) (closed circles) and in the presence of 2 \( \mu \)M ssDNA 60-mer (squares) (0.1 \( \mu \)M \( \phi 12 \) P4 in 20 mM Tris buffer, pH 7.5, 75 mM NaCl, 7.5 mM MgCl\(_2\), at 28°C). Triangles represent kinetics of AMP-PNP hydrolysis. Lines represent fits to Equation 2, yielding parameters in Table I. B, rate of 1 mM ATP hydrolysis as a function of poly(C) concentration (closed circles). Rate of 1 mM ATP hydrolysis as a function of poly(C) concentration in the presence of 0.5 mM ADP (open circles). Lines represent fits to Equation 10. C, rate of 1 mM ATP hydrolysis by 0.12 \( \mu \)M (-0.02 \( \mu \)M hexamer) \( \phi 12 \) P4 (closed circles) and 3'-MANT-3'-dATP (open circles) in the presence of 2 \( \mu \)M oligonucleotides of different lengths. Lines represent fits to Equation 6 (\( \phi 12 \) P4: \( k_B = 0.41 \pm 0.04 \) s\(^{-1}\), \( k_{\text{RNA}} = 1.3 \pm 0.4 \) s\(^{-1}\), \( M = 2.1 \pm 0.7\), \( r = 4.1 \pm 0.4\); \( \phi 8 \) P4: \( k_B = 0 \) s\(^{-1}\), \( k_{\text{RNA}} = 0.46 \pm 0.05 \) s\(^{-1}\), \( M = 11 \pm 1\), \( r = 8.5 \pm 0.4\)).

to \( \phi 8 \) P4 (Fig. 1C, open circles), which was stimulated by RNA longer than 8 bases and exhibited higher processivity (\( M \approx 11\)).

2'-MANT-3'-dATP Is a Hydrolyzable Fluorescent ATP Analog—\( \phi 12 \) P4 contains three tryptophan residues that allowed for detection of nucleotide binding using FRET between the tryptophans and MANT-labeled nucleotides. MANT-ATP was hydrolyzed by \( \phi 12 \) P4 with \( k_{\text{cat}} \) and \( K_m \) approximately 1 order of magnitude lower than those for ATP hydrolysis. However, the relative stimulation by RNA and the apparent cooperativity were identical as for the hydrolysis of unlabeled ATP (Fig. 2A and Table I). Traces of MANT-ADP fluorescence upon mixing with P4 were single-exponential, whereas the traces corresponding to MANT-ATP binding had to be fitted by two exponential terms (Fig. 2, B and C). This suggested that MANT-ATP binding is either a two-step process or that there are two distinct classes of MANT-ATP binding sites. Alternatively, we considered the possibility that the two phases in MANT-ATP binding could arise from the two isomers of MANT-ATP present in the commercial preparation (i.e., the MANT group linked to the 2'- hydroxyl versus the 3'-hydroxyl group). Such an effect has been observed for MANT-ATP binding to the transcription termination factor Rho (8). Therefore, we investigated the interactions of P4 with two additional MANT-nucleotide derivatives, where the fluorophore was attached specifically to the 3'-hydroxyl (3'-MANT-2'-dATP) or the 2'-hydroxyl (2'-MANT-3'-dATP) of the deoxyribose ring. Both MANT-dATP analogs bound to P4 in a single kinetic phase. Only the 2'-MANT-3'-dATP was hydrolyzed by \( \phi 12 \) P4 (Fig. 2A and Table I). Thus, 2'-MANT-3'-dATP constitutes a suitable analog to study ATP binding, whereas 3'-MANT-2'-dATP can be used as a fluorescent ATPase inhibitor.

2'-MANT-3'-dATP Binding Is a RNA Independent, Single Step Second-order Process—ATP binding kinetics was determined using the 2'-MANT-3'-dATP analog (Fig. 3). Rate of FRET intensity change increased linearly with the 2'-MANT-3'-dATP concentration (Fig. 3A). Thus, 2'-MANT-3'-dATP binding was a single step second-order, reaction. No significant difference in 2'-MANT-3'-dATP binding in the absence and presence of RNA was detected. Concentration dependences of the measured apparent rate constants yielded the second-order rate constants and dissociation constant: \( k_{\text{OFF}} = 64 \pm 2 \) s\(^{-1}\), \( k_{\text{ON}} = 0.25 \pm 0.02 \mu \text{M}^{-1} \) s\(^{-1}\), and \( K_d = 256 \pm 30 \mu \text{M} \) in the absence of RNA, and \( k_{\text{OFF}} = 64 \pm 4 \) s\(^{-1}\), \( k_{\text{ON}} = 0.30 \pm 0.03 \mu \text{M}^{-1} \) s\(^{-1}\), and \( K_d = 213 \pm 39 \mu \text{M} \) in the presence of RNA. FRET amplitudes (Fig. 3B) yielded a similar value for the dissociation constants \( K_d = 210 \pm 51 \mu \text{M} \) in the absence and \( K_d = 265 \pm 52 \mu \text{M} \) in the presence of poly(C). Binding of the non-hydrolyzable 3'-MANT-2'-dATP (Fig. 3) appeared to be a single step, second-order association with \( K_{\text{OFF}} = 18.5 \pm 0.6 \) s\(^{-1}\) and \( k_{\text{ON}} = 0.102 \pm 0.006 \mu \text{M}^{-1} \) s\(^{-1}\) giving the dissociation constant \( K_d = 181 \pm 18 \mu \text{M} \).
MANT-ADP Binding Is a RNA Independent, Single Step Second-order Process—

Although the MANT-ADP used in this study was a mixture of 2'- and 3'-isomers, single exponential binding kinetics were obtained for all concentrations. This could be the result of identical binding of the two isomers or lack of binding or FRET signal for one isomer. Fig. 4A shows the apparent rate of FRET intensity increase upon MANT-ADP mixing with P4 as a function of MANT-ADP concentration. Linearity of this dependence (up to 200 μM MANT-ADP, i.e. about five times more than $K_d$ for MANT-ADP) suggests that MANT-ADP binding is a single step second-order association.

Fig. 4B represents the FRET amplitude upon MANT-ADP binding to P4 as a function of MANT-ADP concentration. The apparent dissociation constants were independent of RNA ($K_d = 36 \pm 5$ and $41 \pm 8$ μM in the absence and presence of ssRNA, respectively). Similarly, inorganic phosphate had no effect on MANT-ADP binding ($K_d = 43 \pm 9$ μM in the presence of ssRNA and 50 mM inorganic phosphate). AMP-PNP (non-hydrolyzable ATP analog) competed with MANT-ADP binding ($K_d = 114 \pm 19$ μM in the presence of ssRNA and 1 mM AMP-PNP) demonstrating that MANT-ADP and ATP bind to the same set of sites.

Mg$^{2+}$ Inhibits Nucleotide Binding—Under typical cellular conditions more than 99% of all ATP and ADP is found in a complex with Mg$^{2+}$ (9). As shown previously for $\phi 6$ P4 (10, 11) and more recently for $\phi 12$ P4 (4), Mg$^{2+}$ at concentrations...
higher than 1 mM inhibits ATP hydrolysis. To shed light on this phenomenon we studied the influence of Mg\(^{2+}\) on nucleotide binding (Fig. 5A). The plot of the 2'-MANT-3'-dATP dissociation constant as a function of Mg\(^{2+}\) concentration was linear, suggesting a competitive inhibition of substrate (Mg:2'-MANT-3'-dATP) binding by free Mg\(^{2+}\) ions. The slope of the line revealed the inhibition constant \(K_I = 5 \pm 1\) mM. The extrapolated Mg:2'-MANT-3'-dATP dissociation constant at zero free Mg\(^{2+}\) concentration is 95 ± 8 \mu M. Similarly, Mg:MANT-ADP binding was competitively inhibited by free Mg\(^{2+}\) ions (Fig. 5A) having the same inhibition constant \(K_I = 5 \pm 2\) mM. The extrapolated Mg:MANT-ADP dissociation constant at zero free Mg\(^{2+}\) concentration was 15 ± 4 \mu M. Thus the inhibition by Mg\(^{2+}\) is because of competition for the same binding site.

**Nucleotide Affinities**—P4 affinity for different nucleotides was measured using competition against the non-hydrolyzable fluorescence ATP analog 3'-MANT-2'-dATP. Fig. 5B shows the FRET intensity decrease as a result of increasing competition with ATP and UTP. Dissociation constants determined for different nucleotides are listed in Table II. P4 exhibited similar affinity for ATP, ADP, and AMP-PNP. Thus, under the cellular conditions, where ATP concentration is millimolar and ADP concentration is micromolar, most of the nucleotide binding sites would be occupied by ATP. The UTP binding was approximately 1 order of magnitude weaker than ATP binding. This is consistent with the previously reported purine specificity of \(\phi 12\) P4 (4).

**ATPase Inhibition Experiments Revealed Sequential Catalytic Mechanism**—Considering the similar values of dissociation constants for ATP, ADP, and AMP-PNP, ADP and AMP-PNP should inhibit ATP hydrolysis. Detailed characteristics of the inhibition could shed light on the catalytic mechanism. Therefore we measured steady-state kinetics of ATP hydrolysis at different concentrations of ADP or AMP-PNP and in the absence as well as in the presence of RNA (Fig. 6). In the absence of RNA, ADP inhibited hydrolysis competitively (unchanged \(k_{cat}\), increased \(K_m\)), whereas AMP-PNP inhibited non-
competitively (decreased \(k_{\text{cat}}\), unchanged \(K_m\)). In the presence of RNA the inhibition by ADP became a mixed type (decreased \(k_{\text{cat}}\), increased \(K_m\)), whereas the inhibition by AMP-PNP remained noncompetitive. These results demonstrated that some configurations of ATP and AMP-PNP within the hexamer (and some configurations of ATP and ADP in the presence of RNA) led to inactive protein. To find which configurations were inhibitory we have performed a titration experiment.

Fig. 7A shows all possible configurations (microstates) of the ATP and the inhibitor within the hexamer. Abundance of a particular microstate is equal to \(A = q^i(1-q)^{6-i}\), where \(q = [I]/([ATP] + [I])\) and parameter \(i\) is the number of subunits occupied by the inhibitor (in Fig. 7A, \(i = 0\) for the first column, \(i = 1\) for the second column, etc.). As discussed above only some of the microstates would lead to hydrolysis. For example, if three neighboring subunits are required to bind ATP before one of them is hydrolyzed, then only the configurations, highlighted in gray, will be active. Fig. 7B shows the predicted inhibition curves for several model cases. In the “trimer of dimers” model ATP is hydrolyzed by every other subunit, in a fashion similar to F\(_r\)-ATPase mechanism (12). The model “one in the row” describes ATP hydrolysis going in sequential steps around the P4 hexamer with only one of binding sites participating in ATP hydrolysis at a time (i.e., no cooperativity). “Two in the row” model requires that two neighboring subunits cooperate in ATP hydrolysis, whereas the rest of the hexamer may bind inhibitor without any loss of activity. In analogy, the “three in the row” model assumes cooperation of three neighboring subunits. An extreme case is the concerted ATP hydrolysis model of simultaneous binding and hydrolysis of all six ATPs, which was recently proposed for the SV40 large T antigen (13).

The experimental data obtained for P4 and AMP-PNP as inhibitor compared well with the three in the row model, whereas ADP inhibition was approximated by the two in the row model (Fig. 7B). This implicates that to get a catalytically active complex three binding sites in the row need to be occupied, two of which must be ATP, whereas one can be ADP. The remaining three subunits are dispensable for hydrolysis and may bind AMP-PNP or ADP. Note that the model is a steady-state approximation and does not take into account effects of nucleotide binding kinetics. That might explain why the match between the experimental data and the model was not perfect (e.g., equilibrium dissociation constant of ATP and ADP are equal but rates of ATP association and dissociation might be different from those of ADP).

**DISCUSSION**

Comparison of \(\phi 12\) P4 and \(\phi 8\) P4—The packaging motor from a related bacteriophage \(\phi 8\) has been characterized previously (6) and allows for detailed comparison. Similarly to \(\phi 8\) P4, the \(\phi 12\) P4 has comparable affinity for ATP and ADP and exhibits RNA-induced cooperativity. Thus, both molecular motors are driven by the difference in cellular concentrations of ATP and ADP. In contrast to \(\phi 8\) P4, RNA binding to \(\phi 12\) P4 has no effect on the kinetics of nucleotide binding and release. ATP hydrolysis is the only step affected by the presence of RNA. Thus, the translocation step seems coupled to hydrolysis in \(\phi 12\). This is in agreement with the translocation mechanism...
inferred from the crystal structures, in which the largest changes were observed between the pre-hydrolysis (P4-AMP-CPP-Mg complex) and post-hydrolysis state (P4-ADP-Mg) (3).

Energetic considerations for ATP-driven motors have demonstrated that most of the free energy available throughout the catalytic cycle is released during ATP binding (14, 15). Thus, the translocation must be energetically coupled to ATP binding. Given that the power stroke of P4 is mechanistically coupled to the hydrolysis step, the ATP binding energy is either stored in a strained conformation of the enzyme or the energy is relayed between neighboring subunits using the cooperative mechanism proposed below.

An RNA pentanucleotide was able to stimulate φ12 P4 activity, whereas the RNA nonamer was required for φ8 P4 stimulation (Fig. 1C). φ12 P4 exhibits much lower RNA affinity and translocation processivity than φ8 P4 (Fig. 1, B and C). We suggest that these differences can be explained by the lower stability of the φ12 P4 ring, which in turn leads to spontaneous opening during translocation. Similar low processivity because of ring opening was observed for the bacteriophage T7 gp4 helicase (16). The spontaneous ring opening will also allow direct binding of short RNA oligonucleotides to the RNA binding site. On the other hand, φ8 P4 employs a ring opening mechanism for RNA loading (17). Consequently, this mechanism requires longer RNA oligonucleotides that bind simultaneously to the primary and secondary binding sites (18). Note that this difference applies only for P4 hexamers in solution. After attachment to the viral procapsid the ring is stabilized and the catalytic activity is regulated (2, 4).

The Origin of Cooperativity and ATP Hydrolysis Coordination—The most striking finding is that P4 exhibits steady-state hydrolysis cooperativity without ATP binding cooperativity. In other words, RNA binding, which induces cooperativity, has no

![Fig. 7. A, schematic of all possible microstates of the ATP and inhibitor bound to the P4 hexamer. Subunits that bind ATP are drawn as open circles, whereas crossed circles represent subunits binding the inhibitor. Configurations with the gray background are able to hydrolyze ATP in the three in the row model. B, simulated inhibition curves in the presence of 1 mM ATP and increasing inhibitor concentrations are represented by lines: one in the row model, solid line; two in the row model, dotted line; three in the row model, dashed line; trimer of dimers model, dash-dot line; concerted hydrolysis model, dash-dot-dot line. The closed circles represent the measured experimental data for AMP-PNP and the open circles for ADP as the inhibitor, respectively. Each point is an average of three independent measurements (0.1 μM φ12 P4, 1 mM poly(C), standard buffer, 28 °C).](image)

![Fig. 8. Model of ATP hydrolysis cooperativity. A, mechanism of ATP hydrolysis in the absence of RNA. Blue squares represent P4 subunits of the hexamer unraveled in the plane. The red symbols designate the nucleotide-binding sites occupied by ATP. The double-headed arrows indicate independent stochastic binding to the nucleotide sites. The lower line shows the randomly attained, three in the row, configuration that permits hydrolysis at the middle site (orange). Note that hydrolysis also requires a correct conformation of the key side chains from the neighboring subunits (e.g. arginine fingers, Gln-278, Tyr-288). Arrows depict the proposed communication of strain between subunits. B, ATP hydrolysis in the presence of RNA. Triangular appendages correspond to the L2 loops. Green symbols mark the bound ADP, whereas the red symbols designate the required ATP molecules. Pink symbols designate the binding sites that may be occupied by ATP, inhibitor, or may be empty. The yellow line and circles represent the RNA sugar phosphate backbone. Arrows depict the proposed communication of strain between subunits.](image)
The stroke (6). In analogy to the F₁-ATPase (20–22) we envision in Fig. 8 the pentamer, which is the shortest oligonucleotide stimulating the state formation. According to the structural model the RNA becomes sequential in the presence of RNA. The sequentially hydrolyzed α in the presence of RNA causes lowering of $K_m$ to a value comparable with $K_a$ (Table I). This is because each hydrolysis cycle requires on average binding of only one additional ATP molecule. The stochastic binding mode dominates the $k_{cat}$ at low ATP concentrations ($\Delta [ATP] < K_a$) and hence the activity is low. At higher ATP concentrations the sequential coupling is efficient. Gradual switching between the two limiting modes leads to the apparent cooperativity.

Kinetic study of hexameric packaging motor P4 from bacteriophage φ12 revealed RNA-induced ATPase cooperativity. We propose a stochastic-sequential cooperativity model to describe the coordination of ATP hydrolysis within the hexamer in the absence of nucleotide binding cooperativity. In this model the apparent cooperativity is a result of hydrolysis stimulation by ATP and RNA binding to the neighboring subunits rather than cooperative nucleotide binding. The translocation step is coupled to ATP hydrolysis. Simultaneous interaction of neighboring subunits with RNA makes the otherwise random hydrolysis sequential and processive. Given the structural and sequence similarity between P4 and hexameric helicases this mechanism may apply to other members within this large family of molecular motors.

Acknowledgments—Denis Kainov is gratefully acknowledged for help with protein expression and purification and for motivating discussions. Prof. George Oster is thanked for stimulating discussions on the mechanism of energy transduction.

REFERENCES

1. Mindich, L. (1999) Microbiol. Mol. Biol. Rev. 63, 149–160
2. Kainov, D. E., Pirttimaa, M., Tuma, R., Butcher, S. J., Thomas, G. J., Jr., Bamford, D. H., and Makeyev, E. V. (2003) J. Biol. Chem. 278, 48084–48091
3. Mancini, E. J., Kainov, D. E., Grimes, J. M., Tuma, R., Bamford, D. H., and Stuart, D. I. (2004) Cell 118, 743–755
4. Kainov, D. E., Lisal, J., Bamford, D. H., and Tuma, R. (2004) Nucleic Acids Res. 32, 3515–3521
5. Mancini, E. J., Kainov, D. E., Wei, H., Gottlieb, P., Tuma, R., Bamford, D. H., Stuart, D. I., and Grimes, J. M. (2004) Acta Crystallogr. Sect. D 60, 558–580
6. Lisal, J., Kainov, D. E., Bamford, D. H., Thomas, G. J., Jr., and Tuma, R. (2004) J. Biol. Chem. 279, 1343–1350
7. Cornish-Bowden, A. (2004) Fundamentals of Enzyme Kinetics, 3rd Ed., Portland Press, London
8. Jeong, Y. J., Kim, D. E., and Patel, S. S. (2004) J. Biol. Chem. 279, 18370–18376
9. Zhang, W., Truttmann, A. C., Luthi, D., and McGuigan, J. A. (1997) Anal. Biochem. 251, 246–250
10. Paatero, A. O., Syvanja, J. E., and Bamford, D. H. (1995) J. Biol. Chem. 270, 6729–6734
11. Juuti, J. T., Bamford, D. H., Tuma, R., and Thomas, G. J., Jr. (1998) J. Mol. Biol. 279, 347–359
12. Senior, A. E., Nadanaciva, S., and Weber, J. (2002) Biochem. Biophys. Acta 1553, 188–211
13. Gai, D., Zhao, R., Li, D., Finkielstein, C. V., and Chen, X. S. (2004) Cell 119, 47–60
14. Antes, I., Chandler, D., Wang, H., and Oster, G. (2003) Biophys. J. 85, 695–706
15. Oster, G., and Wang, H. (2003) in Molecular Motors (Schliwa, M., ed) pp. 287–297, Wiley-VCH Verlag, Weinheim
16. Jeong, Y. J., Levin, M. K., and Patel, S. S. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 7204–7209
17. Lisal, J., Lam, T., Kainov, D. E., Emmett, M. R., Marshall, A. G., and Tuma, R. (2005) Nat. Struct. Mol. Biol. 12, 460–466
18. Skordalakes, E., and Berger, J. M. (2003) Cell 114, 135–146
19. Ricard, J., and Nozi, G. (1985) J. Theor. Biol. 117, 635–649
20. Oster, G., and Wang, H. (2000) Biochim. Biophys. Acta 1458, 482–510
21. Sun, S., Chandler, D., Diller, A. R., and Oster, G. (2003) Eur. Biophys. J. 32, 676–683
22. Wang, H., and Oster, G. (1998) Nature 396, 279–282