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Proton Transfer in the Mechanism of Polyadenylate Polymerase

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Abstract

Polyadenylate polymerase (PAP) is the template-independent RNA polymerase responsible for synthesis of the 3' poly(A) tails of mRNA. To investigate the role of proton transfer in the catalytic mechanism of PAP, the pH dependence of the steady state kinetic parameters of yeast PAP were determined for the forward (adenylyltransfer) and reverse (pyrophosphorolysis) reactions. The results indicate that productive formation of an enzyme-RNA-MgATP complex is pH independent over a broad pH range, but that formation of an active enzyme-RNA-MgPP_i complex is strongly pH dependent, consistent with the production of a proton on the enzyme in the forward reaction. The pH dependence of the maximum velocity of the forward reaction suggests two protonic species are involved in enzyme catalysis. Optimal enzyme activity requires one species to be protonated, and the other, deprotonated. The deuterium solvent isotope effect on V_{max} is also consistent with proton transfer involved in catalysis of a rate determining step. Finally, pK_a calculations of PAP were performed by the multiconformational continuum electrostatic (MCCE) method. Together, the data support that the protonation of residues K215 and Y224 exhibit cooperativity important for MgATP²⁻ and MgPP_i²⁻ binding/dissociation, and suggest these residues function in electrostatic, but not in general acid catalysis.

Keywords

Poly(A) polymerase; solvent deuterium isotope effect; nucleotidyl transfer; pH variation kinetics; cooperativity

INTRODUCTION

Polyadenylate polymerase (PAP) is the key enzyme in the synthesis of the 3' poly(A) tails of mRNA in eukaryotes [1–3], catalyzing the adenylyl transfer reaction:

Yeast PAP and many of the basal polyadenylation factors in yeast, which are associated in a multiprotein complex called the cleavage/polyadenylation factor (CPF), are conserved in higher eukaryotes, including humans [4, 5]. PAP is a member of the Pol X family of nucleotidyltransferases, which includes DNA pol β, many other eukaryotic, bacterial, and poxvirus poly(A) polymerases, CCA adding enzyme, terminal deoxynucleotidyltransferase (TdT), and various antibiotic nucleotidyltransferases [6]. PAP is also an important model system for template-independent nucleic acid synthesis in general, and particularly for a number of closely related enzymes including cytoplasmic poly(A) polymerases, polyU

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polymerases, and terminal uridylyl transferases, which are important for RNA editing and regulation of gene expression [7].

PAP contains three globular domains that move essentially as rigid bodies, the organization of which defines a large active site cleft [8, 9] (Figure 1A). Upon proper binding and recognition of both substrates, the enzyme adopts a closed, active conformation [8, 10]. PAP employs a two-metal (Mg^{2+}) ion mechanism [9, 11, 12], the essential features of which are common to all nucleic acid polymerases and many nucleotidyltransferases [13–16]. The active site, depicted in Figure 1B and C, is characterized by the arrangement of the two Mg^{2+} ions and three conserved acidic residues (D100, D102, and D154 in PAP); the conformation of the bound substrates are also shown. One metal binds as a co-substrate (as MgATP); the other, so-called catalytic metal coordinates both the substrate 3' hydroxyl group of poly(A) and a non-bridging oxygen of the α -phosphate of ATP. Both metals function by providing scaffolding for the reaction and by stabilizing the negative charges that develop on the reactants during catalysis [17]. Upon substrate binding and domain closure, two residues of the PAP middle domain, K215 and Y224, are brought into contact with the γ -phosphate of ATP [10]. Many polymerases have basic or polar uncharged residues that make similar interactions to the incoming nucleotide [18, 19], and the catalytic contribution of these interactions, other than contributing binding energy, is uncertain [20].

Yeast PAP is one of the few template-independent nucleic acid polymerases that have been subjected to extensive kinetic study [10, 21]. The steady state kinetic mechanism is rapid equilibrium random [21] and is described in Scheme 1, and kinetic parameters are defined in Table 1. The terms **A**, **B** and **P** refer to poly(A), $MgATP^{2-}$ and $MgPP_i^{2-}$, respectively; the terms that include E refer to enzyme forms to which poly(A) is bound in the product mode. Previous structural, kinetic, mutagenesis, and steady state fluorescence studies [9, 10] suggest the mechanism includes two rapid-equilibrium isomerization steps (K_{c1} and K_{c2}) that correspond to the conversion between open and closed conformations on either side of the central step. When MgATP is the substrate, the closed, active conformation is favored, whereas when incorrect nucleotides are utilized, this equilibrium favors the open conformation. However, K_{c2} is such that the open conformation is favored, which helps favor product dissociation over the back-reaction under physiological conditions. As a consequence of these isomerization steps, the observed steady state rate constants correspond to the rate constants of the central steps, V_1 and V_2 (Scheme 1), multiplied by the factor, F_c , corresponding to the fraction of enzymes present in the **EAB_(closed)** and **E'AP_(closed)** conformations, respectively. The K_{c1} and K_{c2} terms are also manifested in the K_m terms for the substrates and products, respectively, and can't be directly determined by steady state kinetic techniques [10]. Importantly, the central step actually represents a number of discrete steps involved in chemistry. Consequently, the overall rate of this step (V_1) is governed by one or more rate determining step(s) which may include activation of the nucleophile (i.e., deprotonation of the 3' hydroxyl of RNA), nucleophilic attack and phosphodiester bond formation, and/or P-O bond breakage to form pyrophosphate.

One outstanding question deals with the mechanistic significance of proton transfer during catalysis. Enzyme-catalyzed nucleotidyltransfer requires deprotonation of the 3' hydroxyl group of the nucleic acid substrate. Coordination of this hydroxyl to Mg^{2+} is widely presumed to lower its pK_a [17], and the deprotonation of this group may occur by general base catalysis (Figure 1C). The identity of the catalytic base is unknown, and recent computational studies on different enzymes have investigated several possibilities, including the Mg-coordinated water, a conserved aspartic acid (D154 in PAP), or the α -phosphate oxygen of the nucleotide substrate [22–26]. Additionally, other reaction steps lead to the reorganization of negative charge on the reacting molecules. These steps include charge development on the non-bridging α -phosphate oxygen of ATP in a phosphorane transition

state [13] or charge development on the α,β -bridging oxygen of ATP during bond breakage and subsequent product (PP_i) formation. Thus charge neutralization in the transition state is possibly an important catalytic strategy employed by these enzymes. It has also been suggested that proton transfer to pyrophosphate may promote departure of this leaving group [20].

To investigate the role of proton transfer and acid/base catalysis in PAP, the pH dependence of the steady state kinetic parameters of PAP was determined in both the forward and reverse direction in the pH range of 5.8 to 9.0. The maximal velocity in both directions is sensitive to solution pH, indicating that solvent protons are in equilibrium with groups at the active site in both the enzyme-substrate and - product (Michaelis) complexes. The pH dependence of the forward velocity (V_1) suggests the involvement of two protonic species, where the active enzyme form (i.e., that which undergoes the rate determining step) has one group protonated, and the other, deprotonated. Interestingly, these two species exhibit strong cooperativity, where binding of the first proton to the enzyme increases the affinity for the second proton. Solvent deuterium isotope effect experiments support that proton transfer is involved in catalysis of a rate determining step. The possible catalytic role of K215 and Y224, residues which interact with the MgATP substrate and MgPP_i product, was also investigated computationally by multiconformational continuum electrostatics (MCCE) calculations of PAP. The calculations support that these residues are fully protonated at physiological pH both in the presence and absence of substrates. From both the MCCE calculations and pH kinetic studies, it is concluded that these residues function in electrostatic catalysis, but not in general acid catalysis.

EXPERIMENTAL

Chemicals

Deuterium oxide (99.9% D) was from Cambridge Isotope Laboratories (Andover, MA). Luciferin and luciferase were purchased from Promega (Madison, WI). Oligo RNAs (2'-deprotected, PAGE-purified, and desalted) were purchased from Dharmacon (Lafayette, CO). Nucleotide concentrations were determined by UV absorbance using their published molar absorptivity values. RNA concentrations were determined using their theoretical molar absorptivity values. [2,8-³H]-ATP was purchased from PerkinElmer (Boston, MA) as a 50% ethanol solution. Prior to use, the ethanol was removed by applying a steady stream of air over the surface of the solution.

Poly(A) Polymerase

All experiments utilized a recombinant yeast PAP (62.4 kDa) that had a 32 amino acid C-terminal truncation and included a C-terminal His₆ tag to facilitate purification [21], referred to here as PAP. These modifications have been shown not to alter the catalytic properties of the enzyme [27]. The enzyme was purified by nickel affinity and ion exchange chromatographies, as described previously [21]. Protein concentration was determined by UV absorbance ($\epsilon_{280}^{0.1\%} = 0.99 \text{ L}\cdot\text{g}^{-1}\cdot\text{cm}^{-1}$).

Kinetic assays and pH-dependence studies

The initial rates of polyadenylation (adenylyltransfer) from the reaction of tritium labeled MgATP and an 18-mer oligoadenylate (A_{18}) were determined at 30°C using a discontinuous assay measuring the incorporation of ³H-adenylate into the acid-insoluble fraction as previously described [21]. The initial rates of pyrophosphorolysis from the reaction of MgPP_i and A_{18} at 30°C were determined by the previously described method [21] which measures the amount of ATP formed during the reaction by a discontinuous luciferin/luciferase assay [28]. Assay buffer conditions were 30 mM each bis-Tris/bicine, 1 mg/mL

BSA, 10% glycerol, 10 mM β -mercaptoethanol, 1 mM spermine-4HCl, 0.01% Nonidet-P 40, and 10 mM MgCl_2 , unless otherwise stated. In contrast to previous studies [21], additional NaCl was omitted, which we found to interfere with substrate binding. Substrate concentrations were varied depending on the experiment; these were: A_{18} (4 to 200 μM), MgATP (5 to 300 μM), and MgPP_i (10 to 250 μM). The reactions were initiated by the addition of 1–5 nM PAP, depending on the pH.

The pH dependence studies were conducted in the pH range of 5.8 to 9.0. To confirm the structural integrity of PAP over the pH range investigated in the kinetic studies, thermal denaturation studies of PAP, monitored by circular dichroism (CD) spectroscopy, were performed. The enzyme exhibited essentially identical CD spectra at 30°C from pH 5.8 – 9.0, and showed similar unfolding profiles (proteins unfolded above 40°C) independent of pH (not shown). For the kinetic experiments, a mixed buffer system containing equal parts bis-Tris and bicine was employed, which minimizes the variance of ionic strength due to changes in protonation of the buffer components. The pH was adjusted with HCl. The effective ionic strength, measured using an ion conductivity meter, varied by ~25% over this pH range. The new salt and buffer conditions employed in this study resulted in a small difference in the kinetic parameters compared with our previous studies [21], with the overall catalytic efficiency ($V/K_{ia}K_b$) of the enzyme 3.7-fold higher under the new, low salt condition (pH 7).

Solvent deuterium isotope effect (SIE) experiment

The kinetics of PAP in deuterium oxide was studied in a bis-Tris/bicine buffer system. The bis-Tris/bicine buffer in D_2O was prepared from the aqueous buffer that was adjusted to pH = 7.1 by evaporating the water, and redissolving in D_2O (repeated 3 $\times$). A control experiment measuring the activity versus pL profile ($L = \text{H}$ or D) under V_{max}/K_m conditions demonstrated that the pL of this experiment corresponds to the apparent pH or pD optimum (not shown). Stock solutions of MgCl_2 , spermidine-4HCl, BSA, and oligo- A_{18} were prepared in D_2O from their crystalline powders. Accounting for glycerol and other reagents not prepared in D_2O , the fraction deuterium of the reaction solution in D_2O was 0.83.

Consideration of MgATP and MgPP_i metal complexes and their ionization state

At physiological pH and Mg^{2+} concentrations, ATP and PP_i are present in solution predominantly as magnesium complexes of various protonation states. The concentrations of MgATP^{2-} and MgPP_i^{2-} given a fixed, total concentration of Mg^{2+} (10 mM) and as a function of pH were estimated using the program WinSGW [29] using literature values for the relevant dissociation constants [30, 31], and these calculations are provided as Supplementary Data (Figure S1). Unless stated otherwise, all references in this manuscript to ATP and PP_i refer to MgATP^{2-} and MgPP_i^{2-} , respectively, from these calculations. No additional corrections were made for the effects of other ions (Na^+ , K^+ , Cl^- , etc.) on these equilibria.

Computation of protein pK_a values by MCCE

To calculate residue pK_a values and determine the effect of explicit solvent molecules on protein electrostatics, multiconformational continuum electrostatics (MCCE) simulations on PAP were performed. A detailed description of MCCE is given elsewhere [32, 33]. Briefly, MCCE generates multiple conformers of a protein from a PDB file, where a conformer refers to protein having a particular set of side chain conformational and protonation states. MCCE then uses DelPhi [34] to calculate the reaction field energy and electrostatic pairwise interactions. Finally, the equilibrium distribution of these conformers as a function of pH is determined by Monte Carlo sampling. The pK_a and n_H values are derived by fitting the net residue charge versus pH function to the Henderson-Hasselbalch equation.

MCCE simulations of the free enzyme (open conformation) used PDB 1FA0, (molecule B) [9] from which all bound ligands (Mn^{2+} , nucleotides) were removed. MCCE simulations for the ternary complex (closed conformation) used a slight modification of PDB 2Q66 [10]. These modifications included inserting the D154 side chain and the second Mg^{2+} (both missing in the structure). Also, 5-mer oligoA was removed and the 3' terminal adenylate residue was represented by $\text{H}\cdot\text{AMP}^{1-}$ to account for electrostatic effect on other crystallographically observed residues. Partial atomic charges and atomic radii for ATP^{4-} and $\text{H}\cdot\text{AMP}^{1-}$ were taken from topology files generated by PRODRG [35], and the atomic radii for (tri)phosphate P (2.1 Å) and O (1.7 Å) were used [36]. In both simulations, multiple conformers were generated only for residues within 4 Å of the following active site residues: Y87, S89, D100, D102, D154, R186, K215, Y224, and N226. During the simulation, the relaxation (translational and rotational) of water molecules was allowed.

Kinetic models and data analysis

The steady state kinetic expression for a bireactant enzyme (using the nomenclature of Cleland) is given in Equation 1 [37]. Here, v is the measured initial velocity and V is the apparent maximal velocity, V_1 (forward) or V_2 (reverse). Throughout the manuscript, reactants are represented as follows: $A = A_{18}$, $B = \text{MgATP}^{2-}$, $P = \text{MgPP}_i^{2-}$. When pyrophosphorolysis was studied, Equation 1 was modified by substituting B with P , K_a with K_a' , and K_b with K_p . For competitive (product) inhibition experiments with MgPP_i^{2-} , data were fitted to Equation 2, where V is an apparent maximal velocity and K is an apparent constant. The Michaelis constants, defined in terms of the equilibria described in Scheme 1, are discussed in reference [21] and presented in Table 1.

$$v = \frac{V[A][B]}{K_{ia}K_b + K_b[A] + K_a[B] + [A][B]} \quad \text{Equation 1}$$

$$v = \frac{V[A]}{K(1 + [P]/K_{ip}) + [A]} \quad \text{Equation 2}$$

The initial rates, determined from plots of product formed versus time, at a given pH were simultaneously fitted by nonlinear regression to Equation 1 using the program, gnuplot. For regression analysis of the initial velocity data, each data point was weighted as a fixed percentage of its value. An average of 40 to 50 data points were used for each fit to Equation 1, and the standard deviations for the fitted parameters were estimated to be 5–15%. The errors for the kinetic parameters derived from this analysis, as reported throughout this manuscript, are asymptotic standard errors from gnuplot and approximately correspond to 85% confidence.

The pH-dependence of the kinetic parameters V , V/K_m , or $1/K_i$ was analyzed using the appropriate Equation 3–6, below [38], where Y_{obs} and Y are the pH-dependent and pH-independent parameters, respectively. The term, Y_{lim} , refers to a non-zero limit of the parameter, Y . For example, in Figure 5, Y_{obs} corresponds to the apparent V_1 determined at a given pH, Y is the pH-independent maximum velocity (V_1), and Y_{lim} is the limit of V_1 at high pH (fit to Equation 6). The “ n ” term in the denominator of Equation 4 is the Hill parameter, n_H , which is equal to 1 in the absence of cooperativity. In fitting to Equations 3–6, the kinetic parameters (Y_{obs}) were weighted by their standard errors derived from fits to Equations 1 or 2.

$$Y_{\text{obs}} = \frac{Y}{1 + 10^{(\text{pK}_{a1} - \text{pH})} + 10^{(\text{pH} - \text{pK}_{a2})}} \quad \text{Equation 3}$$

$$Y_{\text{obs}} = \frac{Y}{1 + 10^{n(\text{pH} - \text{pK}_a)}} \quad \text{Equation 4}$$

$$Y_{\text{obs}} = \frac{Y_{\text{lim1}} + Y_{\text{lim2}}(10^{(\text{pH} - \text{pK}_a)})}{1 + 10^{(\text{pK}_a - \text{pH})}} \quad \text{Equation 5}$$

$$Y_{\text{obs}} = \frac{Y + Y_{\text{lim}}(10^{(\text{pH} - \text{pK}_{a2})})}{1 + 10^{(\text{pK}_{a1} - \text{pH})} + 10^{(\text{pH} - \text{pK}_{a2})}} \quad \text{Equation 6}$$

RESULTS and DISCUSSION

Kinetic pH dependence studies of PAP

pH variation studies of enzyme activity are useful in determining the role of protonic groups in substrate binding and catalysis [38]. Previous work has established that PAP exhibits a rapid equilibrium random substrate binding mechanism [21]. For this type of mechanism, the $1/K_i$ terms correspond to the equilibrium formation constants of the enzyme substrate (or product) binary complexes, and V/K_m terms correspond to the apparent second-order rate constants for reaction of a particular substrate or product when the other is saturating [39]. The pH dependence of these parameters will reflect the importance of protonic species in the formation of productive binary ($1/K_i$) or ternary (V/K_m) enzyme complexes [38]. Previous studies of PAP [10] have demonstrated that the K_m terms, K_a and K_b , are also functions of the equilibrium constant for the formation of the closed enzyme conformation, K_{c1} (Scheme 1), in addition to substrate equilibrium dissociation constants; analogous relationships exist for K_p , K'_a , and K_{c2} for the reverse reaction. The pH dependence of V_1 and V_2 will generally reflect protonic groups important in the formation or stabilization of a transition state of a rate-determining step. To understand the mechanistic role of protonic groups in PAP catalysis, the steady state kinetic parameters for both the forward and reverse reactions were determined in the pH range of 5.8 to 9.0. In this work, initial rates were determined at various substrate concentrations and pH values. The initial rate data at a particular pH were simultaneously fit to Equations 1 or 2, as described in the previous section, to determine the steady state kinetic parameters at that pH. The apparent pK_a values were then determined from the pH-dependence of these kinetic parameters using Equations 3 – 6, as described below. These results (Figures 2–7) are discussed below, and the complete tabulated results are reported as Supplementary Data (Table S2).

Poly(A) binding

The pH dependence of the parameters for poly(A) binding in the forward direction is shown in Figure 2. The K_{ia} term, which measures poly(A) binding to the free enzyme, is pH dependent. Since polyadenylate has no titratable groups in this pH range, the dependence is certainly due to titration of an enzyme group. This group has an apparent pK_a of 7.20 ± 0.25 , where the protonated enzyme form binds tighter, and the K_{ia} exhibits limiting values of 36.4 and 181 μM with standard errors of about 20%. In contrast, the plot of V_1/K_a is essentially

invariant with pH in the range between 6.5 and 8. Because of the slight curvature of the plot at high and low pH, the V_1/K_a data were fitted to Equation 3, which yields pK_a values of 5.56 ± 0.14 and 9.20 ± 0.16 , and a maximum rate constant of $5.39 \times 10^5 \text{ M}^{-1} \text{ sec}^{-1}$ ($\pm 5\%$).

The previous crystal structure [10] and the MCCE calculation performed in this study indicate that none of the amino acids directly involved in RNA binding have a pK_a near neutrality. We suspect the pH dependence of K_{ia} is due to the ionization of charged residues located on the inner surface of the active site cleft that do not form direct contacts with poly(A). This is supported by the MCCE calculation for the free enzyme for two such amino acids: H375, $pK_a = 6.96$, and K385, $pK_a = 7.52$. Under conditions of MgATP saturation (corresponding to the V_1/K_a condition), domain closure is favored upon poly(A) binding, consistent with previous studies [10]. As a result, the apparent pK_a of groups that affect poly(A) binding would be perturbed because the acid dissociation equilibrium is coupled to the equilibrium for domain closure, K_{c1} , and this explains the absence of the pK_a near 7 in the V_1/K_a plot. Given that PAP is tethered to the pre-mRNA by virtue of its association with RNA-binding proteins in the context of the CPF [5], the effective poly(A) concentration is large and constant under cellular conditions, thus the pH dependence of poly(A) binding is most likely not physiologically relevant.

MgATP binding

The pH dependence of the kinetic parameters for MgATP binding is shown in Figure 3. The K_{ib} term is essentially pH independent at low pH, but gradually increases ($1/K_{ib}$ decreases) at higher pH. Consequently, the data were fitted to Equation 4, and a $1/K_{ib}$ (limit) of $0.027 \pm 0.003 \mu\text{M}^{-1}$ (or $K_{ib} = 37 \mu\text{M}$), an apparent pK_a of 8.36 ± 0.17 , and a Hill coefficient (n_H) of 0.71 ± 0.18 were determined. A Hill coefficient of less than one suggests negative cooperativity in proton binding between two or more protonic groups involved in MgATP binding. Here, negative cooperativity occurs when the protonation of one group antagonizes the protonation of the second group. The flatness of the plot at low pH is consistent with the notion that the MgATP^{2-} species is the actual substrate and validates the calculation made to correct for the pH dependence of $[\text{MgATP}^{2-}]$, as described in Supplementary Data.

The V_1/K_b term is invariant near neutral pH, but decreases at high and low pH, similarly to the V_1/K_a term (Figure 2). Fitted to Equation 3, the data yield pK_a values of 6.12 ± 0.28 and 9.07 ± 0.30 , and a maximum rate constant of $8.41 \times 10^5 \text{ M}^{-1} \text{ sec}^{-1}$ ($\pm 15\%$). Note that at pH 5.81, the low point on the V_1/K_b plot corresponds to a high point on the $1/K_{ia}$ plot (Figure 2), suggesting formation of nonproductive complexes at low pH; these points were omitted in the analysis. The similar pH dependence of both V_1/K_a and V_1/K_b suggests these terms reflect a common step in Michaelis complex formation, such as a conformational change that occurs after initial ternary complex formation, rather than either MgATP or poly(A) binding, explicitly.

Product binding

The pH dependence of the kinetic parameters for product binding was determined by kinetic studies of the reverse (pyrophosphorolysis) reaction, and the data are shown in Figure 4. The term, K_{ip} , similarly to K_{ib} , appears to increase ($1/K_{ip}$ decreases) gradually at high pH values. Accordingly, the data were fitted to Equation 4, and $1/K_{ip}$ (limit) of $0.20 \pm 0.12 \mu\text{M}^{-1}$, an apparent pK_a of 6.77 ± 1.5 , and a Hill coefficient (n_H) of 0.39 ± 0.19 were determined. This suggests strong negative cooperativity between two or more protonic sites involved in pyrophosphate binding. The observation that K_{ip} approaches a limiting value at low pH is consistent with MgPP_i^{2-} binding to the enzyme as a substrate in the reverse reaction and substantiates the pH correction in determining $[\text{MgPP}_i^{2-}]$ (Supplementary Data). Furthermore, it is unlikely that the observed decrease in $1/K_{ib}$ and $1/K_{ip}$ above pH 7 is due

to an error in calculating the concentrations of MgATP^{2-} and MgPP_i^{2-} , respectively, since the calculated concentrations of these species vary by only 2 and 12 %, respectively, in this pH region, which is insufficient to account for the larger variation in the K_i terms.

Both, V_2/K'_a and V_2/K_p are strongly pH dependent in the pH region tested (Figure 4), exhibiting higher values at low pH. The V_2/K'_a data were fitted to Equation 4 ($n=1$), which yielded a pK_a of 5.96 ± 0.04 and a maximum rate constant of $3.23 \times 10^4 \text{ M}^{-1} \text{ sec}^{-1}$ ($\pm 5\%$). Non-linear regression analysis of the V_2/K_p data using Equation 4 gave excessively poor fits due to the scatter in the data. However, as shown in Figure 4, the data are adequately described by the model when the pK_a is set at 5.96, yielding a maximum rate constant of $1.17 \times 10^5 \text{ M}^{-1} \text{ sec}^{-1}$ ($\pm 15\%$). Because the reaction could not be properly sampled at $\text{pH} < 6$ where the curve is expected to flatten out, the extrapolated maximum values of V_2/K_m should be considered tentative. When the data are plotted on a logarithmic scale, it is evident they approach an asymptote having a slope ($\Delta \log[V_2/K_m]/\Delta \text{pH}$) of -1 , consistent with the consumption of a proton in the reverse reaction.

MCCE calculations support that K215 and Y224 exhibit cooperative proton binding and account for pH-dependence of K_{ib} and K_{ip}

In proteins, amino acids often exhibit acid-titration behavior that differs from the free amino acid due to several factors, including electrostatic effects from nearby residues or the availability of alternate conformations that will either stabilize or destabilize charge on that residue [40, 41]. In the crystal structure of the enzyme substrate ternary complex, direct contacts are observed between 215, Y224 and the γ -phosphate of MgATP [10], and upon reaction, this interaction would be maintained with MgPP_i in the product complex. Therefore, we hypothesized that the ionization of these residues might be cooperative and thus explain the pH dependence of K_{ib} and K_{ip} . This was investigated computationally by performing multiconformational continuum electrostatics (MCCE) calculations for the free enzyme and enzyme-substrate ternary complex [40, 41]. In the free enzyme (which determines the pH dependence of K_{ib} and K_{ip}), the results are, for K215: $\text{pK}_a = 10.50$, $n_H = 0.347$; and Y224: $\text{pK}_a = 11.96$, $n_H = 0.293$. The Hill coefficients of less than one indicate negative cooperativity and are consistent with the kinetic experiments. Because interactions involving K215 and Y224 promote MgATP and MgPP_i binding and stabilize the closed ternary complex, we propose that the observed negative cooperativity is important in weakening these interactions, thus promoting facile opening of the closed conformation and product dissociation between successive rounds of adenylyltransfer. A detailed account of the MCCE results is provided in Supplemental Data.

pH dependence of V_1 and V_2

The pH dependence of $V_{\max}$ generally reflects the protonation of groups involved in stabilizing the transition state of a rate determining step [38, 39]. Our previous work demonstrated that the conversion of central complexes, **EAB** and **E'AP**, is rate determining for PAP [21]. Furthermore, a modest thioeffect on $V_{\max}$ when [α -thio]-ATP is utilized as an alternative substrate further supports that the chemical step is rate determining [21], but does not exclude the possibility that other processes, such as local conformational changes, also limit the rate. Finally, kinetic studies of PAP mutants further suggest that the rate of the forward reaction (V_1) is limited, specifically, by conversion of **EAB**_(closed) to **E'AP**_(closed) since the large domain motion is rapid and K_{c1} favors domain closure [10].

The pH-sensitivity of the $V_{\max}$ terms, V_1 and V_2 , demonstrates that the substrate/product-bound active site is capable of proton exchange with bulk solvent. As shown in Figure 5, the forward rate constant, V_1 , increases as a function of pH to a sharp maximum around pH 7.1, and then decreases, which suggests two protonic species are important in enzyme catalysis,

where maximum activity corresponds to an enzyme form having one species protonated, and the other, deprotonated. The observation that V_1 approaches a non-zero limit at high pH suggests that the reaction may proceed, albeit less efficiently, by an alternative mechanism corresponding to an enzyme form in which both species are deprotonated. Thus, the data were fit to Equation 6; the two pK_a values correspond to *molecular* acid dissociation constants. Although the average of the two pK_a values were accurately determined to be 7.05, the parameters, V_1 , V_1' (non-zero asymptote at high pH), pK_1 and pK_2 , could not be unambiguously determined by fitting all parameters simultaneously. This is because the shape of the curve changes negligibly as a function of the pK_a values when $(pK_2 - pK_1)$ is very small or negative [38, 42]. However, by setting the pK_a values constant, then varying these manually (with the constraint that the average pK_a value is 7.05), and solving Equation 6 for the other parameters, limiting values for all parameters were defined where the goodness of fit (χ^2/ndf) no longer showed improvement upon subsequent manipulation of the pK_a parameters. This analysis yielded: $V_1 \geq 330 \text{ s}^{-1}$, $pK_1 \geq 7.5$, and $pK_2 \leq 6.6$; V_1' was determined to be $28 \text{ s}^{-1} \pm 15\%$. In this model, V_1 corresponds to the theoretical maximum rate of the active protonated enzyme form; the observed maximum velocity (near pH 7.05) is smaller because only a fraction of **EAB** complexes are present in this form under optimal conditions.

The analysis indicates pK_2 is less than pK_1 , which results in the narrow shape of the curve in Figure 5. As described earlier by Dixon [42], this general phenomenon is suggestive of cooperativity between protonic groups. In this case, cooperativity refers to the situation where the addition of the first proton to the enzyme increases the affinity for the second proton. Alternatively, the data were analyzed according to a model involving two independent groups (i.e. no cooperativity), and this result is shown by the dashed line in Figure 5. Here, the best fit results are $V_1 = 113.4 \pm 16.4 \text{ sec}^{-1}$, $V_1' = 14.4 \pm 11.5 \text{ sec}^{-1}$, $pK_1 = 7.0 \pm 0.1$, and $pK_2 = 7.6 \pm 0.1$. This model corresponds to a limiting case where the *microscopic* acid dissociation constants of the two titrating groups are identical [38, 42]. It is apparent that this model gives a significantly poorer fit to the data than does the cooperative model, as measured by the χ^2/ndf parameter (Figure 5 legend). Thus, we conclude the data support the cooperative model. The origin of cooperative proton binding in PAP catalysis is not understood. The significance of this phenomenon is discussed further in Supplementary Data.

The pH dependence of the reverse rate constant, V_2 , is shown in Figure 6. These data fit well to a simple bell-shaped curve (Equation 3) giving observed pK_a values of 6.14 ± 0.27 (pK_1) and 6.41 ± 0.23 (pK_2) and a maximum apparent V_2 of $4.70 \pm 1.92 \text{ sec}^{-1}$. The increase in V_2 as pH decreases is consistent with the V_2/K_m data (Figure 4), and likely corresponds to the protonation of the **E'AP** complex prior to catalysis of the reverse chemical step. The enzyme inactivation at low pH was also observed in preliminary studies of the reverse reaction at low, fixed concentrations of A_{18} and $MgPP_i$ (not shown) and may correspond to the decrease seen in the V_1/K_m plots (Figures 2 and 3) at low pH.

There is an important consideration in the analysis of the V_2 and V_2/K_m data. For the reverse reaction, the central step (V_2 in Scheme 1) is preceded by an unfavorable equilibrium (K_{c2}) corresponding to a conformational step [10]. Because $V_{2(\text{obs})} = F_c \times V_2$, where F_c is the fraction of enzymes in the closed, active conformation, any effect of pH on F_c (which cannot be ascertained directly by steady state measurements) will perturb the pH versus V_2 plot and give pK_a values different from the corresponding microscopic pK_a values. This suggests the possibility that protonation of the **E'AP** complex stabilizes the closed conformation of the enzyme-product complex. Further studies are necessary to test this hypothesis.

pH dependence of K_{eq}

The equilibrium constant of an enzyme catalyzed reaction can be calculated from the steady state kinetic parameters using the appropriate Haldane relationship [37]. For PAP, $K_{eq} = (V_1 K_p)/(V_2 K_b)$. In this study, low catalytic efficiency above pH 7 precluded the determination of the kinetic parameters for the reverse reaction. However, these parameters at higher pH values can be estimated from the best fit solutions to Equations 3 and 4, as presented in Figures 6 and 4, respectively. As shown in Figure 7, $\log(K_{eq})$ versus pH falls along a line with a slope of 1, consistent with the concomitant production of a proton with adenylyltransfer. Only data at or below pH 7.1 were used in fitting, as these values were directly measured. Demonstration that this relationship holds over the entire pH range is compelling evidence that the steady state data are well determined and that our data analysis is sound. Finally, we determined the pH-independent equilibrium constant, $K_{eq} = [MgPP_i^{2-}]/[MgATP^{2-}] = 5.9 \times 10^{-6}/[H^+]$, which is, to our knowledge, the first report of this constant for the nucleotidyltransfer reaction involving single-stranded nucleic acid.

Solvent deuterium isotope effect

Solvent deuterium isotope kinetic experiments can yield insight into the nature and importance of proton transfer events [43]. The steady state kinetic parameters of PAP in the forward direction were determined at 0.00 and 0.83 fraction deuterium at pH 7.1 and the equivalent pL, respectively. The data are presented in Table 2, and a subset of the data is shown in Figure 8. At 0.83 fraction D, there was a solvent isotope effect (SIE) of 1.6 on V_1 , and a SIE on all the substrate binding parameters (K_i and K_m). The SIE on V_1 is consistent with a normal isotope effect on proton transfer in the transition state [43] and is on par with the SIE seen for other DNA and RNA polymerases [20]. Some of the SIE on substrate binding can be rationalized by general effects of D_2O on solvent structure and/or ion stabilities. For example, D_2O often has a stabilizing effect on macromolecular interactions (protein-protein and protein-nucleic acid) due to increased hydrophobic effect from the higher structural order of D_2O [44]. Also, analogous to the relative stabilities of lyonium and lyoxide, the solvation of ions tends to be more favorable in H_2O [43]. The general solvent effects involving ion and macromolecular solubility are expected to be “medium effects,” i.e. the cumulative effect of many protons each having a very small SIE. Because of the small value of the SIE and the relatively high standard error (5–15%) of the fitted parameters, a proton inventory experiment was not done, and it remains unknown whether each SIE is derived from one or more protons. Interestingly, a recent proton inventory study on several pol α -type enzymes suggested that two protons were involved in the catalytic step [20]. In summary, the SIE effect on V_1 is consistent with the involvement of proton transfer in transition state stabilization.

Summary and discussion of kinetic data

The V_1/K_a and V_1/K_b terms reflect two factors: (1) the apparent second-order rate constant for association of each substrate with the appropriate binary complex, and (2) the probability that the ternary complex will proceed through catalysis. In the forward reaction, formation of the **EAB** ternary complex is essentially pH independent near neutrality ($V_1/K_a = 5.4 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ and $V_1/K_b = 8.4 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$, Figures 2 and 3). These values are lower than the diffusion limit of $10^8 \text{ M}^{-1} \text{ s}^{-1}$ by about two orders of magnitude, suggesting that only a small proportion of **EAB** complexes are capable of further reaction. The pH dependence of V_1 also suggests that, at the pH optimum, a relatively small fraction of enzymes are in an active protonation state [compare the observed maximum rate (60 s^{-1}) with the theoretical maximum rate (lower limit 330 s^{-1}) determined from the fit of the data to Equation 6]. This result can be explained by the equilibration of protonic groups in the enzyme ternary complex prior to catalysis of the rate determining step. In the forward reaction, the observed rate is limited by the proportion of enzymes present in the active protonation state. In the

reverse reaction, V_2/K'_a and V_2/K_p , are both strongly pH dependent (Figure 4), indicating the requirement of proton binding for either productive formation of the **E'AP** complex, or for catalysis of the first committed step.

The pH dependence of V_1 gives evidence of two protonic species present on the enzyme substrate complex, where optimal enzyme activity requires one species to be protonated, and the other, deprotonated. Of course, these species cannot be identified solely by kinetic studies. During nucleotidyltransfer, general base catalysis may be employed in deprotonation of the substrate 3'OH. Also, general acid catalysis or hydrogen bond donating groups may neutralize negative charge that develops on the attacking 3'O nucleophile, on the phosphorane intermediate, and/or on the pyrophosphate leaving group [13, 24, 45]. Our hypothesis is that efficient catalysis requires the organization of the active site environment such that these developing charges are stabilized in the transition state. It is probable that the $pK_a \geq 7.5$ corresponds either to the deprotonation of the 3'OH group of the enzyme-bound poly(A) substrate or a general base that accepts this proton during catalysis. This value can be compared with the expected pK_a of 8–10 for a Mg-coordinated water in an enzyme [39]. A recent study [46] of a related enzyme, DNA pol β , reported an apparent pK_a of 8.1 for the enzyme-bound 3'OH, which is comparable to the value reported here. The data also reveal a second species ($pK_a \leq 6.6$) that must be protonated for optimal catalysis. The identity of this group is less certain as it is not apparent from the structural data that any active site residues are poised to participate directly in proton transfer to these substrate atoms during catalysis. Instead, the PAP structure [PDB code 2Q66] [10] indicates a number of buried solvent molecules (Figure 1B) that form specific interactions with substrates, particularly with the triphosphate moiety of ATP, shown schematically in Figure 9. These include two solvent molecules that interact with the α,β -bridging oxygen of ATP and one that interacts with the pro- S_p oxygen atom of the α -phosphate of ATP. Note the backbone amide groups of N226, G233, and V234 position these solvent molecules in a manner that would favor hydrogen bond donation to the triphosphate/pyrophosphate groups. These considerations lead us to speculate that protons may be localized on substrate atoms and/or explicit solvent molecules during catalysis. Exchange of these protons with bulk solvent explains the pH dependence of the kinetic parameters in this study. To substantiate this hypothesis, additional high resolution structures, particularly of the enzyme-substrate and enzyme-product complexes with both Mg^{2+} ions present, are necessary. This will identify any additional ordered solvent molecules near the 3'OH, and determine whether significant structural reorganization of these solvent molecules occurs as the enzyme reaction progresses. A similar mechanism was recently proposed for *Sulfolobus solfataricus* DNA polymerase IV (Dpo4), which also has a solvent-containing active site, wherein proton transfer between explicit water molecules and substrates is hypothesized to occur [26].

The role of K215 and Y224

Others have noted that positively charged active site residues are a common feature of nucleic acid polymerases and are proposed to be involved in catalysis [18, 19]. Here we conclude the pH dependence of K_{ib} (Figure 3) and K_{ip} (Figure 4) reflects cooperative titration of this pair in the free enzyme, consistent with the MCCE calculation. Mutational studies previously showed that the primary function of both residues is to provide structural interactions that stabilize the closed enzyme conformation in addition to substrate and product binding (the Y224F and K215A mutants both exhibited weaker binding of $MgATP^{2-}$ and $MgPP_i^{2-}$). Importantly, K215A showed only a (4.5-fold) effect on V_1 , where Y224F had no effect [10]. This effect is small when compared to the catalytic effects (10^1 – 10^3 -fold) observed upon mutation of supposedly analogous charged residues in other enzymes, notably residue R183 in DNA pol β [47] and K560 in RB69 DNA polymerase

[48]. This suggests that different polymerases may employ distinct mechanisms for proton transfer and/or charge stabilization during catalysis.

We also considered that either K215 or Y224 function as a general acid, consistent with a recent proposal that proton transfer to the pyrophosphate leaving group generally occurs in DNA and RNA polymerases [20]. In such a mechanism, activity in the reverse (pyrophosphorolysis) direction would require the conjugate base form of this residue to accept a proton from MgHPP_i^{1-} . Our results, which show an increase in pyrophosphorolysis activity with a decrease in pH, appear inconsistent with this proposal. Furthermore, the MCCE calculations suggest K215 and Y224 are predominantly protonated below pH 10. From the kinetic data, it is apparent that MgPP_i^{2-} combines readily with the enzyme in the pH range tested. Therefore, in the reverse reaction, the active form of **E'AP** can easily result from combination of poly(A), MgPP_i^{2-} , and an additional proton with the K215- NH_3^+ /Y224-OH form of the enzyme. It is reasonable to hypothesize that, in the forward reaction, pyrophosphate formation on the enzyme is promoted by charge neutralization, but this could occur by electrostatic catalysis or by proton transfer from another site, such as an ordered solvent molecule.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. PAP structure and active site

(A) The domain structure of PAP. The arrangement of the N-, middle, and C-terminal domains, which defines the active site cleft, is depicted. The crystal structures of the open enzyme (PDB 1FA0, *light grey*) and of the closed, ternary complex (PDB 2Q66, *dark grey*) are superimposed with respect to the middle domain to show the open-to-closed domain motion; the trajectory of the N-terminal domain motion is shown by the arrow. The 3' deoxy-nucleotides were removed from 1FA0. The positions of bound Mg²⁺ (*purple*), ATP (*red*), and RNA (*blue*) from 2Q66 are indicated. (B) The active site structure of the closed PAP-MgATP-RNA complex (PDB code 2Q66). The nucleotide is positioned in the active site such that the γ-phosphate of ATP is located nearest to the surface of the enzyme. The arrow indicates the trajectory of nucleophilic attack by the 3' OH. The positions of the ATP-bound Mg²⁺ (*purple*) and several explicit solvent molecules (*aqua*) are shown. Asp154, which was mutated to Ala, and the second (catalytic) Mg²⁺ are absent in the structure. (C) A model of the active site, with the second Mg²⁺ and Asp154 added, shows the organization of the two metals, three conserved aspartic acids, and the bound substrates (R₁ = riboadenosine [of ATP], R₂ = polyA). In the mechanism of nucleotidyltransfer, the 3' hydroxyl group is deprotonated (a hypothetical base, X, is depicted), then serves as a nucleophile in the attack on the α-phosphorus atom of ATP.

Figure 2. pH dependence of kinetic parameters of the adenylyltransfer (forward) reaction: poly(A) binding

The parameters V_1/K_a (●) were fit to Equation 3; and $1/K_{ia}$ (○), to Equation 5. The data point in parentheses was not included in the analysis. The curves represent the best fit to the data.

Figure 3. pH dependence of kinetic parameters of the adenylyltransfer (forward) reaction: MgATP²⁻ binding

The parameters V_1/K_b (●) were fit to Equation 3; and $1/K_{ib}$ (○), to Equation 4 ($n_H = 0.71$). The curves represent the best fit to the data.

Figure 4. pH dependence of kinetic parameters of the pyrophosphorolysis (reverse) reaction
 The parameters V_2/K'_a ($\blacktriangledown$) were fit to Equation 4; V_2/K_p ($\bullet$), to Equation 4 (where pK_a was fixed at 5.96); and $1/K_{ip}$ ($\circ$), to Equation 4 ($n_H = 0.39$). The curves represent the best fit to the data.

Figure 5. pH dependence of V_1

The apparent V_1 data were fit to Equation 6, and the solid line represents the best fit ($\chi^2/\text{ndf} = 1.09$). Limiting values were estimated: $V_1 \geq 330 \text{ s}^{-1}$, $\text{pK}_1 \geq 7.5$, and $\text{pK}_2 \leq 6.6$. The dashed line represents the best fit to an alternative model where the constraint $(\text{pK}_2 - \text{pK}_1) = 0.6$ was imposed ($\chi^2/\text{ndf} = 3.00$); in this model, $\text{pK}_1 = 7.01$, $\text{pK}_2 = 7.61$. Error bars represent the standard error of V_1 from each data set from the fit to Equation 1.

Figure 6. pH dependence of V_2

The apparent V_2 data were fit to Equation 3; error bars represent the standard error of V_2 from each data set from the fit to Equation 1.

Figure 7. pH dependence of K_{eq}

The K_{eq} values at and below pH 7.1 (●) were calculated from the data of the kinetic analysis of both the forward and reverse reactions using the Haldane relationship: $K_{eq} = (V_1 K_p) / (V_2 K_b)$. The K_{eq} values above pH 7.1 (○) were calculated using the data from the analysis of the forward reaction and the calculated parameter, V_2/K_p , from the nonlinear regression analysis of the data shown in Figure 4. The line represents the best fit of the data *between pH 5.8 and 7.1* to the function, $\log(K_{eq}) = n \cdot \text{pH} + C$, where the slope, n , equals the number of protons consumed (n is negative) or generated (n is positive) in the reaction. Here, the slope (n) was fixed at 1; given this solution, $K_{eq} = 1$ at pH 5.23. When the slope was determined by linear regression analysis of the data *between pH 5.8 and 7.1*, $n = 1.08 \pm 0.06$.

Figure 8. Solvent deuterium isotope effect for PAP-catalyzed adenylyltransfer

Kinetic data were measured in aqueous (●) and deuterated (○, 0.83 fraction D) solutions at pH 7.1 or the equivalent pD, respectively. This subset of data demonstrates the SIE on the maximum velocity and substrate binding. The $[MgATP^{2-}]$ was $319.3 \mu M$ (roughly $3 \times K_m$), and the curves were generated using Equation 1 and the parameters listed in Table 2. Error bars represent the standard deviation from duplicate data sets.

Figure 9. PAP active site: position of crystallographic waters

A schematic view of the network of solvent molecules (W) at the active site and their relation to reactants and enzyme functional groups is shown. The heavy atom distances (Å) from PDB 2Q66 are given. The Mg-coordinated water, **W** (left), is depicted in bold. Not depicted are the heavy atom distances between the γ -phosphate and the side chains of S89 (2.71 Å), K215 (2.85 Å), and Y224 (2.55 Å), and the distance between the ϵ -amino nitrogen of K215 and the hydroxyl oxygen of Y224 (2.75 Å).

Scheme 1.
Steady State Kinetic Mechanism of PAP (proton independent).

Table 1

Steady State Kinetic Parameters of PAP

<u>Polyadenylation Direction</u>	<u>Pyrophosphorolysis Direction</u>
$K_a = K_4/(1 + K_{c1})$	$K_{a'} = K_6/(1 + K_{c2})$
$K_{ia} = \frac{1}{(1/K_1 + 1/K_7)}$	$K_p = K_5 \cdot (1 + K_7/K_1)/(1 + K_{c2})$
$K_b = K_3 \cdot (1 + K_1/K_7)/(1 + K_{c1})$	$K_{ip} = K_8$
$K_{ib} = K_2$	

Table 2

Solvent Deuterium Isotope Effect on Steady State Kinetics of PAP.

	0.00 D	0.83 D	H/D
V_I (sec^{-1})	60.8 ± 5.3	37.7 ± 2.3	1.61
K_{ia} (μM)	45.6 ± 7.4	26.2 ± 4.2	1.74
K_a (μM)	134.9 ± 16.8	95.7 ± 9.0	1.41
K_{ib} (μM)	28.8 ± 3.5	19.7 ± 2.6	1.46
K_b (μM)	85.3 ± 14.2	72.0 ± 9.1	1.18
$K_a K_{ib}$ (μM^2)	3885.1	1885.3	2.06
$V_I/K_a K_{ib}$ ($\text{sec}^{-1} \cdot \mu\text{M}^{-2}$)	1.56×10^{-2}	2.00×10^{-2}	0.78