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Exploring the mechanism of DNA polymerases by analyzing the effect of mutations of active site acidic groups in Polymerase β

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Abstract

Elucidating the catalytic mechanism of DNA polymerase is crucial for a progress in the understanding of the control of replication fidelity. This work tries to advance the mechanistic understanding by analyzing the observed effect of mutations of the acidic groups in the active site of Polymerase β as well as the pH effect on the rate constant. The analysis involves both empirical valence bond (EVB) free energy calculations and considerations of the observed pH dependence of the reaction. The combined analysis indicates that the proton transfer (PT) from the nucleophilic O3' has two possible pathways, one to D256 and the second to the bulk. We concluded based on calculations and the experimental pH profile that the most likely path for the *wild-type* (WT) and D256E and D256A mutants is a PT to the bulk, although the WT may also use a PT to Asp 256. Our analysis highlights the need for very extensive sampling in the calculations of the activation barrier and also clearly shows that *ab initio* QM/MM calculations that do not involve extensive sampling are unlikely to give a clear quantitative picture of the reaction mechanism.

Graphical Abstract

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I. Introduction

The accurate action of DNA polymerases (pol) is essential for the replication of all life forms. [1] These enzymes catalyze the bond formation of phosphodiester by many orders of magnitude relative to the corresponding reaction in water.[2] Elucidating the nature of this rate acceleration and reaching an understanding of the detailed reaction mechanism are essential for a clear analysis of the origin of replication fidelity. A significant progress has been made by experimental (e.g., refs. [1–3]) and theoretical (e.g., refs. [4–7]) studies of the nature of the chemical steps, as well as considerations of the possible roles of conformational changes (e.g., refs. [5, 8]) and protein motions.[9] However, the quantitative advances in these key areas are still at a relatively early stage (for general review see ref. [10]).

Recent experimental studies [11, 12] that explored the effects of mutations of the acidic groups in the active site of Pol β (Fig. 1) have provided (at least in principle) possible clues about the reaction mechanism. Here we focus on analyzing the effects of these mutations and on relating it to the reaction mechanism. A part of our analysis considers the assumption [11, 12] that the mechanism of Pol β must involve a proton transport (PT) to D256 or E256 and that this is established by the pH profile and structures of the WT and the mutants. We note that this appear to be a clear implicit assumption, although ref. [11] mentioned the examination of another option but obtained for it extremely high barrier (see discussion section) and thus considered in details only the PT to D256. In our view, it is unjustified to restrict the detailed considerations to mechanisms that involve PT to D256 and it is absolutely essential to also consider in a quantitative way the likely mechanism of a proton transfer to the bulk (especially in some of the mutants). In the present analysis we keep in mind the factors that determine the effective pK_a of the proton donor and acceptor, which ultimately determine the barriers for the overall reactions.

It seems to us that a careful analysis of the mutational experiments must include attempts to evaluate the energetics of the different feasible mechanisms, as well as attempts to examine the meaning of the corresponding pH profiles. Thus we like to examine the validity of the assumptions, made by Refs. [11] and [12] in their analysis of the effect of mutations of the active site acidic groups, that we have a PT to 256 (see above). However, our main focus is on trying to increase the consistency of the analysis of the proton transfer steps. In doing so, we will consider the effects arising from the mutation of the acidic groups (D256 or D192) [12]. We will illustrate that the effects of these mutations reflect the effect of the negative charge of the acidic residues, as well as the mutation induced changes in the position of the Mg_{cat} . Another point that will be considered in our analysis is the fact that ref. [12] found about 4 orders of magnitude change in the rate constant for the chemical step, k_{pol} , (where the corresponding change in activation barrier is about 5 kcal/mol) upon the D192E mutation and a larger barrier for the D192A mutant (not active) and assumed that this change is due in part to the interaction with Arg 258. In this respect, we note that reaching such a conclusion should require reliable quantitative calculations that could actually reproduce the mutational effects (see below). Thus, it is unjustified to accept the premise [11] that the amino acid substitutions explain the change in catalysis. Of course, the mutational experiments provide major and crucial information, whose interpretation is a major challenge. However, the interpretation and the corresponding calculations should reproduce the observed effects.

II. Computational Methods

II.1 General Considerations

Our evaluation of the free energy barriers has been based on using the method, a mixed quantum mechanics/molecular mechanics (QM/MM) method that has been described extensively elsewhere (e.g., refs. [13, 14]). This method describes reactions by mixing resonance states (or more precisely diabatic states) that correspond to classical valence-bond structures, which represent the reactant, intermediate (or intermediates), and product states.

The EVB approach evaluates the free-energy function g that is needed for the calculations of the activation energy, $g^\ddagger$, by using a free energy perturbation (FEP) /umbrella sampling (US) method which has been described in details elsewhere. [13]

The EVB calculations are calibrated using information about the reaction mechanism and potential surface of the corresponding solution reaction. In the case of the reaction catalyzed by Pol β , there is still debate (and some confusion) on whether phosphodiester hydrolysis reaction follows an associative, dissociative or concerted mechanism (these mechanisms are depicted in Figure 2). Thus we have emphasized for a long time the need for a careful analysis of the relevant experimental and theoretical information about this type of reaction as well as related phosphate monoester hydrolysis reactions in solution (e.g., refs. [10, 15]). Our studies demonstrated repeatedly that the available experimental information (including linear free energy relationships (LFER) studies) cannot tell what is the mechanism, but that all careful and consistent computational studies (that in fact reproduce the available experimental results) lead to associative and/or concerted surfaces, unless we deal with extremely strong leaving group. Our most careful recent ab initio solution study of phosphate diester hydrolysis [15] produced the surface shown in ref. [16], where the reaction

proceeds through a concerted path. Using a similar surface to calibrate our EVB we concluded previously[4] that an associative mechanism, with some concerted character, is the most likely reaction mechanism for correct nucleotide insertion. Thus for chemistry in the active site of Pol β , we assumed the reaction takes place in the three generic steps (see Figure 2); a proton transfer from the primer 3'OH is followed by in-line nucleophilic attack of 3'O⁻ to the dNTP α -phosphate, and the reaction is completed by the departure of the pyrophosphate leaving group (as discussed here, these steps can involve a concerted process). These generic steps are indicated in the Tables and Figures of this work as PT, NA, LGD, respectively. For the proton transfer step there are also several possibilities: the proton of the primer 3'OH could either transfer to Asp256, 5'-oxygen or a non-bridging oxygen of the dNTP α -phosphate, or a surrounding water. Based on previous simulations, it seems most likely that the dominant mechanism involves a proton transfer to Asp256 or to the bulk [4, 17] followed by nucleophilic attack on P $_{\alpha}$ by the deprotonated 3'OH and formation of a pentavalent intermediate in an associative/concerted mechanism. The concerted EVB surface calibrated on the ab initio solution reaction appears to reproduce the main features of this surface as shown in ref. [16].

The calculations of pKa were done by the PDLD/S-LRA approach which has been found to be very effective in studies of electrostatic energies in proteins [18].

II.2. Simulation conditions and protocol

The starting structures for the simulations were generated from the x-ray structures of human pol β on native (PDB: 2FMS) from ref. [19] and D256E (PDB: 4JWM) from ref. [11]. The simulated DNA-protein-substrate complex was immersed in 18 Å sphere of water molecules subject to the surface-constraint all-atom solvent (SCAAS)[20] type boundary conditions. The geometric center of the reactive atoms is defined as the center of the simulations sphere. Positions of all atoms beyond 24 Å (from the center of solvent sphere) were fixed at their crystallographic positions, and their nonbonded interactions with the atoms within the simulation sphere were turned off. The structure of the simulated system was first relaxed by 20 ps step MD simulation at 300 K with a step size of 1.0 fs. Harmonic position restraints of 200 kcal/mol Å² were applied initially to all the protein and DNA atoms. This allowed the water molecules to adjust in and around the protein environment. The initial simulation was then followed by another 20 ps simulation run, applying the 200 kcal/mol Å² harmonic constraint only on the protein atoms forming the first coordination shell of the two Mg²⁺ ions. The harmonic constraint on these atoms was gradually decreased in next twenty 10 ps step simulations. Finally the complex system was equilibrated for another 40 ps without any constraints with a step size 1.0 fs at 300 K. This served as a starting structure for the FEP calculations. The FEP mapping procedure involved the use of 21 frames (20 ps each) for moving along the reaction coordinate and all the simulations were done at 300 K with a time step of 1 fs. Thus we performed a total of about 500 ps simulation run for each system studied and in addition examined simulations that start from different starting points as well as longer simulations and obtained an error range of about 2 kcal/mol.

The EVB and FEP calculations were done with the LRF long range treatment[20] (which is equivalent to having no cutoff for the electrostatic interactions). All the simulations were

performed using the MOLARIS simulation package[20, 21]. The nucleic acid bases were represented by AMBER charges[22] and ENZY MIX vdW parameters (after validation that these parameters give reliable solvation energies and base-pairing energies and structures). A single center model with the vdW parameters $r^* = 1.30$ and $\epsilon = 0.06$ kcal/mol[4] was used for the Mg^{2+} ions.

The effect of the ionizable groups was taken into account as follows: (i) by performing EVB calculations where only the ionizable residues within the 27 Å region from the geometric center of the EVB atoms are ionized (the 27 Å distance completely satisfies the representation of the protein ionized groups as was demonstrated in a previous study of Pol β [23]), and then (ii) evaluating the effect of the ionizable groups beyond the 27 Å from the geometric center of the EVB atoms by using a macroscopic treatment with a large dielectric constant for charge-charge interaction.[18] The ionization states were evaluated by using our Monte Carlo (MC) approach. [18] The overall effect of the ionizable groups beyond the 10 Å region was found to be quite small (i.e., a change of ± 1.5 kcal/mol in the activation free energy).

The parameters for the stepwise mechanism were taken as the same parameters used in the evaluation of the free energy surface for the nucleotide transfer reaction of T7 pol.[4] The calibrated EVB surface for the solution reaction is depicted in Figure S1. The barriers and the reaction free energies associated with each step of the solution reaction were reproduced by adjusting the off diagonal element H_{12} and the gas phase shift α_0 as described in the study of T7 pol[4]. The corresponding EVB parameters obtained are given in Table S1.

Although the solution surface is most likely concerted,[16] we found out that we can obtain very similar results (see ref. [16]) with a stepwise surface that allow for significantly more efficient calculations and thus more effective sampling. More specifically, here we used a stepwise associative path which starts with an initial proton transfer to both bulk water and Asp 256. The corresponding EVB resonance structures are depicted in the figure 3. The atoms shown in the figures are those included in the EVB (QM) system. Both calibrated EVB surfaces were forced to reproduce the barrier of the solution surface and can be used for studies in proteins by replacing the solvent with the protein environment. The stepwise associative mechanism is described by seven different VB states (Figure 3): the reactant state (I), the intermediate containing both the nucleophile and Asp256 are deprotonated (II), the intermediate containing protonated Asp256 and the deprotonated nucleophile (V), the pentacoordinated phosphorane intermediates (III and VI), and the product states (IV and VII).

The modeling of the two mechanistic options is described in details in the results section below.

As stated above, the pKa calculations were performed with the PDLD/S-LRA approach [18], with the standard MOLARIS procedure [20, 21], averaging the PDLD/S-LRA results over 20 configurations generated from 10 ps runs. The calculations were done with several values of ϵ_p to take into account missing relaxation.

III. Results and Analysis

III.1 Exploring the Proton Transfer Mechanism

One of the central questions addressed by this paper is the nature of the proton acceptor in Pol β . In our previous studies of T7 pol and Pol β , we compared the energetics of several alternative proton acceptors in selecting the most likely mechanism (that appeared to be a PT to the bulk). Thus we find it unjustified to follow the approach of ref. [11] and to assume that the proton acceptor is D256. The nature of the proton donor should be judged by both the observed pH profile and free energy calculations, as well as the consistency between both sets of results. Of course, in judging the likelihood of different mechanistic options we may also get hints from the structural changes upon mutations. We also like to note that, in considering the energetics, we have to determine the total chemical barrier and not only the energetics of the step after the proton transfer.

III. 1a. Energy considerations—The free energy profile for the PT to the bulk and the PT to D256 were evaluated by PDL/D-S-LRA and EVB calculations respectively. The calculations indicate that, for the native enzyme, the PT to bulk is energetically favored, as was found previously in ref. [16] (see also Figure 4). However, as will be discussed below, the likelihood of the PT to the bulk should be examined more carefully, looking for the upper limit of the energy of the PT step. The PT to the bulk mechanism was also considered in the evaluation of the free energy profile of the D256E, D256A, and D192A mutants. The details of our analyses of each case are provided below.

In trying to evaluate the energetics of a PT to the bulk, we focused on the proton transfer reaction energy, $G_{PT,bulk}$, rather than on the activation energy, $G_{PT,bulk}^\ddagger$, since this step is not likely to be the rate limiting in the nucleotide incorporation reaction of Pol β (see refs. [5] and [24], and the discussion below). Thus it is sufficient (in most cases) just to calculate the $G_{PT,bulk}$, which in turn requires calculating the pK_a shift of the proton donor.

The pK_a shift of the ribose 3'OH group in the protein satisfies the relationship[16]

$$\Delta pK_a = \Delta \Delta G_{sol, OH \rightarrow O^-}^{wat \rightarrow pro} / 2.303RT \quad (1)$$

$G_{PT,bulk}$ is then calculated as

$$\Delta G_{PT,bulk} = 2.303 RT (pK_{a,3'OH} - pH) \quad (2)$$

The pK_a of the ribose 3'OH in water is taken as 13.[25] Here $pK_{a,3'OH} = 13 + pK_a$ and $pH=7$ are used in all the calculations.

The calculations of the pK_a of the 3'OH can be performed by ab initio QM/MM FEP and by the EVB approach. However, the PDL/D-S-LRA approach [18] is very effective in pK_a calculations and was used here. For the PT to the bulk in the WT, at $pH=7$, we obtained a pK_a between 9 and 10.9 (for ϵ_p of 4 and 8, respectively, where the use of different values of

ϵ_p , reflects the missing effect of complete convergence of the protein structure and missing water penetration effect). Using Eq. 2 we obtain $G_{PT,bulk}$ of 2.8–5.5 kcal/mol. These calculated results can be compared to the observed effective pK_a of 8.1 in the protein (see ref. [11]), where Eq. 7 gives $G_{PT,bulk} = 1.38 \cdot (8.1 - pH) = 1.5$ kcal/mol. Thus the calculated and observed values are close, although in the case of the WT it is not completely certain (see below) that the experimental pK_a corresponds to the pK_a of the 3'OH. The PT free energies for the mutants are analyzed in a similar way and are given in Table 1.

In order to establish our assertion that in the case of PT to the bulk we do not have to consider the PT barrier (since it is not rate limiting) we examined a PT to the bulk from 3'OH through a chain of water molecules (see Fig. 5). As established in many of our works (e.g., refs. [5], [24]) the barrier is not much different than the endothermicity (as long as the donor and acceptor are not separated by more than 4 Å). Thus it is enough to determine the PT energy considering the free energy of moving the proton to the different sites of the proton conducting chain. Now, for PT from 3'OH to the bulk, we have to consider a PT along the chain $(OH^-)_{bulk}H_2O_{(n)}-H_2O_{(3)}H_2O_{(2)}H_2O_{(1)}(3'OH)$, starting with a PT from $(OH^-)_{bulk}$ to a surface H_2O ($H_2O_{(n)}$) and then a PT along the chain until we reach the PT from 3'OH to OH^- on site 1. With this in mind, we evaluated the free energy of PT to the bulk from the key sites obtaining 6.4, 13 and 13 kcal/mol for sites 1, 2 and 3. This yields around 14 kcal/mol barrier for PT to the bulk (we assumed that the barrier is pushed up above the PT energy by about 3 kcal/mol and then pushed down by about 2 kcal/mol once the calculations include an off diagonal EVB coupling (see related work in ref. [26])). We also examined the PT from a protonated D256 to a nearby OH^- (taken as the OH^- in site 1) and obtained about 1 kcal/mol barrier. If this path is being used we get an effective free energy of PT to the bulk through D256. However, what is needed in this case is the total free energy of the PT through the given chain (rather than the barrier) and in this case the lowest free energy is PT to the bulk (regardless that the path involves PT through D256).

In addition to the PT to the bulk a major candidate for being the proton acceptor is D256, and this option should be explored. Arguably, at present, the most rigorous calculation of the PT step from the 3'OH to a Mg bound OH^- in pol β has been performed by the FDFT study reported in ref. [5] (giving about 5 kcal/mol free energy), as this study involved ab initio calculations with a converging in the free energy mapping. However, this approach is very expensive and, as much as PT between groups in proteins is concerned, we believe that the calibrated EVB provides arguably the most reliable tool, (e.g., ref. [26]).

At any rate, in order to explore PT from 3'OH to D256 by the EVB approach, we used a reference reaction that considered the proton transfer from methanol to an acetate ion in water. In the corresponding calculations we also included the catalytic Mg^{2+}_{cat} ion. The Mg^{2+}_{cat} ion was constrained to the observed position in the protein by a force constant of 10.0 kcal/mol Å² while applying a force constant of 1.0 kcal/mol Å² to the rest of the reacting atom in the model solution reaction. However, no constraints were used in the protein calculations. It should be noted that our calculations can be done while considering the pK_a 's of the isolated 3'OH and D256, but such calculations must also reflect the effect change of the pK_a of the donor (or acceptor) due the change in ionization state of the second group. Thus we focused on comparing the actual PT profile in water and in the protein

(using the profile in water for the calibration of the EVB in the protein). The results of the calculations of the PT to D256 (or E256) and the bulk are summarized in Table 1, as well as in Figure 4.

III. 1b. Analyzing the pH effects—Obviously our calculations are not perfect due to convergence difficulties, in the complex electrostatic environment generated by the Mg ions. Thus it seems to us that current free energy calculations are not sufficiently reliable, and the most compelling and effective way of judging the mechanism is to look critically at the pH profile. More specifically, the pH dependence measured by Wilson and coworkers[11] is schematically shown in Figure 6. Now, if we assume that the mechanism involves a PT to the bulk, our overall barrier should follow $1.38 (\text{pK}_a(\text{OH}) - \text{pH})$, which is what we observed in D256A, where we clearly have a PT to the bulk. We also have a very similar observed dependence in D256E and even in some respects for the WT. Thus just looking at the pH profile we can conclude that the most likely mechanism is a PT to the bulk in the D256E and the D256A mutants. In the WT mechanism, it is harder to draw unique conclusions from the pH profile, which has smaller slope than that of D256A (see Fig. 6) and can be interpreted as having a combination of PT to D256 and PT to the bulk.

The above considerations lead us to conclude that the D256E mutant involves most likely a PT to the bulk. We note, however, that reproducing this effect by free energy calculations is quite challenging, since we have major compensating electrostatic effects due to the repulsion between the negatively charged E256 and the negatively charged 3'O-, where the proper relaxation requires very extensive sampling. Fortunately, in considering the action of the mutant the repulsion by the ionized Glu or the attraction by the Mg will partially disappear in the nucleophile attack step.

III. 1c. Proton Transfer through two water molecules—A mechanism of a PT to the phosphate has been considered in our previous works for phosphomonoesters (e.g., refs. [27, 28]). This type of mechanism can operate in polymerases[4] and a version of this mechanism has been used recently in a QM/MM energy minimization study of Pol β [7, 8], considering PT from the O3' to the O1 $_{\gamma}$ of pyrophosphate through two water molecules. It was further argued that this mechanism might be the most favorable pathway even for other polymerases.[8, 29] Unfortunately, the study of refs. [8, 29] and the corresponding conclusions involve significant problems. First, the energetics of the PT process has not been calibrated or validated by any pK_a or related calculations in solution. It is also crucial to compare the calculations with several waters to those obtained with a single water. At present, the most systematic study of the competition between one water and two water mechanism in phosphate hydrolysis has been reported in ref. [30]. Performing similar analysis in Pol β may be quite instructive. Second, the evaluation of the PT barriers and paths by energy minimization has been shown by us (e.g., ref. [26] and related works) to be problematic, including cases that were studied with relatively careful treatments (e.g., refs. [31, 32]). Apparently it is still possible that the reaction involves a PT to the phosphate, but establishing such a mechanism must involve major sampling of the ab initio QM/MM surface. Alternatively, one should study the QM/MM surface in solution using careful free energy evaluation and then calibrate the EVB on the calculated surface and use the

calibrated surface in exploring the pathway in the protein (the use of such strategy is demonstrated in ref. [33]). In this respect, we note that ref. [34] performed a CP QM/MM calculations of the reaction of the Human Flap Endonuclease with relatively careful sampling but this did not involve a comparison of the energetics of one and several water molecules.

III.2 Additional mutations of active site acidic groups

The study of ref. [12] has explored the substantial change (3 kcal/mol increases) in the chemical barrier of the D192E mutant and the larger effect for D192A (no detected activity), assuming [12] that the resulting observed effect (see Table 1) reflects the D256 as a base mechanism. It was also argued that the Asp192–Arg258 salt bridge plays a crucial role in the prechemistry. The assumption of a D256 as a base mechanism in the D192E(A) on D256 systems dismisses the possibility of a PT to the bulk (through OH⁻ or even through other elements), which is, at least partially, supported by the observed pH profile of the wt.

In order to explore the effect of mutating D192, we focused on the D192A mutant, since it gave more stable results than those obtained for the D192E mutant, but still reflects the same electrostatic effect. In order to understand the nature of the electrostatic effect of D192 mutations, we compared the EVB calculations of the effect of the D192A mutation on the movement to the TS to a qualitatively equivalent charging thermodynamic cycle (Fig. 7). That is, we designed a thermodynamic cycle for the pentavalent intermediate formation, when the aspartate charge of D192 is set to zero. The cycle comprises the pentavalent formation (the NA step) in the WT enzyme, together with the charging/uncharging of the D192 residue on the end points of the NA step, i.e., reactant and pentavalent intermediate. This cycle yielded an energy increase of 4.4 kcal/mol for the movement to the mutant from the WT. The cycle indicates that the change in the chemical step upon mutation of D192 is mainly associated with the neutralization of the negative charge.

At this point we note that in both PT to the bulk and PT to D256, the D192A mutant leads to a large increase in the activation barrier (Fig. 4 and Table 1). As seen from the figure, we reproduce approximately the anticatalytic effect of the mutation. This reflects, as illustrated in Fig 7, the reduction in the stabilization of the attacking 3' O⁻ by the Mg_{cat}.

III.3 The role of the Mg ions

A major part of the catalytic effect on Pol β and other polymerases is provided by the Mg ions. As shown in the first attempt to quantify the catalytic effect [35] and subsequent studies (e.g., ref. [36]), the Mg ions effect is mainly electrostatic. Of course, getting this electrostatic effect quantitatively is very challenging, since it involves the compensating effects of the ligands. Furthermore, including the Mg in the QM system gives very large charge transfer (CT) contributions, which would be obtained as electrostatic contributions when the Mg is included in the MM part. That is, when the Mg is included in the EVB region we can recover the CT by mixing ionic and covalent states, where the ionic state is more stable and the degree of mixing is controlled by the corresponding off diagonal element. Frequently we can get very similar energies in polar solvents, with and without the mixing term, where the energy of the mixed state is not much lower than the ionic state..

In this study we found as expected that the Mg contribution is compensated largely by the ligands' motion. More specifically, the positions of both Mg ions were tracked along the phosphoryl transfer reaction and the overlay structures of the active site of the native and D256A mutant on the reactant, pentavalent intermediate, and product states. The corresponding results are shown in Figure 8 that takes as reference three distances to Mg_{cat} (d1, d2, and d3 in Fig. 8). Looking at the distances we see that in addition to the displacement of the substrate with respect to the native, in the mutant, the Mg ions are displaced too, keeping the same distance to the substrate in native and mutant for the three states (d2 and d3) but larger distance to E256 in the mutant (d1). This means that the divalent metal ions and their concomitant displacement along with the substrate are necessary to better stabilize the transition state associated to the phosphoryl transfer reaction. Motions of the Mg ions were also reported in refs. [37, 38]. Interestingly, we reported more than 25 years ago[39] the same behavior for the Staphylococcal Nuclease catalyzed reaction mechanism on which the catalytic calcium ion is displaced in the D21E mutant explaining the increase of its free energy barrier due to a loss of the Ca^{2+} stabilizing effect at the transition state. Nonetheless, the case of DNA polymerase is more complex, since the so-called catalytic triad (in reference to the 190, 192, and 256 acidic groups) is also playing a critical role in the catalysis.

In DNA polymerase, the non-bridging phosphoryl oxygens of the substrate can interact with the metal ions, thus increasing the dipole moment of the phosphorus-oxygen bond, i.e., the electrophilicity of the phosphorus atom is enhanced by the Lewis acid effect of the Mg ions. Hence, the D190 and D192 residues can synergically support the catalytic effect of the Mg ions through stabilization of the partial positive charge on the phosphorus of the α -phosphate before and after the nucleophilic attack. Figure 8B shows the comparison of the relative distances between D190 and Pa in the pentavalent state of both native and D256E mutant. At any rate, the substrate displacement and concomitant larger distance (compare 3.89 and 4.28 in Fig. 8B) to the acidic residue suggest a loss in the stabilization of the Pa by D190 and D192. This can be better appreciated in the scheme of Figure 9, which represents the model of the phosphoryl transfer catalyzed by DNA Polymerase in terms of the key electrostatic interactions in the active site. In this model, we see that: (a) the Mg^{2+} ions stabilize the negative charges of the nucleophile and leaving group, (b) the D190 and D192 residues stabilize the electrophilic (partially positively charged) phosphorus, and (c) the electrostatic interaction between D256 and Mg^{2+} ion keeps the substrate in place, i.e., optimally aligned with D190 and D192. The latter seems relevant to further discuss the protonation state of D256, since that this residue acting as proton acceptor of the proton transfer would trigger the displacement of the substrate (along with the metal ions) out of its optimal position for catalysis due to the lack of the corresponding negative charge to anchor the Mg ion and thus, at the end, the increase in activation barrier in D256E would most likely arise from this effect and not from the switch of mechanism in the PT step, suggested in ref. [11]. Even though, the change in pH dependence on the D256E mutant could eventually reflect a competition of both PT mechanisms as was discussed above.

It is important to note that works that do not realize the compensating nature of electrostatic effect (which is basically the dielectric effect for the charge-charge interaction) by the part of the system that is considered as the surrounding can be trapped in problematic analysis.

Thus for example refs. [40, 41] concluded that the Mg path plays a major role in catalysis. Overlooking the fact(s) that what counts is the overall free energy along the least energy path rather than magical barriers or specialized fluctuations; confusing the obvious fact that the potential energy changes drastically upon moving the catalytic metals (see ref. [42]).

The nature of the metals catalysis of DNA Pol has been explored computationally first in our early work [43], where we examined the general two-metal-ion model proposed by Steitz and coworkers (e.g., ref. [44]). This work assumed that the intermetal distance (ca. 4 Å) has an important chemical role in the catalysis through stabilization of the TS (or intermediate) geometry via a strain mechanism (that stabilizes the pentacordinate geometry). This assumption was shown to be problematic by our work, where it was found [43] that the TS stabilization is electrostatic in nature. Thus, the present work is in-line with the electrostatic stabilization by the Mg^{2+} ions and provides further insight, explaining the anti-catalytic effect in D192 and D256 mutants.

IV. Discussion

This work explored the information obtained from recent mutational studies, focusing on the nature of the initial PT. In doing so we kept in mind that computational proposal of PT process in proteins has to be calibrated or validated by pK_a calculations or related calculations in solution, and also validated by a demonstrated ability to evaluate pK_a 's in proteins.[45] Furthermore, the evaluation of PT barriers and paths by energy minimization approaches has been shown by us to be problematic.[24] Therefore, one needs to critically assess different possible pathways (comparing the relative heights) before adopting any of them. Proposals that are based on a PT through a single or several water molecules[7, 8, 29] are questionable, since they have neither been validated by pK_a calculations nor based on proper free energy simulations, extensive relaxation and careful comparison of alternative paths. We note that this appears to be a clear implicit assumption, although ref. [11] mentioned examination of other options but obtains an extremely high barrier of 40 kcal/mol for the PT to water. This estimate is most clearly a significant overestimate due to the inability of energy minimization approach to provide sufficient relaxation and water penetration. Furthermore, PT to H_2O even in water costs $2.3RT (\text{pK}_a(\text{base}) - \text{pK}_a(\text{H}_3\text{O}^+)) = 1.38(13 - (-1)) = 19.3$ kcal/mol and thus the more relevant path for PT to the bulk involves transfer to OH^- (as it is done in our study). We also note that ref. [11] recognized that the pK_a of D256A is similar to that of the 3'OH in water, but did not comment on the significance of this finding any further.

The implicit implications of refs. [11, 12], that mutational studies prove that Asp 256 is the base in the PT step of DNA Pol (see above), is problematic and must be examined by simulating the effect of mutations on the reaction mechanism. Furthermore, a careful analysis of structural changes should explore the corresponding energetics by starting the simulations from the observed structures, using very extensive relaxation. Looking, for example, at the structure of the D256E mutant (Figures 1 and 8) one can note that the Mg_{cat} is in a larger distance from the carboxylate than in the WT (3.7 Å relative to 2.2 Å), where in this case the Asp charge is not localized and that of the 3'O⁻ is localized. However, it is quite difficult to obtain a very accurate position for the Mg ions, and thus, we have to be

careful in judging different type of information. Hence, it is useful to start from the observed structure and to try to evaluate the PT energies and the activation barriers (Table 1).

The analysis of the active site mutations presented in this work indicates that the reaction rate is determined by the barrier of the chemical step and not by the prechemical steps (this is based in part on the reasonable correlation between the calculated and observed chemical barrier). The issue of the nature of the rate determining step is relevant to the poorly defined “dynamical type effects” that have been implicated to play a major role in the control of replication fidelity by DNA polymerases. This includes the idea that the difference in the selectivity of the correct and incorrect (R and W, respectively) base pairs are due to pre-chemistry barriers, that are, in an absolute scale, lower than the chemical barrier. This idea considered “checkpoints” as crucial factors in the control of the fidelity (see discussion in ref. [16] and references cited therein). Although we have clarified that this is a problematic concept using well-defined free energy considerations (see ref. [46]).

Considering the above checkpoint idea it might be instructive to note a recent criticism by ref. [47] of our arguments in ref. [46], which, instead of actually addressing our very clear energy diagrams, has focused on the names used in some of the discussion. That is, while we did use the term “*rate*” when we meant “*specificity*” (in the caption of Figure 1 of ref. [46]) and “*rate determining*” when we again meant “*specificity*” (in the abstract), it should be clear from all our figures that we meant that the fidelity is determined by the changes in the highest barrier, relative to the unbound state. Thus, the arguments provided in ref. [47] that, in contrast to our work, prechemistry *can* contribute to fidelity even if it is not rate-limiting, have grossly distorted what was shown in our actual paper (that there is no scenario where the prechemistry step contributes to the fidelity *if it does not present the highest barrier* in the catalytic cycle). Obviously, Figure 4 of ref. [46] shows that the fidelity *can* be controlled by prechemistry steps, when these present the highest barriers. Of course in most cases the barrier for the prechemistry step is lower than the chemical barrier (measured relative to the unbound state).

It is important to address here previous QM/MM studies,[6, 48] which obtained concerted reaction path in DNA Pol β . The calculated path is probably quite reasonable. However, as a general note for workers in the field, we would like to point out that the surface obtained by energy minimization is a potential surface rather than a free energy surface (this clearly leads to problems in determining the prechemistry barrier) and there are other potential problems in using energy minimization approaches (see ref. [49]). However, our main concern, which is clearly not limited to the work of refs. [6, 48], is the fact that most of the works do not mention calibration or validation by reproducing the energetics of the reference reaction in solution and have also not been explored by proper sampling.

An interesting issue that was addressed in ref. [11] is the nature of the interaction between D256 and R254. In considering the effect of Arg254 we must allow for a major relaxation of the environment (which is hard to obtain with the ONIOM approach [50]). Thus it seems to us the results reported in ref. [11] involve unrealistic electrostatic energies, where the corresponding electrostatic interaction has been largely overestimated by Batra et al.[11] For example, it is hard to see how can Arg254 be destabilizing the TS of the WT (by 33 kcal/

mol) while stabilizing the TS of D256E. In judging the validity of our view, one should consider our long time studies in the energetic of ion pair in proteins, which focused on reproducing the observed energetics (e.g., see review in ref. [18]). In other words, one must assess the validity of QM/MM minimization approaches by exploring cases where the energy of the given ion pair energy is known experimentally. Doing so will show that the effective dielectric is very large even in protein interiors.

Another possible source for problems is the assumption that the charge transfer (CT) effect, obtained when the metals are included in the QM region, provides a major new insight. In fact, although the CT is a real effect, it can be captured by properly calibrated QM/MM models (note that including the metal alone without the ligand in the QM region leads to a major unbalance in the calculation), as well as EVB calculations that include the Mg in the EVB region. In fact, it seems that in many cases it is better to represent the metal by electrostatic model (in particular a model with dummy atoms [51]). Interestingly, ref. [34] used our classical dummy atoms model in CP calculations. Approaches that just include the metal in the QM region usually increase the instability of the calculations and the chance to have a carefully calibrated model. In our view, the size of the QM region is not the most important issue, in particular when one compares the effects of mutations or the effect of the wt relative to the same system in water.

The catalytic role of the magnesium ions in the DNA pol β has been considered in ref. [41], using path sampling approach, starting with a Monte Carlo procedure from initial configurations generated by targeted Molecular Dynamics (TMD). This study has considered just the prechemistry step in the enzyme and found that different energy barriers arise from different initial positions of the two metal ions. It was argued that the energetically favored ion route in the displacement of both magnesium ions along the conformational change from the open to closed state should give a total energy barrier for the conformational pathway (of about 10 kcal/mol) of the WT protein. While these findings are clearly interesting, they involve a problematic perspective, since in principle one would like to have a stable converging potential of mean force (PMF) for the conformational and chemical pathways. In doing so the most important challenge is finding the least free energy path and the corresponding free energy. In such cases, the different coordinates (including those of the Mg positions) should be “optimized” so that the movement along the real global reaction coordinate has a minimal barrier. In this respect, it is not clear how relevant are the different free energy along the different pathways described in Fig. 2 of ref. [41], since it is not clear whether allowing all the coordinates to relax will lead to a single PMF. In our view, it would be advantageous to have the reaction path along the two dimensional surface of the Mg motion and all the other coordinates.

Interestingly, Elber and coworkers [52] determined a single TS from the minimum free energy path between the open and closed states of DNA Pol in contrast to the several metastable states proposed by ref. [41], suggesting that there was no evidence favoring the more complex model [52].

The above issues become more critical when one tries to explore the catalytic role of the metals (see section III,3). In this case, it is not justified the proposed mechanistic role to the

metals (see ref. [53]). That is, the metal do change their position during the reaction in repose to the change of the substrate charges position during the reaction and the same occurs for other active site charge residues (this type of situation has already been discover in our studies of metal catalyzed reactions (see ref. [39]). We note in this respect that some studies (e.g., ref. [54]) of the general two-metal-ion mechanism, proposed by Steitz [44], have suggested a cooperative motion of the two metal ions, where upon moving from the reactant state to the TS the metals get closer to stabilize the TS and then move apart to facilitate the product release [54]. While this structural change is expected and makes sense in terms of the electrostatic energy of the system, the real question that has to be resolved is not so much the motion of the metal ion but its effect in stabilizing the TS, which of course should be assessed with a fully relaxed metal position. In fact ref. [44] suggested metals play a steric role, which turned out (see discussion in section III.3) to be problematic.

Overall the present work can serve as a warning that in exploring the effect of mutations of charged groups in the active site of polymerases must involve very careful sampling and validation on reproducing pH dependence and the energetics of the solution reactions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
The active site residues for WT (left) and D256E mutant (right).

Figure 2.

Reaction mechanism for the nucleotide addition by DNA polymerases. The initial step corresponds to a proton transfer to either the Bulk or an Aspartate. The next step is a nucleophilic attack to form a phosphodiester bond in the corresponding pentavalent structure followed by the departure of the pyrophosphate as leaving group. The three plausible mechanisms are shown (see main text for further details).

Figure 3.
The EVB states considered in this work.

Figure 4.

(A) Energy profile for the nucleotide incorporation on native and mutant of DNA Polymerase β for the WT (PDB: 2FMS), the D256E mutant (PDB: 4JWM), and the D192A mutant (modified WT structure, PDB: 2FMS). (B) Energy profile of PT mechanisms for WT: PT to Bulk (in black) and PT to Acid (in brown). (C) Energy profile of PT mechanisms for D256E: PT to Bulk (in red) and PT to Acid (in green). The calculations are done for pH = 7 except for D192A where it is taken for pH = 8. The free energies of the PT to the bulk reflect the average of two estimates (reflecting two values of ϵ_p , see Table 1).

Figure 5.

Showing the nature of a conduction path for PT from 3'OH to the bulk. For this process we have to consider a PT along the chain $(\text{OH}^-)_{\text{bulk}}\text{H}_2\text{O}(\text{n})\text{--}\text{H}_2\text{O}(3)\text{H}_2\text{O}(2)\text{H}_2\text{O}(1)\text{ (3'OH)}$ starting with a PT from $(\text{OH}^-)_{\text{bulk}}$ to a surface H_2O ($\text{H}_2\text{O}(\text{n})$) and then a PT along the chain until we reach the PT from 3'OH to OH^- on site 1.

Figure 6.
The pH profile of the rate constants of D256 and its mutants (reproduced schematically from the results of ref. [11]).

Figure 7.

Reaction Free Energy for the formation of the pentavalent intermediate in the enzyme with deactivated D192 (i.e., uncharged residue). This free energy was obtained from the thermodynamic cycle for the uncharging/charging of D192. The cycle is given in the figure. Energies in kcal/mol. The uncharging process is qualitatively similar to the D192A mutation.

Figure 8.

The Positions of the Mg ions in the WT and in D256E for the reactant (A), pentavalent intermediate (B), and product (C). Three distances are shown as references (d1, d2, d3) in an overlay of the native and mutant structures, which were aligned in their D190 and D192 residues. Distances in Angstroms. The distances from the Pentavalent intermediate to the D190 residue in WT and D256E are shown in (B).

Figure 9. Model representing the electrostatic nature of the phosphoryl transfer reaction in DNA Polymerases. The key electrostatic interactions between the Mg ions, the catalytic residues, and the substrate are depicted.

Table 1

Calculated and observed $\ddagger$ activation and reaction free energies for individual steps for the different possible reaction mechanisms for pol β . $\ddagger$

PT	DNA Pol β	$G^{\circ}(\text{PT})_{\text{calc}}$ $\ddagger$	$G^{\circ}(\text{PT})_{\text{obs}}$	G (NA) _{calc}	$G^{\circ}(\text{NA})_{\text{calc}}$	G (LGD) _{calc}	G (rxn) _{calc}	G (rxn) _{obs $\S$}
PT Bulk	WT	4.2	1.5 $\pm$ 0.07	14.9	12.8	14.6	14.9	16.6
PT Bulk	D256E	4	3.4 $\pm$ 0.1	18.6	14.7	16.6	18.6	21.5
PT Bulk	D256A(no Mg _{cat})	9.2	7.0 $\pm$ 3.2	n/a	n/a	n/a	n/a	22.9
PT Bulk	D256A(2Mg ²⁺)	4.3	7.0 $\pm$ 3.2	n/a	n/a	n/a	n/a	22.9
PT Bulk	D192A	2.7	n/a	18.1	12.5	21	21	IN #
PT 256	WT	6.1	n/a	19.5	17.6	21.5	21.5	16.6
PT 256	D256E	6.4	n/a	20.9	16.5	18.7	20.9	21.5

$\ddagger$ Energies in kcal/mol. Calculations with the WT structure (PDB: 2FMS), D256E mutant structure (PDB: 4JWM), D256A mutant structure (PDB: 4JWN), and modified WT structure for D192A. G (rxn)_{calc} designates the highest barrier (either G (NA)_{calc} or G (LGD)_{calc}). The barriers are taken at pH=7 except for D192A where it is taken for pH=8. The nucleophile attack step of the D256A was not calculated since this system loses one Mg and undergoes major structural changes.

$\S$ Average of the values obtained using $\epsilon_p=4$ and $\epsilon_p=8$ for the WT, D256E, and D192A; while for D256A we used the average of the values obtained with $\epsilon_p=8$ and $\epsilon_p=20$.

$\S$ Estimated energies from the observed kinetics in Ref. [11] for the WT, D256E, and D256A.

Inactive (Ref. [12]).