Supporting Information

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SI Results and Discussion

The Changes Introduced at ϕ29 DNAP Residue Asp^{121} Impair the Stabilization of dsDNA Substrates at the Polymerization Active Site. Considering the difficulties shown by mutants D121A and D121E in translocating on DNA after the dNMP insertion step, the affinity for primer/template DNA molecules of the wild-type and ϕ29 DNAP mutants was directly studied by EMSA, essentially as described in *Materials and Methods*. As shown in Fig. S1, both the wild-type enzyme and mutant D121N gave rise to a single retardation band that has been interpreted as a stable complex competent for polymerization, in which the primer terminus was stabilized at the polymerization active site (1). Mutants D121A and D121E did not give a stable retarded band, indicating binding defects at the polymerization active site (Fig. S1).

The Global Structure of the Polymerase Is Not Affected by the Mutations Introduced. To rule out that the defects observed in both the 3′-5′ exonuclease and polymerization activities of mutants D121A and D121E were due to a global misfolding of these proteins, we obtained experimental information about the structure of the polymerase by fluorescence spectroscopy with the wild-type enzyme and with mutant D121A, which is the most affected one, to confirm its correct folding.

The fluorescence emission spectrum of the wild-type ϕ29 DNAP showed a maximum at 341 nm (Fig. S2*A*). The protein has 11 tryptophan residues and 37 tyrosine residues either buried or exposed to solvent (PDB ID code 1XHZ) (2, 3). Those residues are sensitive to their environment, which changes when protein folds/unfolds. One of the most common ways of unfolding a protein is a rise in temperature. Fluorescence emission decreases upon heating up to 36 °C, undergoing successive signal increase until 44 °C, from which point emission decreases again successively (Fig. S2*B*). The emission maximum red shifts reached values over 350 nm, which are indicative of an increase of solvent-exposed tryptophan residues, compatible with protein denaturation (Fig. S2*B*). The variation of the intensity ratio at the wavelengths of the emission maxima upon heating shows an apparent transition midpoint at around 40 °C under the tested conditions (Fig. S2*D*). Similar fluorescence spectra and apparent transition midpoint values were observed for ϕ29 DNAP mutant D121A (Fig. S2 *C* and *D*). It is clear from the complex behavior in the evolution of the spectra that, upon heating, a number of processes, in addition to denaturation, are occurring, making interpretation not straightforward. However, as both samples present a similar trend, our conclusion is that the mutation does not substantially affect the protein folding and conformational stability and that the biochemical results obtained are not due to a general misfolding of the protein.

SI Materials and Methods

DNA Gel Retardation Assay with dsDNA. The interaction of either the wild-type or the ϕ29 DNAP mutant with the primer/template structure was assayed using as substrate the 5′-labeled 15mer/21mer. The incubation mixture contained (in a final volume of 20 μL) 12 mM Tris·HCl (pH 7.5), 1 mM EDTA, 20 mM ammonium sulfate, 0.1 mg/mL BSA, 10 mM $MgCl_2$, 0.7 nM sp1/sp1c+6 (15mer/21mer) DNA molecule, and the indicated amount of wild-type or mutant DNAP. After incubation for 5 min at 4 °C, the samples were subjected to electrophoresis in precooled 4% (wt/vol) polyacrylamide gels [80:1 acrylamide/bisacrylamide (wt/wt)] containing 12 mM Tris·acetate (pH 7.5) and 1 mM EDTA, and run at 4 °C in the same buffer at 8 V/cm (4). After autoradiography, ϕ29 DNAP/DNA stable interaction was detected as a shift (retardation) in the migrating position of the labeled DNA.

Fluorescence Spectroscopy. Intrinsic fluorescence emission spectra on the ϕ29 DNAP and the mutant D121A (~5 μM) were recorded in buffer A [20 mM Tris·HCl (pH 7.5), 150 mM NaCl, and 1 mM DTT] using a Fluoromax 4 spectrofluorimeter (Jobin-Yvon, Inc.) equipped with a Peltier module for temperature control. Excitation and emission wavelengths were calibrated using the Xe emission line at 467 nm and the water Raman signal. Fluorescence emission spectra of wild-type and mutant samples were recorded at 4 °C within the range of 270–380 nm after excitation at 280 nm. Two individual experiments were performed for each sample. Thermal denaturation was followed by recording fluorescence emission spectra every 1 °C and 2 °C, respectively, for each sample (wild-type and mutant). For clarity, Fig. S2*D* shows spectra acquired every 10 °C. In all cases, equilibration time before the acquisition of a fluorescence spectrum was 1 min. The slit widths were 2.0 nm for excitation and emission in all spectra.

1. Méndez J, Blanco L, Lázaro JM, Salas M (1994) Primer-terminus stabilization at the psi 29 DNA polymerase active site. Mutational analysis of conserved motif TX2GR. *J Biol Chem* 269:30030–30038.
2. Kamtekar S, et al. (2004) Insights into strand displacement and processivity from the crystal structure of the protein-primed DNA polymerase of bacteriophage φ29. *Mol Cell* 16:609–618.
3. Abril AM, Marco S, Carrascosa JL, Salas M, Hermoso JM (1999) Oligomeric structures of the phage phi29 histone-like protein p6. *J Mol Biol* 292:581–588.
4. Carthew RW, Chodosh LA, Sharp PA (1985) An RNA polymerase II transcription factor binds to an upstream element in the adenovirus major late promoter. *Cell* 43:439–448.

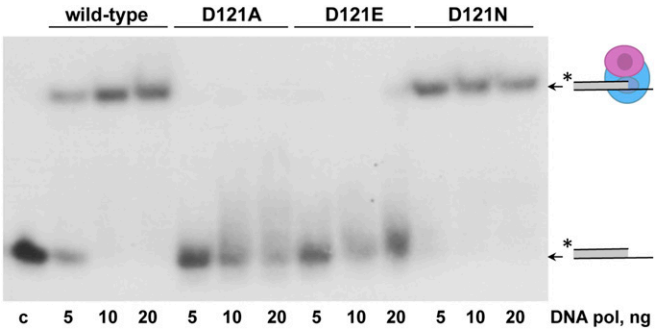

Fig. S1. dsDNA binding of wild-type and mutant ϕ29 DNAPs. The assay was carried out as described in *SI Materials and Methods*, using the 5′-labeled hybrid sp1/sp1c+6 (15mer/18mer) as substrate, in the presence of the indicated amounts of either wild-type or mutant ϕ29 DNAPs. After nondenaturing gel electrophoresis, the bands corresponding to free ssDNA and to the DNAP/DNA complex were detected by autoradiography. c, control lane without enzyme. Asterisks indicate the 5′-^{32}P-labeled end of the primer strand.

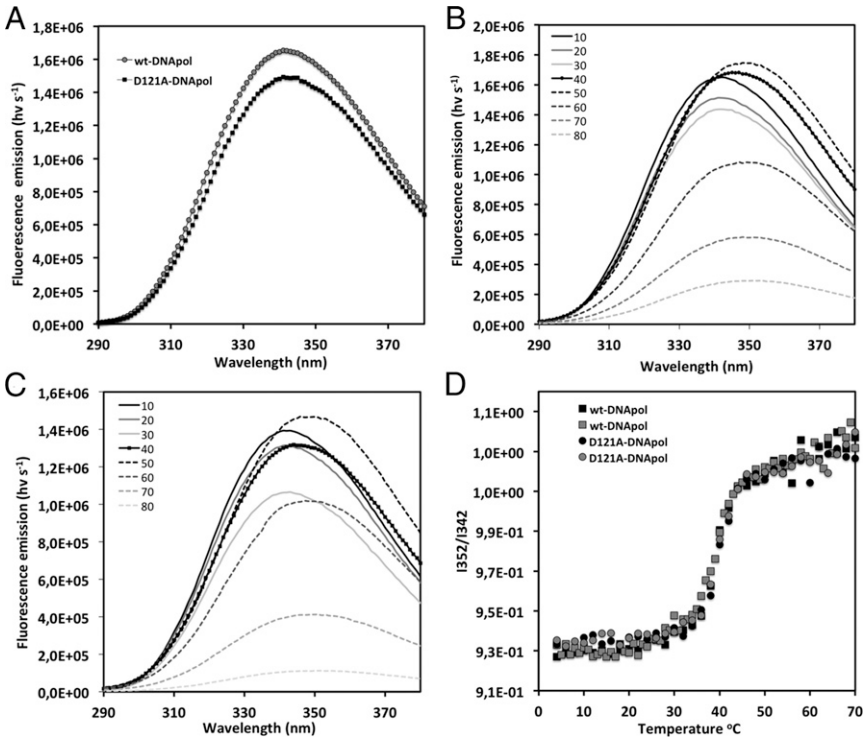

Fig. S2. Fluorescence spectroscopic characterization of wild-type and D121A mutant ϕ29 DNAPs. (*A*) Fluorescence emission spectra of wild-type (wt) and mutant ϕ29 DNAPs. Fluorescence spectra (λex = 280 nm) were recorded at 4 °C and at a protein concentration of 5 μM in 20 mM Tris·HCl (pH 7.5), 100 mM NaCl, and 1 mM DTT. Both wt and mutated samples displayed emission maxima at 342 (±1) nm. Protein thermal denaturation of wt DNAP (*B*) and mutant D121A ϕ29 DNAP (*C*) monitored by fluorescence emission is illustrated as described in the main text. Fluorescence spectra recorded at several temperatures are shown. (*D*) Dependence of *I*352/*I*342 on temperature. A similar behavior for wt and mutated samples is observed, showing an apparent transition midpoint at around 40 °C. Black and gray dots show experiments performed every 1 °C and 2 °C, respectively. hv, photon energy.