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A Light-Activated DNA Polymerase**

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DNA polymerases are key enzymes that replicate DNA strands by catalyzing the addition of deoxyribonucleotides to the 3′ end of a primer chain to form a complementary strand to a DNA template. From sequence comparisons and crystal-structure analyses, DNA polymerases can be categorized into at least five families (pol I, pol α, RT, pol β, and pol III).[1] The pol I polymerase family has been the subject of extensive biochemical and structural studies. This family includes enzymes such as *Escherichia coli* DNA polymerase I and *Thermus aquaticus* polymerase (*Taq*).[2] The latter has found broad application in the polymerase chain reaction (PCR), arguably one of the most important reactions developed to date.[3] The biotechnological importance of *Taq* polymerase prompted us to investigate the possible light regulation of this enzyme through the incorporation of a light-activated non-natural amino acid.

Taq polymerase, like other DNA polymerases, features architectural domains resembling the conformation of a palm, a thumb, and fingers in the shape of a right hand.[4] The mechanism of polymerase action was proposed to follow an open–closed model.[5] After the duplex DNA binds to the polymerase, the thumb domain of the polymerase closes around the DNA to switch the enzyme into an open conformation. The subsequent binding of a matching deoxynucleotide triphosphate (dNTP) induces a rotation of the finger domain to form a closed conformation and bury the active center. Rapid catalysis of the phosphoryl transfer then leads to a relaxation to the open conformation of the enzyme–product complex to enable the discharge of pyrophosphate (PPi) and translocation of the template strain.

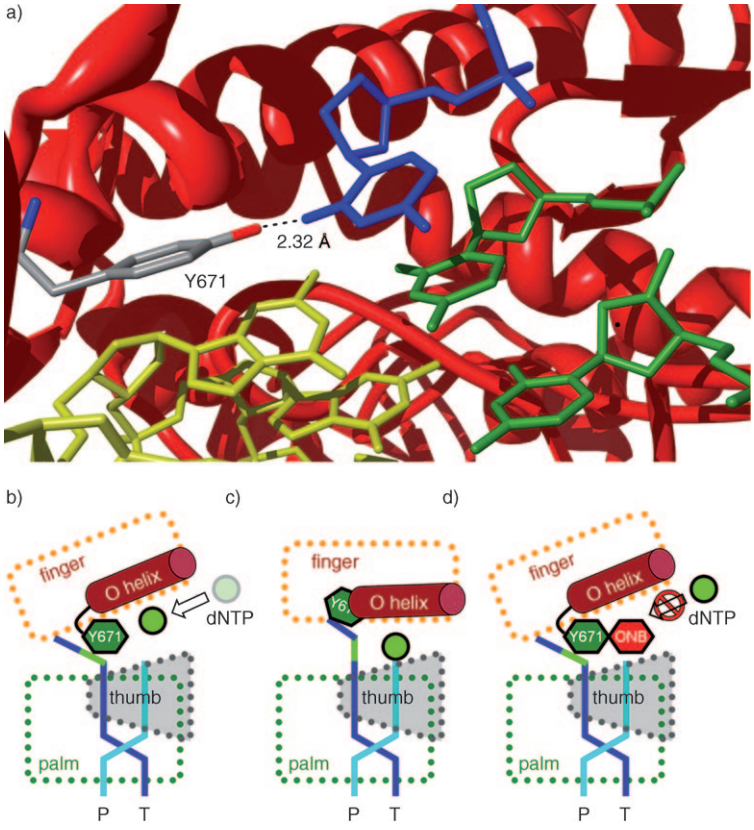

Figure 1. a) Rendered image of the active site of DNA polymerase I from *Thermus aquaticus* (PDB: 2 KTQ). The protein is shown in red, the DNA template in yellow, the growing DNA chain in green, and the ligand dideoxycytidine triphosphate in blue. An ONB group introduced on the hydroxy group of the Tyr671 residue may well extend into the space reserved for the incoming dNTP and thus disrupt a crucial hydrogen bond (dotted line). b) Model of the *Taq* polymerase open complex. c) Model of the *Taq* polymerase closed complex. d) Hypothetical model of inactive caged *Taq* polymerase. P = DNA product, T = DNA template.

At the end of the cycle, the polymerase–DNA complex is ready for the next round of dNTP incorporation.[6]

During the critical open–closed conformational change, the O-helix motif in the finger domain plays a crucial role by positioning the dNTP in the open complex and wedging the amino acid Tyr671 against the DNA template; in this way, the primer–template pair can position itself in the active site (Figure 1). In a subsequent conformational change, the rigid-body motion of the O-helix releases the side chain of Tyr671 from its stacking arrangement against the 5′ end of the template base and enables the first DNA base of the template to line up with the incoming dNTP.[6] Mutations revealed that the Tyr671 residue is critical in this step, as its replacement with other amino acids (except structurally similar phenylalanine) resulted in a loss of function.[7]

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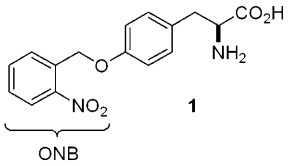
We speculated that the introduction of the caged tyrosine derivative **1**, which contains an *ortho*-nitrobenzyl (ONB)



group, at the Y671 position would inactivate *Taq* polymerase by occupying the space reserved for the incoming dNTP with the sterically demanding ONB group (Figure 1d). The removal of the ONB caging group through brief irradiation with UV light of 365 nm would generate wild-type *Taq* polymerase and thus restore DNA-polymerization activity.

An in vivo approach was used to incorporate the caged tyrosine derivative **1**.[8–10] A mutant *M. janaschii* tyrosyl tRNA synthetase (*Mj*ONBTyrRS) that specifically recognizes the non-natural amino acid **1** (and none of the 20 common amino acids) was added to the biosynthetic machinery of *E. coli* along with its cognate tyrosyl tRNA (MjtRNA$^{Tyr}_{CUA}$) through the introduction of the pSupONBTyr-tRNA plasmid encoding the synthetase and six copies of the tRNA.[11] The tRNA delivers **1** in response to an amber stop codon, TAG, and thus produces mutant protein caged at the desired amino acid position. The engineered *E. coli* cells were then transformed with the pTaqY671TAG plasmid encoding a *Taq* polymerase mutant containing the TAG stop codon at the Tyr671 position. TOP10 cells harboring both plasmids were grown in Luria–Bertani (LB) medium (supplemented with amipicillin and chloramphenicol) in the presence of **1** (1 mM) to an optical density of 0.6 at 600 nm (OD_{600}) of 0.6 and were subsequently induced through the addition of isopropyl-β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM. After 16 h, the cells were harvested, and the *Taq* polymerase protein was purified by denaturing all mesophilic proteins through heat treatment at 75 °C for 20 min. No *Taq* polymerase was observed when pTaqY671TAG was expressed in the absence of **1**. In the presence of **1**, a protein band corresponding to wild-type *Taq* polymerase was observed on an SDS-PAGE gel stained with Coomassie Blue (Figure 2). Further purification of the heat-treated caged *Taq* polymerase (ca. 1 mg per liter of bacterial cell culture) by anion- and cation-exchange chromatography provided the enzyme with the same purity as that of commercially available NEB *Taq* polymerase (see the Supporting Information).

We investigated the light regulation of DNA-polymerase activity by irradiating wild-type *Taq* (4.5 ng/20 μL) and an equivalent amount of *Taq*Y671ONBY (4.8 ng/20 μL) in quartz cuvettes with a 25 W UV transilluminator (UVP) at 365 nm for 0, 5, 10, or 20 min, followed by PCR. Electrophoresis on agarose gels revealed that light irradiation did not inhibit the activity of wild-type *Taq* (Figure 3a). Most importantly, the caged enzyme was completely inactive before UV irradiation, and could be activated readily by exposure to UV light for 5 min. Longer irradiation times of 10 or 20 min did not increase the amount of the final PCR

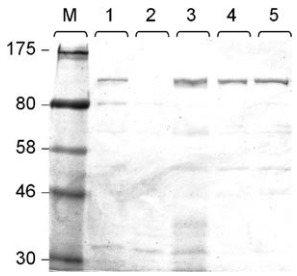


Figure 2. Expression of the photocaged *Taq* DNA polymerase Y671ONBY. M: NEB broad-range prestained marker; lane 1: heat-treated wild-type *Taq* polymerase; lane 2: heat-treated protein expressed from pTaqY671TAG in the absence of **1**; lane 3: heat-treated protein expressed from pTaqY671TAG in the absence of **1**; lane 4: purified and dialyzed caged *Taq* polymerase (18 μg mL^{-1}); lane 5: commercially available NEB *Taq* polymerase (25 μg mL^{-1}).

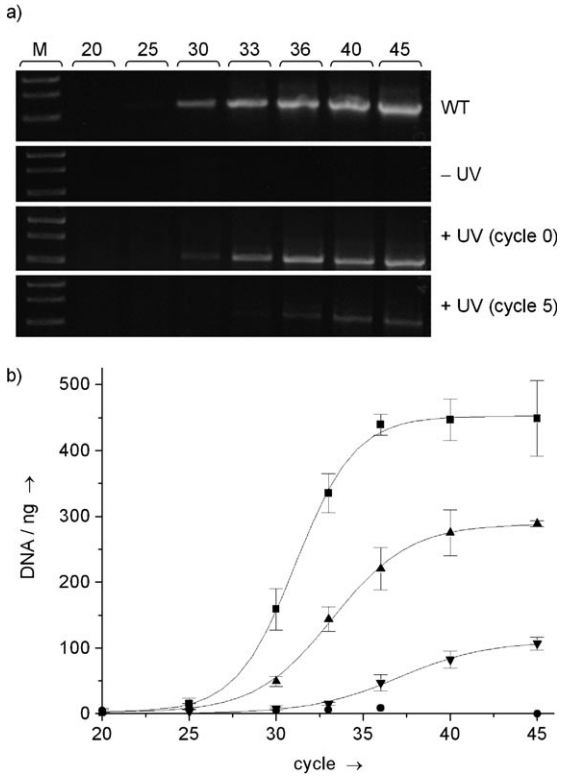


Figure 3. Induction of polymerase activity by the decaging of *Taq*Y671ONBY with light. a) PCR product generated by using caged and noncaged *Taq* polymerase (after cycles 20, 25, 30, 33, 36, 40, and 45). WT: wild-type *Taq* polymerase (4.5 ng/20 μL); −UV: *Taq*Y671ONBY (4.8 ng/20 μL) without decaging; +UV (cycle 0): *Taq*Y671ONBY with decaging for 5 min (365 nm, 25 W, transilluminator); +UV (cycle 5): PCR reaction mixture with *Taq*Y671ONBY as the polymerase with decaging for 5 min after 5 cycles in the thermocycler. b) Quantitative analysis with ImageQuant 5.2. ■ WT, ● −UV, ▲ +UV (cycle 0), ▼ +UV (cycle 5).

product (data not shown). The amount of PCR product was plotted on a log scale against the number of cycles, and the slopes for the early exponential-amplification stage were compared. This analysis demonstrated a 71% restoration of the activity of the caged *Taq* polymerase upon UV irradiation (see the Supporting Information).

These experiments confirmed that *Taq* DNA polymerase can be engineered by the incorporation of a non-natural amino acid to be light-activatable. The enzyme containing **1** at position Y671 is completely inactive, and catalytic activity can be restored by brief UV irradiation. Moreover, temporal control could be gained over polymerase activity by irradiating the PCR mixture containing *Taq*Y671ONBY after the fifth cycle of the PCR program (Figure 3).

Next, we applied the caged *Taq* polymerase in a light-triggered hot-start PCR. The hot-start PCR strategy has been used to diminish nonspecific DNA amplification until the reaction temperature reaches a level at which the nonspecific annealing of primers to the template DNA can not occur.[12] A hot-start PCR can be triggered in a variety of ways, for example, by an antibody-conjugated polymerase,[13] by a chemically modified polymerase,[14] and by a variety of polymerase mutants which are inactive at lower temperatures.[15] However, no light-activated hot-start PCR has been described previously. The template (mouse genomic DNA; Promega), the primers (B-UP and 3D), and the PCR program (94 °C for 1 min, 45 cycles of 94 °C for 40 s, 56 °C for 40 s, 68 °C for 5 min; final extension at 72 °C for 5 min) were modified from the hot-start PCR study conducted by Kermekchiev et al.[15] The use of a commercially available *Taq* polymerase (New England Biolabs) led to the formation of multiple DNA products and resulted in a visible smear of bands on an agarose gel (Figure 4, lane 4). In contrast, when the PCR

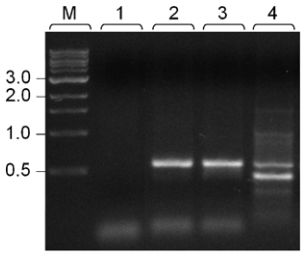


Figure 4. Hot-start PCR with the B-UP/3D primer pair and mouse genomic DNA.[15] M: DNA markers; lane 1: *Taq*Y671ONBY only (29 ng per 40 μL of reaction mixture); -UV; lane 2: *Taq*Y671ONBY (29 ng/40 μL), +UV, hot start; lane 3: NEB *Taq* polymerase (10 ng/40 μL), hot start; lane 4: NEB *Taq* polymerase (10 ng/40 μL), room-temperature start.

mixture containing caged *Taq* was heated to 90 °C and irradiated for 5 min with a 45 mW UV LED 365 fiberoptics system (Prizmatix), only a single product band was visible (Figure 4, lane 2). This result is comparable to that of a hot-start PCR with NEB *Taq* polymerase (Figure 4, lane 3) and thus validates the applicability of the light-triggered hot-start PCR methodology. Different quantities of the NEB *Taq* polymerase were used to compare the activity of the decaged and commercially available polymerases.[16] Approximately 43 % of the theoretical activity was recovered in the UV-initiated hot-start experiment (see the Supporting Information).

In conclusion, we have developed a light-activatable *Taq* DNA polymerase through the in vivo incorporation of a photocaged tyrosine amino acid. Rational design of this engineered enzyme was based on the mechanistic information derived from crystal structures. The introduction of the photocaged tyrosine derivative at position Y671 interferes with the accommodation of dNTPs in the binding pocket of the enzyme. It is also conceivable that the extensive mechanical movement of the O helix and the finger domain may be obstructed by the incorporation of the *ortho*-nitrobenzyl group. Since the selected tyrosine residue is also conserved in many other monomeric DNA and RNA polymerases,[17] this approach may be generally applicable to the photochemical regulation of these enzymes and enable a more detailed investigation of their polymerization mechanism (e.g. through time-resolved X-ray crystallographic analysis). We also developed a light-triggered hot-start PCR method involving facile UV irradiation in a benchtop PCR machine. Beyond this temporal control of DNA polymerization, light activation also offers the possibility of spatial control of locally induced polymerase activity to facilitate the design of novel nanodevices and biotechnological applications, such as on-chip real-time PCR detection.[18]

Experimental Section

Gene manipulation, bacterial culture, and protein purification: The *E. coli* strain BL21 was transformed with the plasmid pTaq and used to express wild-type *Taq* polymerase induced with 0.5 mM IPTG. All primers were synthesized by IDT DNA. The point mutation of the pTaq Y671 codon TAA to TAG was introduced by using a QuikChange II kit (Stratagene). The resulting plasmid was designated as pTaqY671TAG, and the coding region was sequenced. The plasmid was cotransformed with pSupONBY into *E. coli* TOP10 cells. The transformed cells were cultured in LB medium containing ampicillin (50 $\mu g\,mL^{-1}$) and chloramphenicol (34 $\mu g\,mL^{-1}$). The caged tyrosine derivative **1** was added to the medium to a final concentration of 1 mM, and the culture (0.5 L) was incubated at 37 °C (250 rpm). A 100 mM IPTG solution (2.5 mL) was added when the OD_{600} value reached 0.6, and protein expression was continued for 16 h. Cells were pelleted and stored at −80 °C. Subsequent purification was carried out according to a previously reported protocol.[16] For the hot-start PCR, purified caged *Taq* DNA polymerase was used (see the Supporting Information for the purification protocol).

Light-induced PCR: *Taq*Y671ONBY (6 μL) was added to Millipore water (222 μL) in a quartz cuvette for decaging on a transilluminator (UVP). A quantity of 15.2 μL of the decaged enzyme solution was mixed with Master mix (4.4 μL) consisting of Thermopol buffer (20 μL), the forward primer (8 μL), the reverse primer (8 μL), 10 mM dNTP Mix (4 μL), and the template (4 μL; 100 pg μL^{-1}). The following PCR program was used: 94 °C for 2 min, 40 cycles of 94 °C for 30 s, 54 °C for 30 s, 72 °C for 1.2 min; final extension at 72 °C for 5 min.

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