Erschienen in: ChemBioChem ; 15 (2014), 12. - S. 1735-1737
https://doi.org/10.1002/cbic.201400051

Modulating the pK_a of a Tyrosine in *KlenTaq* DNA Polymerase that Is Crucial for Abasic Site Bypass by in Vivo Incorporation of a Non-canonical Amino Acid

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It is estimated that about 10000 abasic sites are formed per day per cell. Abasic sites impose a significant challenge for bypass synthesis by DNA polymerases. Recently, a tyrosine in KlenTaq DNA polymerase has been highlighted as being crucial for nucleotide selection opposite abasic sites. Structural data indicated a hydrogen bond between the tyrosine's hydroxy group and the N3 of an incoming ddATP opposite the abasic site. In order to further investigate abasic site bypass, we incorporated the unnatural amino acid 2,3,5-trifluorotyrosine at the position of the crucial tyrosine of KlenTaq DNA polymerase. Fluorine substitution at the tyrosine decreased the pk_a value of the tyrosine's hydroxy group and allowed its protonation state to be modulated. Single-nucleotide-incorporation experiments revealed reduced activity for the KlenTaq mutant compared to the wild-type when bypassing an abasic site analogue. The finding stresses the involvement of this tyrosine and its hydrogen bonding in abasic site bypass.

DNA is continuously damaged by endogenous and exogenous agents. Under physiological conditions the most frequent type of DNA damage is the abasic site, which results from spontaneous hydrolysis of the bond that connects the sugar to the nucleobase (Figure 1 A).[1] It has been estimated that approximately 10000 abasic sites are formed in a human cell each day.[2] These lesions can stall DNA synthesis, as they present a major challenge for replicative DNA polymerases.[3] Furthermore, they are noninstructive, as genetic information is lost by cleavage of the nucleobase.[2a,4] However, members of DNA polymerase families A and B were observed to preferentially insert dATP opposite an abasic site, a phenomenon termed the "A-rule".[2a,4,5]

In vitro studies of *KlenTaq* DNA polymerase (family A polymerase) showed that this enzyme also follows the A-rule in the presence of the stabilized tetrahydrofuran abasic-site analogue F (Figure 1 B).[6] Crystal structures of this enzyme in complex with a template containing an abasic-site analogue F showed an "amino acid templating" mechanism, facilitated by a tyrosine residue at position 671 (Y671).[6] The tyrosine residue mimics the shape and size of a six-membered pyrimidine nucleobase in the template strand, thus directing purine incorporation opposite the abasic site, by providing an optimal geometric fit for the active site (Figure 1 C). Furthermore, structural and functional data indicate a hydrogen bond interaction between the incoming nucleotide at N3 of adenine and the hydroxy group of Y671 (Figure 1 C).[6a]

The importance of the hydrogen bond between Y671 and the incoming nucleotide was demonstrated by mutational analysis as well as by substituting the N3 of adenine with a nonpolar CH-group.[6a] Herein, we studied this hydrogen bond by modulating the pK_a value of the Y671 hydroxy group. Fluorinated tyrosine analogues have already been employed as valuable tools for the investigation of acid–base catalyzed reactions[7] and of biologically generated tyrosine radicals used for catalysis in proteins.[8] Furthermore, fluorotyrosine analogues provide an opportunity to probe hydrogen bonding interaction networks in proteins.[7,9] The fluorine atom combines isosteric properties comparable to those of hydrogen with increased electronegativity, thereby resulting in lower pK_a values for the fluorinated analogues.[7,8] Thus, the correct choice of a fluorotyrosine analogue in combination with an appropriate re-

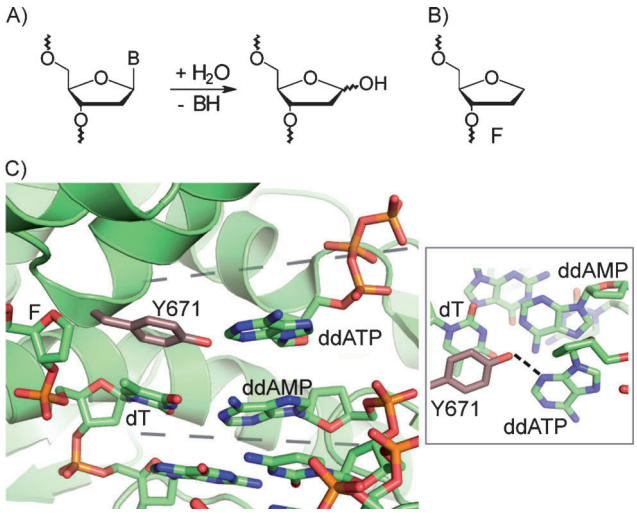

Figure 1. A) Hydrolysis of the glycosidic bond results in formation of an abasic site (B: base). B) Chemical structure of the abasic site analogue F. C) Crystal structure of the active site of wild type *KlenTaq* with ddATP oppo site the abasic site F (PDB ID: 3LWL).[6a] The penultimate base pair, the in coming ddATP, and Y671 are shown. Detail shows hydrogen bond between Y671 and N3 of the incoming ddATP (dashed line).

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action pH allows the protonation state of the tyrosine hydroxy group to be modulated. Site-specific incorporation of fluorotyrosines into proteins offers the possibility to elucidate and validate hydrogen bonding interactions in proteins, for example, in the polymerase active site where this hydroxy group is involved.

We report the site-specific incorporation of 2,3,5-trifluorotyrosine (F_3Y) into *KlenTaq* DNA polymerase at position 671 and its application to validate the impact of a specific hydrogen bond in abasic site bypass. F_3Y was employed because its pK_a value ($pK_a = 6.4$) is much lower than that of natural tyrosine ($pK_a = 9.9$).[8b]

The F_3Y amino acid was enzymatically synthesized from pyruvate, ammonia, and 2,3,6-trifluorophenol, by using the enzyme tyrosine phenol-lyase (see Scheme S1 in the Supporting Information, Figure 2 A).[10] Mass spectrometric analysis confirmed the identity of F_3Y. The synthesized amino acid was incorporated into *KlenTaq* Y671TAG (generated through site-directed mutagenesis) by non-canonical amino acid mutagenesis, by using a mutated *M. jannaschii* tyrosyl aminoacyl tRNA synthetase and its cognate $tRNA^{Tyr}_{CUA}$.[8c] SDS-PAGE analysis demonstrated that protein expression was only observed in the presence of F_3Y (Figure 2 B). Additionally, the expressed protein was digested with trypsin, and fluorinated tyrosine incorporation was confirmed by MALDI-MS/MS analysis (Figure S1). We termed the *KlenTaq* mutant harboring the fluorinated tyrosine analogue at position 671 "*KlenTaq* F_3Y" (Figure S2).

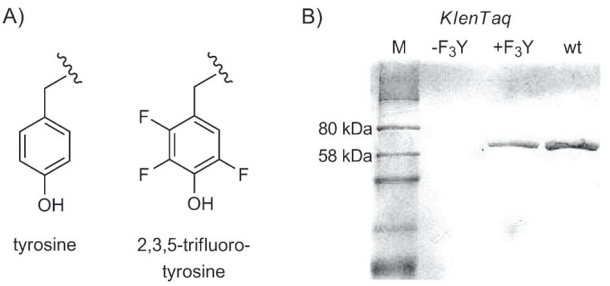

Figure 2. Incorporation of F_3Y into *KlenTaq* DNA polymerase. A) Chemical structures of natural and fluorinated tyrosine side chains. B) SDS PAGE analy sis showing the incorporation of F_3Y into *KlenTaq*. M: Marker; $-F_3Y$: *KlenTaq* expressed without F_3Y; $+F_3Y$: *KlenTaq* expressed with F_3Y; wt: wild type *KlenTaq*.

First, we tested whether the *KlenTaq* mutant follows the A-rule in single primer-extension experiments with a 5′-^{32}P-radiolabeled DNA primer (23 nt) annealed to a template containing an abasic-site analogue, F (Figure 3 A). Single nucleotide incorporation opposite F was conducted with all four nucleotides. All reactions were performed at pH 7.5 and with an incubation time of 120 min. Subsequent analysis by denaturing PAGE showed preferential incorporation of dAMP for both wild-type *KlenTaq* and *KlenTaq* F_3Y, with incorporation of dGMP also detected in both cases (Figure 3 B). Thus, preferential incorporation of purines was shown to be retained in the F_3Y mutant (primer elongation with pyrimidine substrates was not observed under these conditions). Next, we compared the activity of the enzymes when incorporating dAMP opposite a natural dT in the template strand or opposite the abasic-site analogue, F. The polymerases were diluted in a stepwise manner. This assay showed slightly reduced activity for *KlenTaq* F_3Y (compared to wild-type) when using a natural template, but there was more pronounced activity loss when dAMP incorporation opposite F was measured (Figure S3). As dAMP incorporation opposite the lesion was reduced substantially for both enzymes, the reaction time was adjusted to 30 min as opposed to 5 s for the natural template. To further compare the difference in the incorporation efficiency opposite the abasic-site analogue, we performed further single-nucleotide incorporation studies over time, with an excess of enzyme (relative to primer/template) to negate potential different binding efficiencies of the enzymes. As observed previously, there was lower incorporation efficiency for *KlenTaq* F_3Y, as almost full conversion was achieved after 5 min for the wild-type *KlenTaq*, but 30 min were required for the F_3Y mutant (Figure 3 C). We propose that the tyrosine hydroxy group in wild-type *KlenTaq* ($pK_a = 9.9$) is protonated at pH 7.5, thus allowing formation of a hydrogen bond between the hydroxy group and N3 of the incoming adenine. However, reaction conditions presumably favor the deprotonated state of the hydroxy group of fluorotyrosine in *KlenTaq* F_3Y because of the lower pK_a (6.4) of the non-canonical amino acid. Thus, stabilization of the incoming nucleotide in the active site decreases when the hydrogen-bond donor is altered. The possibility of the presence of a counterion in the active site cannot be ruled out, and this might further hamper interactions between the tyrosine hydroxy group and N3 of the incoming dATP.

In summary, we site-specifically introduced 2,3,5-trifluorotyrosine into *KlenTaq* DNA polymerase by in vivo incorporation of a non-canonical amino acid.[8c] We confirmed the importance of hydrogen bonding in abasic-site bypass, especially the impact of the hydrogen bond between the N3 of adenine

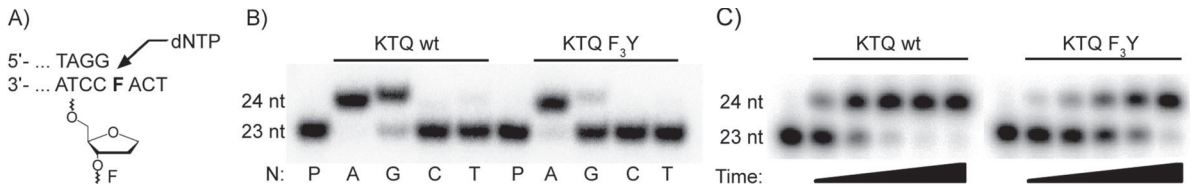

Figure 3. Nucleotide incorporation opposite abasic site F. A) Partial sequence of primer/template. B) Single nucleotide incorporation opposite F in 120 min by wild type *KlenTaq* (KTQ wt) and *KlenTaq* F_3Y (KTQ F_3Y). N: respective dNTP; P: primer. C) Time course experiment of dATP incorporation opposite abasic site F: 30 s, 2 min, 5 min, 10 min, or 30 min reaction time; left lane: primer alone.

and the hydroxy group of Y671. Previous site-directed mutagenesis studies suggested the importance of a potential hydrogen bond between N3 of adenine and the hydroxy group of Y671, as a Y671F mutant showed reduced ability to bypass the abasic site, and reduced incorporation efficiency was observed for the incorporation of 3-deaza-dATP.[6a] Our results corroborate our previous findings and confirm that the hydrogen bond formed between the tyrosine hydroxy group and N3 of the adenine plays an important role in stabilizing the active site. Furthermore, they demonstrate the utility of fluorinated tyrosine analogues in studies investigating hydrogen bond formation at protein active sites.

Experimental Section

Proteins and oligonucleotides. Oligonucleotides were purchased from Metabion (Martinsried, Germany) or Biomers (Ulm, Germany). Wild-type *KlenTaq* was expressed and purified as described previously.[11] To express *KlenTaq* F_3Y, the plasmids pEVOL-3Fy-E3 and pGDR11-Y671TAG (see Scheme S2) were transformed into *Escherichia coli* BL21-Gold (DE3) cells (Agilent Technologies), and the cells were plated on lysogeny broth (LB) agar containing ampicillin (100 $\mu g\,mL^{-1}$) and chloramphenicol (25 $\mu g\,mL^{-1}$), and incubated (37 °C, overnight).

A colony was picked and grown (37 °C, overnight) in 2×YT medium (5 mL) with ampicillin (100 $\mu g\,mL^{-1}$) and chloramphenicol (25 $\mu g\,mL^{-1}$). Expression of *KlenTaq* F_3Y was carried out in 2×YT medium (100 mL) with ampicillin (100 $\mu g\,mL^{-1}$), chloramphenicol (25 $\mu g\,mL^{-1}$), and F_3Y (1 mM), by inoculation with the overnight culture. At OD_{600} 0.6, the cultures were induced with L-arabinose (0.2%) and isopropyl β-D-1-thiogalactopyranoside (IPTG, 1 mM). Cells were further incubated to express protein (6 h, 37 °C), then centrifuged in a Thermo IEC Multi RF model 120 centrifuge (5200 rpm, Rotor 8850, 4 °C, 10 min), and stored (−80 °C). The cell pellet was then suspended in lysis buffer (16 mL, Tris·HCl (500 mM, pH 9.2), NaCl (300 mM), $MgCl_2$ (2.5 mM), Triton X100 (0.1%), lysozyme (0.1 $mg\,mL^{-1}$), and protease inhibitor cocktail (Sigma, 0.1%). After incubation (1 h, 4 °C), the cells were lysed by sonication (Branson Sonifier S-450A, 40% output, power level: 2 15 s, 3 15 s, 4 45 s), heated (85 °C, 30 min), and centrifuged (15000 rpm, Rotor 8850, 4 °C, 15 min). The supernatant was removed and Ni-NTA resin (200 μL) was added. After incubation to bind protein (1 h, 4 °C) the mixture was centrifuged (500 rpm, Rotor 8850, 4 °C, 5 min). The resin was removed by pipetting and placed into a filter spin-column (0.45 μm), and centrifuged (10000 rpm, Rotor 8850, 4 °C, 0.5 min) to eliminate residual supernatant. The resin was then washed with 250 μL of lysis buffer, wash buffer (4:1 ratio of lysis/elution buffer), and eluted in elution buffer (Tris·HCl (100 mM, pH 8), $MgCl_2$ (5 mM), Tween 20 (0.2%), imidazole (200 mM)). The buffer was exchanged by dialysis (Tris·HCl (50 mM, pH 9.2), $(NH_4)_2SO_4$ (16 mM), $MgCl_2$ (2.5 mM), Tween 20 (0.1%), and glycerol (50%)), and the protein was stored at −80 °C. The purified protein was loaded onto a 12% SDS-PAGE gel, electrophoresed (60 V/15 min, 150 V/45 min) and stained with Brilliant Blue G protein stain (Sigma).

Single nucleotide incorporation experiments: For radioactive labeling, primer (400 nM) was incubated in the presence of γ-^{32}P-ATP (400 $nCi\,\mu L^{-1}$, Hartmann Analytic), T4 polynucleotide kinase (0.4 $U\,\mu L^{-1}$, Fermentas) in supplied 1× reaction buffer A for 60 min at 37 °C (50 μL scale). The reaction was terminated (95 °C, 2 min), and the labeled primer was purified by gel filtration (Sephadex G25). Unlabeled primer (20 μL) was added to obtain a final stock concentration of 3 μM. Incorporation opposite F was tested with all four nucleotides. Reaction mixtures (20 μL) contained radioactively labeled primer (100 nM, 5′-d(CGTTGG TCCTGA AGGAGG ATAGG)-3′), F-containing template (130 nM, 5′-d(AAATCA FCCTAT CCTCCT TCAGGA CCAACG TAC)-3′), the respective dNTP (100 μM), and the respective *KlenTaq* DNA polymerase (25 nM), in Tris·HCl (20 mM, pH 7.5), NaCl (50 mM) and $MgCl_2$ (2 mM). Reaction mixtures were incubated at 37 °C and terminated at 120 min by addition of stop solution (45 μL, formamide (80%, *v/v*), EDTA (20 mM), Bromophenol Blue (0.25%, *w/v*), xylenecyanol (0.25%, *w/v*)). After denaturation (95 °C, 5 min), reaction mixtures were separated on a 12% denaturing PAGE gel. Visualization was performed by phosphoimaging.

Assay for incorporation opposite F against time: experiments were performed as described above with the respective *KlenTaq* DNA polymerase (1 μM). Incubation times are given in the figure legend.

Acknowledgements

We thank the staff at the Functional Genomics Center Zürich (FGCZ) for the mass measurements and Dr. Douglas D. Young (College of William & Mary) for advice. This work was funded in part by the National Science Foundation (award no. 1200160). We also acknowledge support by the Konstanz Research School Chemical Biology.

Keywords: abasic site · DNA replication · fluorotyrosine · protein engineering · unnatural amino acid mutagenesis

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