3CAPS - A structural AP-site Analogue as A Tool to Investigate DNA Base Excision Repair

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SUPPLEMENTARY MATERIAL AND METHODS

Preparation of substrates

Oligonucleotides harbouring the synthetic AP-site 3CAPS, the 8-oxo-G as well as the control and complementary oligonucleotides used for the reductive crosslinking and cleavage inhibition assays with bacterial FPG were made on a Polygen DNA-Synthesizer (Polygen GmbH, Langen, Germany) using the phosphoramidite approach. The 8-oxo-G-phosphoramidite was from GlenResearch (Sterling VA, USA) and incorporated according to the manufacturer's guide. Purification was done with ion exchange HPLC (Dionex DNA Pac PA 200; Thermo Fisher Scientific, Reinach, Switzerland) and desalted on SepPak columns (Waters Corporation, Milford MA, USA), followed by drop dialysis on Millipore MF-membrane filters. Oligonucleotides with the sequence d(GCTTCGAGTA A**X**TTGGACAC TATC) (49), where X is 3CAPS in the inhibitor, 8-oxo-G in the substrate and G in the negative control (C-), were annealed to the complementary strand of DNA, bearing a dC at the position opposite X. Where required the single strand was labelled with γ-^{32}P-ATP and used without further purification.

Endo IV incision assay

AP-site incision by recombinant *E.coli* Endonuclease IV (NEB, Ipswich MA, USA) was assessed in NEB3 buffer supplemented with 100 µg/mL BSA according to the supplier's recommendation. Reactions were done in 10 µL volumes containing 0.5 pmol (50 nM) DNA substrate and indicated amounts of recombinant protein at 37°C for 30 min and stopped by the addition of an equal volume of 100 mM Tris-HCl pH 8, 1% SDS, and 200 mM $NaBH_4$.

Reductive crosslinking with bacterial FPG

In a total reaction volume of 40 µL, 40 fmol of γ-^{32}P-labelled double-stranded DNA substrate were incubated with varying amounts of FPG (0, 20, 40 units) at 37°C for 30 min in the reaction buffer according to the supplier's recommendation supplemented with 0.1 M $NaBH_4$. Then, the samples were drop dialysed to remove excess $NaBH_4$ and subjected to a 15% SDS PAGE. The gel was then exposed to FujiFilm Life sciences imaging plates at 4°C overnight. The phosphor screen was then analysed using a FujiFilm FLA-300 IP-reader and AIDA Image Analyzer 4.2 Software (Raytest Isotopentestgeräte GmbH, Straubenhardt, Germany).

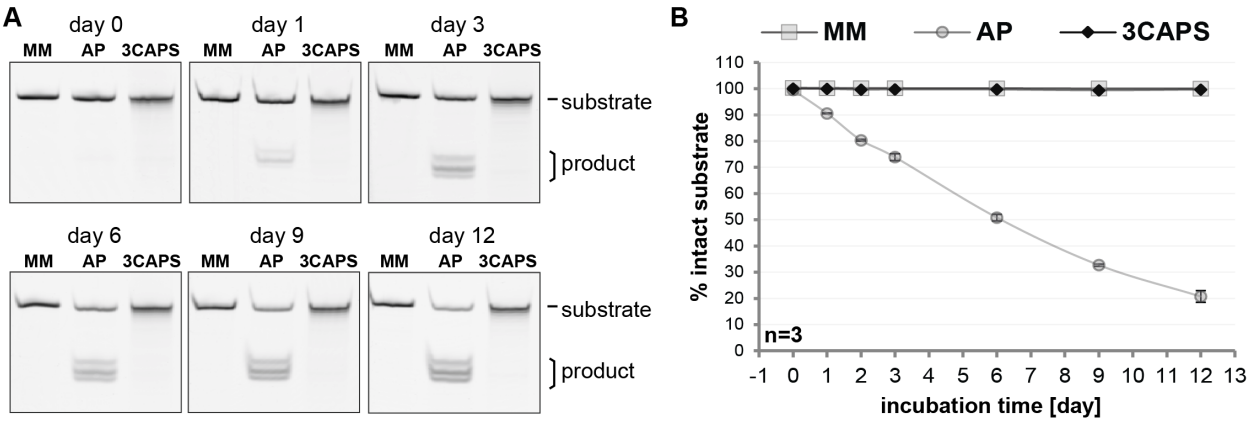
Cleavage inhibition of FPG

In a total reaction volume of 40 µL with buffer conditions according to the supplier's recommendation, 22.5 fmol of γ-^{32}P-labelled 8-oxo-G substrate and varying concentrations (0-0.75 µM) of either the inhibitory substrate DNA with 3CAPS or the intact homoduplex as negative control. Reaction was started by the addition of 2.5 mU of FPG (app. 3.2 fmol). After incubation at 37°C for 1 h, the samples were denatured at 90°C for 10 min and analysed by 20% PAA/8 M urea/1x TBE PAGE. Non enzymatic cleavage of the substrate was analysed in parallel and treated as background.

k_M of FPG for the different substrates was determined by a Hanes-Wilkinson plot and competitive inhibition $IC_{50} = ([S]/k_M + 1)k_i$ was calculated, plotting the percentage of cleaved substrate against the inhibitor concentration.

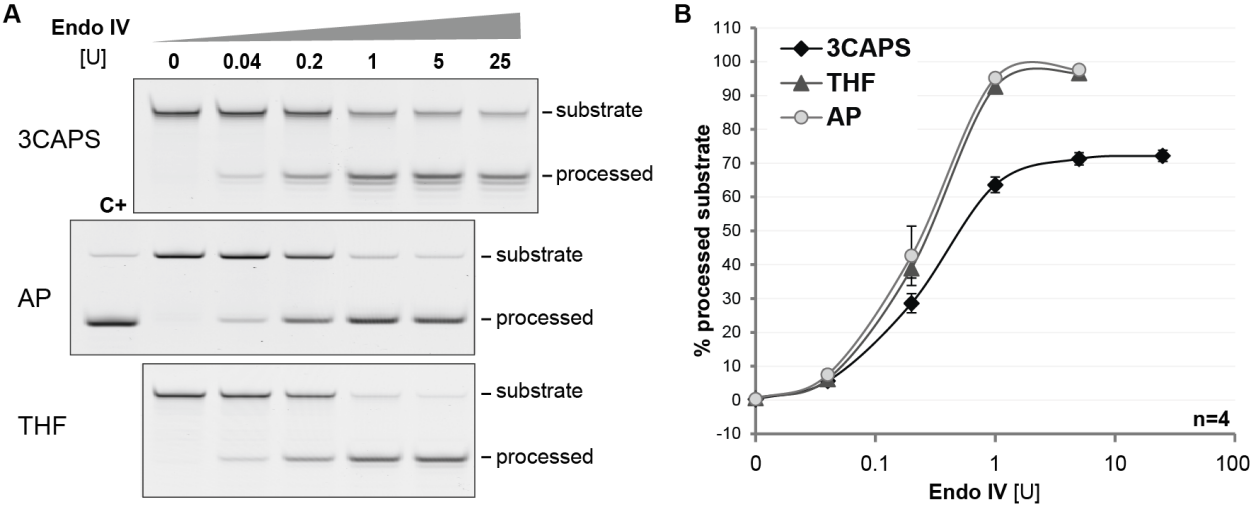
SUPPLEMENTARY FIGURES

Supplementary Figure S1



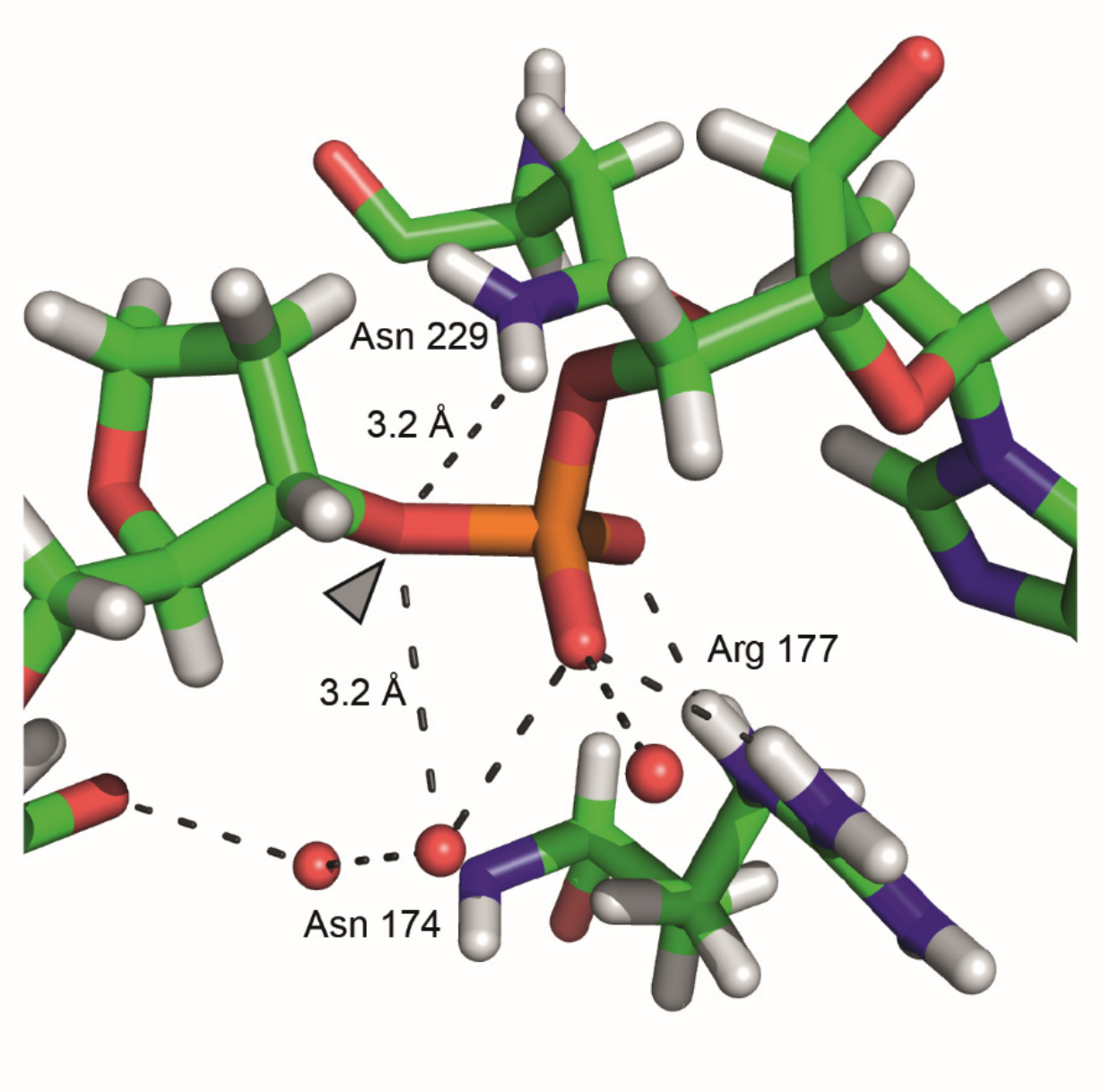
Suppl. Figure S1. Analysis of chemical stability of AP-sites in physiological buffer. (**A**) DNA substrates containing either G•U mismatches (MM), enzymatically produced abasic site (AP) or the synthetic analogue (3CAPS) were incubated in physiological buffer conditions (50 mM Tris-HCl pH 8, 50 mM NaCl, 10 mM $MgCl_2$, 0.5 mM EDTA) at 37°C for up to 12 days. Strand cleavage at AP-sites was detected by denaturing gel electrophoresis, giving rise to 3 distinct products: β- and δ-elimination and spontaneous hydrolysis. (**B**) The spontaneous degradation of the natural and the 3CAPS AP-sites was quantified and remaining substrate was plotted against the incubation time. Error bars, SEM of 3 experiments.

Supplementary Figure S2



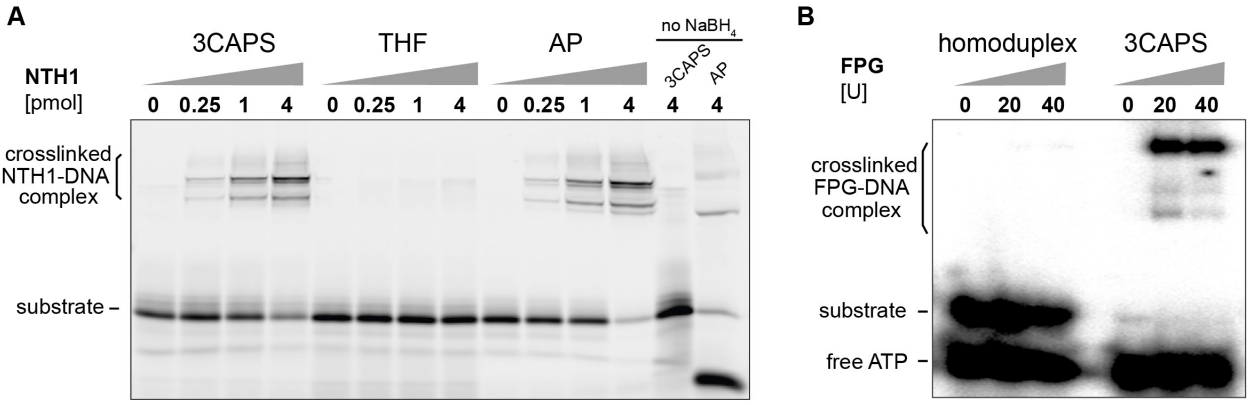
Suppl. Figure S2. The synthetic abasic site analogue 3CAPS is processed by the *Escherichia coli* Endonuclease IV. (**A**) 0.5 pmol of substrate DNA containing enzymatically produced natural abasic sites (AP), the synthetic analogues 3CAPS and THF were incubated with increasing enzyme units of recombinant Endonuclease IV (Endo IV) for 30 min and analysed by denaturing gel electrophoresis. (**B**) Quantitative analysis of AP-site processing with increasing amounts of Endo IV. Lane C+ shows the degradation of the enzymatically produced natural AP-site substrate induced by heating to 99°C in alkaline conditions for 10 min.

Supplementary Figure S3



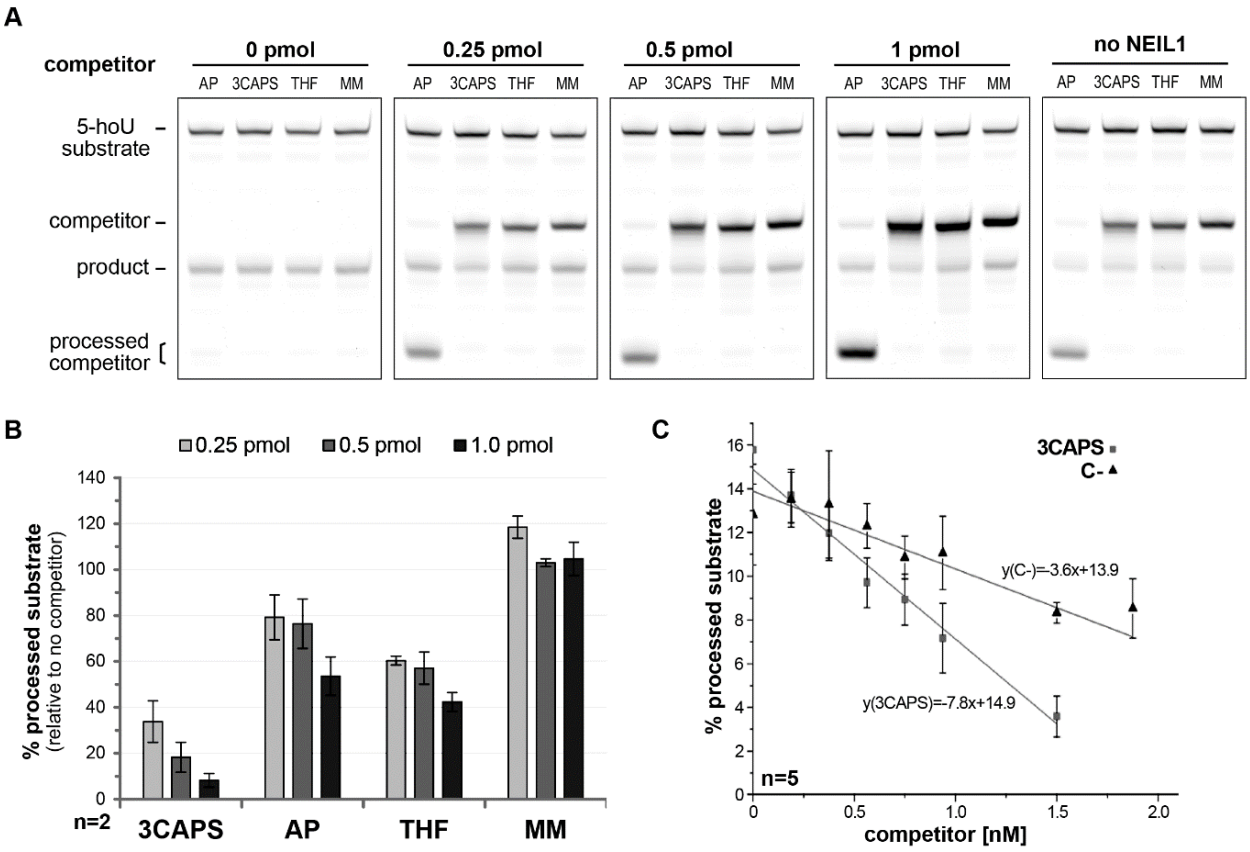
Suppl. Figure S3. Putative interaction model between 3CAPS and APE1. Model of 3'-phosphate group of the abasic site and its interaction with Asn-229, Asn-174, and Arg-177 of APE1 (PDB entry 4IEM) (22), involving a structural water network (red spheres) with hydrogen bonds (dotted lines). The oxygen replaced for a methylene group in the 3CAPS is indicated by the grey arrowhead. In this structure, the 3'-oxygen of the AP-site is engaged in two long range contacts to Asn-229 and to a structural water molecule, which is involved in a hydration network to other water residues. The two non-bridging oxygen atoms of the phosphates are tightly bound by an arginine clamp. Replacing the 3'-oxygen by a CH_2 group, as in 3CAPS, would abolish these weak contacts and is thus expected to disrupt the structural water network, which ultimately may be the cause of the loss of affinity.

Supplementary Figure S4



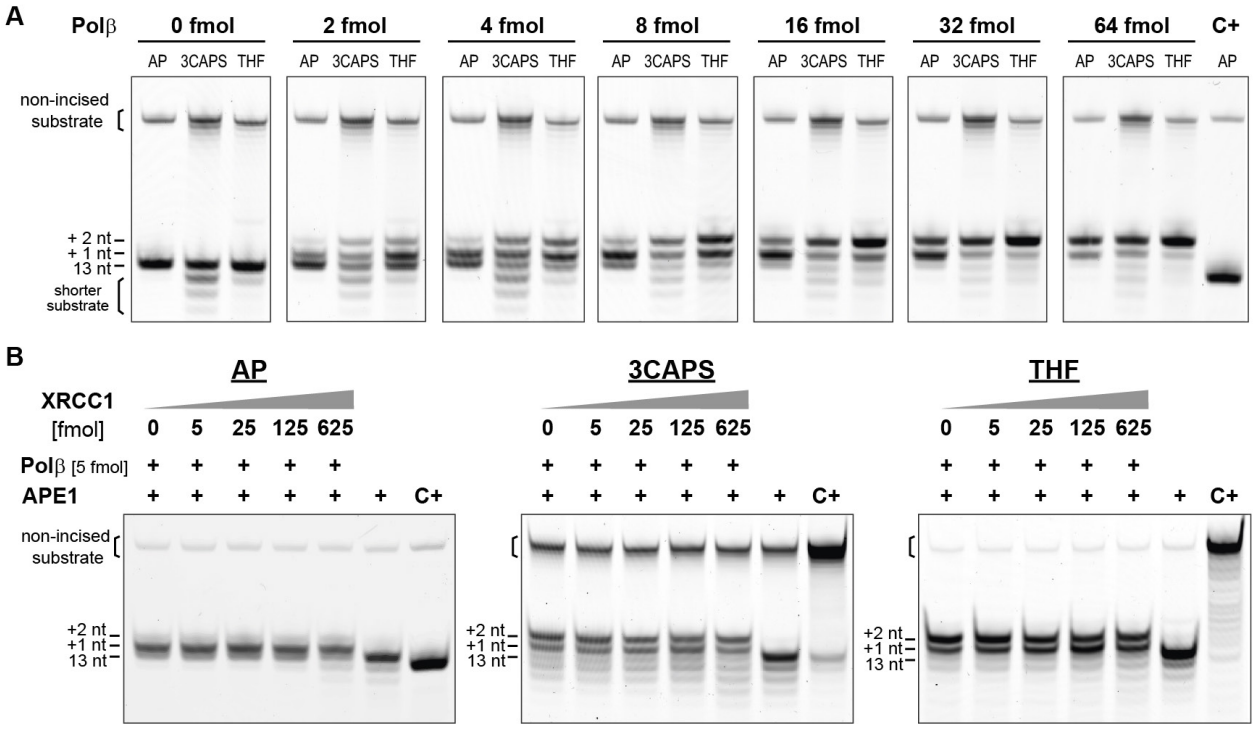
Suppl. Figure S4. Bifunctional DNA glycosylases form a Schiff base interaction with 3CAPS. The functional interaction between human NTH1 (**A**) and bacterial FPG (**B**) with AP-site substrates was assessed by reductive crosslinking with sodium borohydride ($NaBH_4$). (**A**) 1 pmol of fluorescein-labelled substrate DNA was incubated at 37°C with increasing amounts of human NTH1 for 30 min in reaction buffer supplemented with 50 mM $NaBH_4$. (**B**) Increasing concentrations of FPG were incubated with 40 fmol of radioactively-labelled 3CAPS substrates or control homoduplex DNA at 37°C for 30 min in the presence of 100 mM $NaBH_4$. Samples were separated by 15% SDS-PAGE and the formation of covalently linked protein-DNA complexes was detected.

Supplementary Figure S5



Suppl. Figure S5. Competitive inhibition of DNA glycosylase activity by 3CAPS-containing substrates. (**A**) The DNA glycosylase activity of human NEIL1 on 0.5 pmol of 5-hoU substrate was assessed in the presence of increasing amounts of enzymatically generated natural AP-site (AP), the analogues 3CAPS and THF or G•U mismatched (MM) heteroduplex DNA. After 30 min incubation at 37°C, AP-sites were chemically cleaved by heating in alkaline conditions. Reactions without hNEIL1 served as negative controls. (**B**) Shown are the percentage of processed 5-hoU substrates relative to samples without competitor after subtraction of background signal in negative controls. (**C**) Increasing amounts of 3CAPS AP-site analogue containing or homoduplex (C-, negative control) DNA were added to reactions with 2.5 mU bacterial FPG to assess the inhibition of 8-oxo-G processing in 22.5 fmol of radioactively labelled substrate DNA. The percentage of cleaved 8-oxo-G substrate was plotted against the inhibitor concentration to calculate the competitive inhibition IC_{50} value: 1.0 nM for 3CAPS and 1.9 nM for the negative control. In the applied experimental condition, even homoduplex DNA (C-) exhibits a certain inhibitory effect on the cleavage of 8-oxo-G from a double-stranded substrate, most likely owing to FPGs ability to interact with DNA non-specifically (50). However, the FPG inhibition of was much more pronounced when 3CAPS DNA was used as a competitor. Based on measured enzyme constant K_m = 0.7 nM for the 8-oxo-G substrate, the dissociation constant of the inhibitors K_i of 0.6 and 1.1 nM for the 3CAPS and the C- oligonucleotides, respectively, was calculated.

Supplementary Figure S6



Suppl. Figure S6. Stimulation of strand extension and displacement activity of Polβ in 3CAPS- and THF-containing substrate. (**A**) Increasing amounts of Polβ (0-64 fmol) were deployed to analyse strand extension and displacement during repair DNA synthesis. 0.5 pmol of AP-site, 3CAPS and THF substrates were incised with bacterial endonuclease IV (Endo IV). In the presence of 250 fmol XRCC1/Lig3 complex and dCTP/dGTP (50 µM each), allowing for strand extension of two nucleotides, DNA synthesis was induced by the addition of the indicated amount of Polβ. After 10 min of incubation at 37°C, reactions were stopped and analysed. Chemically induced *βδ*-elimination of AP-sites (C+) were used as migration control (13 nt long oligonucleotide with 3'-phosphate end). (**B**) Strand displacement during repair DNA synthesis was analysed under limiting Polβ (5 fmol) conditions and with increasing amounts of XRCC1 (0-625 fmol). In the presence of Polβ, 0.5 pmol of AP-site, 3CAPS and THF substrates were incised with 100 fmol of APE1 for 5 min. DNA synthesis was induced by the addition of XRCC1 and dCTP/dGTP, allowing for primer extension of two nucleotides and assessed after 10 min.