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A thermostable D-polymerase for mirror-image PCR

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ABSTRACT

Biological evolution resulted in a homochiral world in which nucleic acids consist exclusively of D-nucleotides and proteins made by ribosomal translation of L-amino acids. From the perspective of synthetic biology, however, particularly anabolic enzymes that could build the mirror-image counterparts of biological macromolecules such as L-DNA or L-RNA are lacking. Based on a convergent synthesis strategy, we have chemically produced and characterized a thermostable mirror-image polymerase that efficiently replicates and amplifies mirror-image (L)-DNA. This artificial enzyme, dubbed D-Dpo4-3C, is a mutant of *Sulfolobus solfataricus* DNA polymerase IV consisting of 352 D-amino acids. D-Dpo4-3C was reliably deployed in classical polymerase chain reactions (PCR) and it was used to assemble a first mirror-image gene coding for the protein Sso7d. We believe that this D-polymerase provides a valuable tool to further investigate the mysteries of biological (homo)chirality and to pave the way for potential novel life forms running on a mirror-image genome.

INTRODUCTION

All known life forms exclusively use D-configured nucleotides to encode their genetic information in nucleic acids and L-configured amino acids to assemble proteins by ribosomal synthesis. This peculiarity is described as the 'homochirality of life' (1). Already in 1893, Emil Fischer recognized that natural enzymes show stereospecific substrate recognition and formulated the lock-and-key model (2). Reciprocal chiral recognition was demonstrated by Kent and co-workers with the chemically synthesized L-HIV1- and D-HIV1-proteases: the L-enzyme cleaved only L- but no D-peptides, whereas the D-enzyme cleaved only D-but no L-peptides (3). On the level of nucleic acids, reciprocal chiral recognition was corroborated with a D-RNA aptamer binding to mirror-image L-adenosine and its corresponding L-RNA aptamer (so-called Spiegelmer®) comparably recognizing D-adenosine (4). Since compounds made of mirror-image building blocks show high biostability and low immunogenicity, L-nucleic acid aptamers and D-peptides are now being developed as therapeutic modalities (5,6).

The dream of creating a living cell in the lab *de novo* has been around for some time (7,8) and significant progress such as the synthesis of a whole genome now controlling viable cells (9) has been achieved. Also, the vision of mirror life as an object of investigation, e.g. for questions on the origin of life or as a tool to produce valuable polymers of mirror-image chirality has been expressed (10,11). Minimal life based on L-nucleic acids and D-proteins would require at least a minimal L-DNA genome, mirror-image polymerases to copy and transcribe the genome, as well as mirror-image ribosomes and translational factors to translate L-mRNA transcripts into D-proteins.

Several advances in peptide ligation techniques have enhanced the scope of full-length proteins accessible through convergent chemical synthesis strategies (12), the Kay group currently holds the record with a 312-mer (11). The mirror-image configuration of polymerase X from African swine fever virus, the shortest known polymerase (174 amino acids), has recently been demonstrated to elongate an L-DNA primer with L-dNTPs (13,14) and a functional 56-mer L-DNAzyme was made within 36 hours (14). This poses an important proof of concept, however, polymerase X is a thermo-labile repair enzyme (15,16) and its catalytic activity does not meet the requirements for a standard PCR (14).

Here, we describe the preparation of a thermostable mirror-image polymerase that is able to amplify L-DNA in a classical PCR reaction and can even be used to assemble an L-DNA gene from L-DNA oligonucleotides. This artificial enzyme (Figure 1) is a mutant of DNA polymerase IV from *Sulfolobus solfataricus,* a Y-family polymerase consisting of 352 amino acids (17), to our knowledge the longest protein made by chemical synthesis thus far. Furthermore, with an additional single point mutation, this DNA polymerase can be tuned to accept also ribonucleotides as sub-

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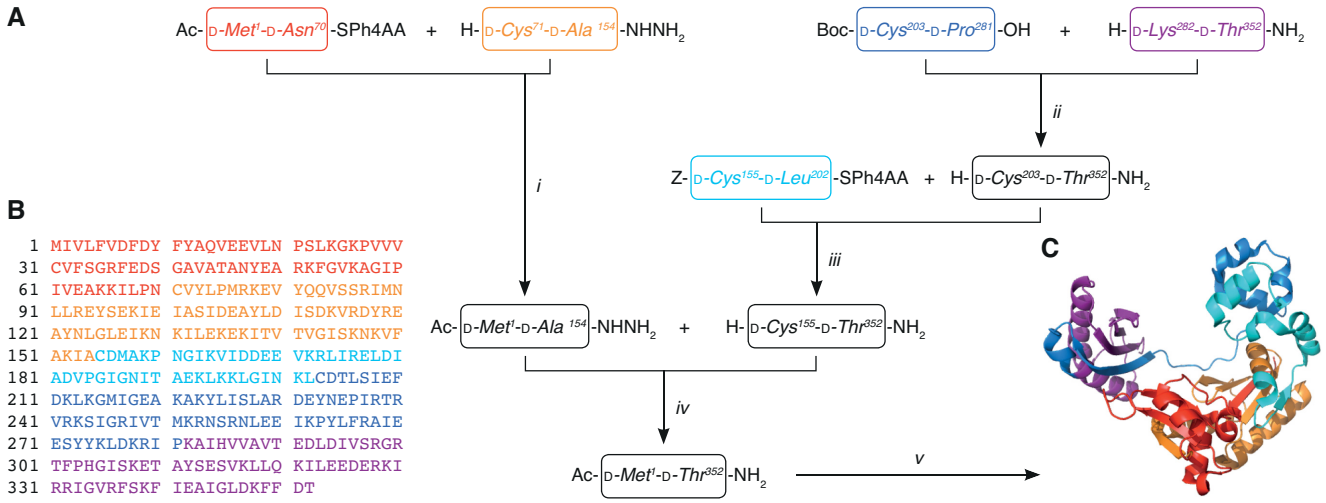

Figure 1. Synthesis strategy for D-Dpo4-3C. (**A**) five fragments were synthesized and assembled as follows: (i) native chemical ligation (NCL) of fragments 1 and 2. Isolated yield ≈ 18%. (ii) Segment condensation of fully protected fragments 4 and 5 followed by deprotection. Isolated yield ≈ 15%. (iii) NCL of fragments 3 and 4•5 followed by Z-deprotection. Isolated yield ≈ 25%. (iv) Thioester-conversion of fragment 1•2 and NCL with fragment 3•4•5. Isolated yield: 10%. (v) Folding. (**B**) sequence of D-Dpo4-3C; coloring as in panel A. (**C**) folded D-Dpo4-3C (artist impression based on PDB 3PR4 (31)).

strates with reasonable efficiency. Thus, this enzyme may be hijacked to act as a DNA-dependent RNA polymerase to prepare longer stretches of L-RNA.

MATERIALS AND METHODS

Chemicals for solid phase peptide synthesis

All chemicals used were of highest available purity. For the solid-phase peptide synthesis, the following amino acid derivatives were purchased from Bachem (Bubendorf, Switzerland): Fmoc-D-Ala-OH, Fmoc-D-Cys(*t*Bu)-OH, Boc-D-Cys(Boc)-OH, Fmoc-D-Asp(OMpe)-OH, Fmoc-D-Glu(O*t*Bu)-OH, Fmoc-D-Phe-OH, Fmoc-Gly-OH, Fmoc-(Dmb)Gly-OH, Fmoc-D-Ile-OH, Fmoc-D-Lys(Boc)-OH, Fmoc-D-Leu-OH, Fmoc-D-Met-OH, Fmoc-D-Asn(Trt)-OH, Fmoc-D-Pro-OH, Fmoc-D-Gln(Trt)-OH, Fmoc-D-Arg(Pbf)-OH, Fmoc-D-Ser(*t*Bu)-OH, Fmoc-D-Thr(*t*Bu)-OH, Fmoc-D-Val-OH and Fmoc-D-Tyr(*t*Bu)-OH.

N-Methyl pyrrolidone (NMP), dimethylformamid e (DMF), hydroxybenzotriazol (HOBt), piperidine, 1,1,1,3,3,3-hexafluoroisopropanol (HFIP), 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-b]pyridinium-3-oxid hexafluorophosphate (HATU) were purchased from IRIS Biotech (Marktredwitz, Germany).

TentaGel-R-Trt resin with a substitution level of 0.22 mmol/g was purchased from Rapp Polymere (Tübingen, Germany). Fmoc-Sieber rink amide NovaSynTG, PyBOP and Z-OSu were from NovaBiochem (now Merck Millipore, Darmstadt, Germany).

N,N-Diisopropylethylamine (DIPEA), trifluoroacetic acid (TFA), 1,2-ethanedithiol (EDT), trifluoroethanol (TFE), trimethylsilyl bromide (TMSBr), acetic anhydride, *para*-acetamidothiophenol (HSPh4AA), mercaptophenyl-acetic acid (MPAA), acetonitrile (ACN; gradient grade and UPLC grade), water (UPLC grade), methanol (UPLC grade), triscarboxyethylphosphine (TCEP) were purchased from Sigma-Aldrich (Munich, Germany). Dithiothreitol (DTT) was from Applichem (Darmstadt, Germany).

Sodiumdihydrogenphosphate dihydrate was from Carl Roth (Karlsruhe, Germany). Guanidine hydrochloride was from Fisher Scientific (Schwerte, Germany).

Analytical UPLC

Analytical UPLC runs were done using a Waters (Eschborn, Germany) Acquity system consisting of a sample manager FTN, quaternary solvent manager, PDA detector, column manager and column manager aux. The following Waters Acquity UPLC® columns were used: BEH C4 2.1 × 100 mm 300 Å 1.7 µm, BEH C8 2.1 × 100 mm 130 Å 1.7 µm, BEH C18 2.1 × 50 mm 130 Å 1.7 µm and BEH C18 2.1 × 100 mm 300 Å 1.7 µm. Separation was achieved using a gradient of solvent A (0.1% TFA in H_2O) and solvent B (0.1% TFA in ACN) from 5% B to 95% B in 2.5 min at a flowrate of 0.613 ml/min for 50 mm columns or in 5 min at a flowrate of 0.5 ml/min for 100 mm columns.

Preparative HPLC

For purification of peptides and peptide derivatives, a Waters preparative HPLC consisting of a 1525 pump module, 2707 autosampler, fraction collector III and a dual wavelength detector 2489 was used. The mobile phase was H_2O/ACN containing 0.1% TFA (v/v). Columns Jupiter C18, 250 × 21.2 mm, 300 Å, 10 µm; Jupiter C18, 250 × 10.0 mm, 300 Å, 10 µm and Jupiter C4, 250 × 10.0 mm, 300 Å, 10 µm (all from Phenomenex, Aschaffenburg, Germany) were used as stationary phases.

LC–MS analyses

Samples were separated on an Acquity UPLC BEH300 C4 column (2.1 × 100 mm, 300 Å, 1.7 µm; Waters) attached

to an Agilent 1290 chromatographic system (Agilent Technologies, Waldbronn, Germany). The column temperature was set to 30°C, the flow rate was 0.2 ml/min and the gradient of solvent A (0.01% TFA in H_2O) and solvent B (0.01% TFA in ACN) was 5% B from 0–3 min, 5–95% B during 3–18 min, hold 95% B from 18–21 min, re-equilibrate column with 5% B from 21.1 to 28 min. From 6 to 28 min, the flowpath was switched to an online coupled ESI–QTOF 6520 (Agilent Technologies) mass spectrometer. MS data were recorded in positive mode and evaluated using the MassHunter software (Agilent Technologies).

Protein gel electrophoresis

NCL reactions were analysed by SDS-PAGE. Therefore, 2 μl sample were diluted with 28 μl of 100 mM TCEP and 3 μl of the diluted sample were mixed with 7 μl H_2O and 5 μl NuPAGE® LDS sample buffer. After 5 min heat denaturation at 95°C, samples were applied to NuPAGE® novex 10% Bis–Tris gels that were run in MES-SDS running buffer in an XCell SureLock electrophoresis chamber for 35 min at 200 V. Gels were stained using SimplyBlue™ SafeStain. Precision Plus Protein™ Dual Xtra Prestained Protein Standards were from Bio-Rad (Munich, Germany), all other materials were obtained from Fisher Scientific (Schwerte, Germany).

Chemical synthesis of peptide fragments

*Synthesis of fragment 1 (Ac-*D*-Met^{1}-*D*-Asn^{70}-SPh4AA).* 0.1 mmol TentaGel-R-Trityl resin was loaded with Fmoc-D-Asn(Trt)-OH as described previously (18): 0.10 mmol resin was incubated with 0.6 mmol thionylchloride twice for 30 min and subsequently washed with DCM. Following this, the resin was incubated with 0.6 mmol Fmoc-D-Asn(Trt)-OH, 2.4 mmol DIPEA in 6 ml DCM for 90 min. Afterward, the resin was blocked using a solution of 10% MeOH (v/v), 10% DIPEA (v/v) in DCM three times for 10 min each and washed with DCM. Automated synthesis was done using an ABI 433 with the FASTmoc protocol. 10 eq. amino acid were activated using 9 eq. HATU and 20 eq. DIPEA in NMP. Coupling time was 45 min and Fmoc-deprotection was performed with 20% piperidine (v/v) in NMP three times for 7 min each. Double coupling was performed after 40 amino acids. Acetylation of the *N*-terminus was performed with 10% acetic anhydride (v/v) and 10% DIPEA (v/v) in DMF three times for 10 min each. The cleavage of the fully protected peptide acid was achieved by incubating the peptidyl resin twice in 10 ml 30% HFIP (v/v) in DCM for 2 h. The peptide was filtered to remove the resin, the solvent was evaporated and the residue was precipitated using ice cold diethyl ether. The precipitated peptide was isolated and dried. Yield: ≈ 600 mg.

5 μmol fully protected peptide, 5 eq. PyBOP and 100 eq. *para*-acetamidothiophenol were dissolved in 6 ml DCM. After addition of 10 eq. DIPEA the mixture was stirred for 4 h. Following this the solvent was evaporated and the residue precipitated by ice cold diethyl ether. The precipitated peptide ester was dried and subsequently protection groups were cleaved off using 2.5% EDT (v/v), 2.5% water (v/v), 2.5% TIS (v/v) in TFA for 2 h. Following the evaporation of TFA, the peptide was precipitated with ice cold diethyl ether. Reversed phase HPLC purification of the peptide ester was performed on a C18 column using an H_2O/ACN gradient. Fractions that contained desired product with a purity of at least 90% were combined and freeze dried. After conversion and purification of the entire 0.1 mmol synthesis, the yield is ≈ 63 mg.

*Synthesis of fragment 2 (H-*D*-Cys^{71}-*D*-Ala^{154}-NHNH$_2$).* 0.1 mmol TentaGel-R-Trityl resin was loaded with hydrazide as described previously (19,20). Therefore, 0.10 mmol resin were incubated twice with 0.6 mmol thionylchloride for 30 min and subsequently washed with DCM and 50% DMF (v/v) in DCM. Following this, the resin was incubated with 5% NH_2NH_2 (v/v) in DMF twice for 30 min. Afterward, the resin was blocked using a solution of 10% MeOH (v/v), 10% DIPEA (v/v) in DCM three times for 10 min each and washed with DCM. Coupling of the first amino acid was achieved by incubating the resin with 1 mmol Fmoc-D-Ala-OH, 0.9 mmol HATU, 2 mmol DIPEA dissolved in NMP for 1 h. Automated synthesis was done as described for fragment 1; double coupling was performed after 43 amino acids.

Cleavage of the peptide hydrazide from the resin was achieved by incubating the peptidyl resin in 2.5% EDT (v/v), 2.5% H_2O (v/v), 2.5% TIS (v/v) in TFA for 2 h. Following the evaporation of TFA, the peptide was precipitated with ice cold diethyl ether. Yield: ≈ 800 mg. Reversed phase HPLC purification of the peptide ester was performed on a C18 column using an H_2O/ACN gradient. Fractions that contained the desired product with a purity of at least 90% were combined and freeze dried. Yield from an entire 0.1 mmol synthesis: ≈ 62 mg.

*Synthesis of fragment 3 (Z-*D*-Cys^{155}-*D*-Leu^{202}-SPh4AA).* 0.1 mmol TentaGel-R-Trityl resin was loaded with Fmoc-D-Leu-OH as described previously (18): 0.10 mmol resin was incubated with 0.6 mmol thionylchloride twice for 30 min each and subsequently washed with DCM. Following this, the resin was incubated with 0.6 mmol Fmoc-D-Leu-OH, 2.4 mmol DIPEA in 6 ml DCM for 90 min. Afterward, the resin was blocked using a solution of 10% MeOH (v/v), 10% DIPEA (v/v) in DCM three times for 10 min each and washed with DCM. Automated synthesis, cleavage and workup were done as described for fragment 1. Yield: ≈ 550 mg.

5 μmol of the *N*-terminally Z- and completely side chain protected peptide was dissolved in 6 ml DCM. After addition of 5 eq. PyBOP, 10 eq. DIPEA and 100 eq. *para*-Acetamidothiophenol, the mixture was stirred for 4 h. Then the DCM was evaporated, the peptide was precipitated and washed with ice-cold diethyl ether. The side chain protecting groups were removed by treatment with 2.5% EDT, 2.5% water, 2.5% TIS in TFA for 2 h. After the evaporation of TFA the peptide was precipitated and washed with ice cold diethyl ether. The peptide-*para*-acetamidothiophenylester was then purified by reversed-phase HPLC. Fractions that contained the desired product with a purity of at least 90% were combined and freeze dried. Yield: ≈ 68 mg calculated for the entire 0.1 mmol synthesis.

Synthesis of fully side chain protected fragment 4 (Boc-D-Cys^{202}-D-Pro^{281}-OH). 0.1 mmol TentaGel-R-Trityl resin was loaded with Fmoc-D-Pro-OH following an earlier protocol (18): 0.1 mmol resin was incubated with 0.6 mmol thionylchloride twice for 30 min and subsequently washed with DCM. Following this, the resin was incubated with 0.6 mmol Fmoc-D-Pro-OH, 2.4 mmol DIPEA in 6 ml DCM for 90 min. Afterward, the resin was blocked using a solution of 10% MeOH (v/v), 10% DIPEA (v/v) in DCM three times for 10 min each and washed with DCM.

Automated synthesis was done as described for fragment 1; double coupling was performed after 41 amino acids. Cleavage and workup were done as described for fragment 1. After the synthesis protocol for this fragment was established, production of the fully-protected peptide was outsourced to Bachem, Bubendorf, Switzerland and 10 g of the fully protected, unpurified material were obtained (full-length product content ≈ 20%).

Synthesis of fully side chain protected fragment 5 (H-D-Lys^{282}-D-Thr^{352}-NH$_2$). 0.1 mmol Fmoc-Sieber rink amide NovaSynTG resin was Fmoc-deprotected and loaded with Fmoc-D-Thr(tBu)-OH using 5 eq. amino acid, eq. 4.9 eq. HATU and 10 eq. DIPEA in 6 ml NMP for 45 min. Automated synthesis was done as described for fragment 1; double coupling was performed after 42 amino acids. Cleavage and workup were done as described for fragment 1. After the synthesis protocol for this fragment was established, production of the fully-protected peptide was outsourced to Bachem, Bubendorf, Switzerland and 20 g of the unpurified, fully protected material were obtained (full-length product content ≈ 20%).

Assembly of full-length D-Dpo4 from peptide fragments

Native chemical ligation of fragments 1 and 2. Fragment 1 (Ac-D-Met^1-D-Asn^{70}-SPh4AA) and fragment 2 (H-D-Cys^{71}-D-Ala^{154}-NHNH$_2$) were dissolved 2 mM in ligation buffer A (50 mM NaH_2PO_4, 6 M guanidine HCl, 200 mM MPAA, 20 mM TCEP, pH 7.0). The reaction mixture was shaken at 30°C for 24 h. HPLC-purification was carried out using a preparative C4 column and a gradient of 30–95% ACN (0.1% TFA) in 30 min. Product containing fractions were combined and freeze dried. Yield: 18%

Segment condensation of fragments 4 and 5. 5 μmol fragment 4 (Boc-D-Cys^{202}-D-Pro^{281}-OH) and 1 eq. fragment 5 (H-D-Lys^{282}-D-Thr^{352}-NH$_2$) were dissolved in 25% (v/v) TFE in DCM according to Kuroda *et al.* (21). After addition of 5 eq. PyBOP and 10 eq. DIPEA, the mixture was stirred overnight. After evaporating the solvent, the peptide was precipitated using ice cold diethyl ether and filtered off.

Removal of the side chain protection groups of the peptide was performed with 2.5% EDT, 2.5% water, 2.5% TIS in TFA for 2 h. Following the evaporation of TFA, the peptide was precipitated with ice cold diethyl ether. Reversed phase HPLC purification of the crude unprotected peptide was performed on a C18 column using an H_2O/ACN gradient. Fractions that contained the desired product were combined and freeze dried. Yield: 15%. Note that the starting materials are unpurified and contain ∼20% of the desired full-length educts. The impurities in the starting material are not considered as educts in the calculation of the yield.

Native chemical ligation of fragments 3 and 4●5. Fragment 3 (Z-D-Cys^{155}-D-Leu^{202}-SPh4AA) and Fragment 4●5 (H-D-Cys^{202}-D-Thr^{352}-NH$_2$) were dissolved in ligation buffer B (50 mM NaH_2PO_4, 6 M guanidine HCl, 200 mM MPAA, 10 mM TCEP, pH 7) to a final concentration of 2 mM each. The reaction mixture was shaken at 30°C overnight. Afterwards, the mixture was purified by reversed-phase HPLC and product containing fractions were combined and freeze dried. For removal of the *N*-terminal Z-protecting group following Kiso *et al.* (22), the peptide was dissolved in 270 eq. TFA and 50 eq. thioanisol and shaken at room temperature for 4 h. After evaporation of TFA, the peptide was precipitated and washed with ice cold diethyl ether and purified by reversed-phase HPLC. Yield ≈ 25%.

Native chemical ligation of fragments 1●2 and 3●4●5. Oxidation of the hydrazide functionality of fragment 1●2 (Ac-D-Met^1-D-Ala^{154}-NHNH$_2$), transformation into a thioester and native chemical ligations was done following the protocol from Zheng *et al.* (20): fragment 1●2 (Ac-D-Met^1-D-Ala^{154}-NHNH$_2$) was dissolved 2 mM in denaturing sodium phosphate buffer (100 mM NaH_2PO_4, 6 M guanidine-HCl, pH 3) and placed in a –15°C ice-bath. Oxidation was achieved by adding $NaNO_2$ to a final concentration of 10 mM. After 15 min, the solution was mixed with an equal volume of a 2 mM solution of fragment 3●4●5 (H-D-Cys^{155}-D-Thr^{352}-NH$_2$) in ligation buffer C (200 mM NaH_2PO_4, 6 M guanidine–HCl, pH 7, 400 mM MPAA, 20 mM TCEP). The reaction mixture was kept shaking at 30°C for overnight. The reaction was quenched by adding 50 μl 400 mM TCEP to 200 μl NCL reaction and incubating for 10 minutes at room temperature. The final product was then purified over a C18 column (Phenomenex, 250 × 4.6 mm, 5 μm, 300 Å) using a gradient of 30% to 90% ACN in H_2O with 0.1% TFA. Yield ≈ 10%.

Refolding of D-Dpo4-3C

Ac-D-Met^1-D-Thr^{352}-NH$_2$ was dissolved to a final concentration of 2 mg/ml in folding buffer (10 mM Tris–HCl, 6 M guanidine–HCl, 10 mM KCl, 1 mM DTT, 0.1 mM EDTA, pH 7.4). After incubating at 95°C for 5 min, the mixture was transferred into a dialysis tube with a molecular weight cut-off of 4000 Da and dialyzed against decreasing guanidine HCl concentrations (4 M, 2 M, 1 M and twice 0 M) in the same buffer for at least 3 h each. Afterward, the mixture was transferred into a reaction vessel and stored at 4°C.

Synthesis history

The initial synthesis strategy was based on eight fragments with a length of around 50 amino acids. We tested several different ligation sites and also different ligation methodologies such as clostripain-mediated ligation (23) or kinetically controlled NCL (24). Where possible, we first validated the strategies using recombinantly expressed fragments of Dpo4 before synthesizing the corresponding fragments as described (13). The most important advances that eventually allowed the synthesis of useful amounts of material

were the synthesis of longer fragments in order to reduce the number of ligations, and use of the peptide–hydrazide method (19,20) for ligation of the two major fragments, which led to less side-product formation and allowed for a simplified purification.

Recombinant expression of L-Dpo4 and mutants thereof

Since the codon usage of *Sulfolobus solfataricus* differs from *Escherichia coli*, an *E. coli*-codon-optimized synthetic gene for wild-type *S. solfataricus* polymerase Dpo4 was purchased from GeneArt (Regensburg, Germany). The synthetic gene sequence was provided in pENTRY-IBA10 vector (IBA GmbH, Göttingen, Germany). The codon-optimized open reading frame including is given in Supplementary Table S1.

The synthetic gene was subcloned into the expression vector pASG-IBA5, thereby adding a Strep-Tag to the N-terminus of the protein and two amino acids to the C-terminus. The encoded amino acid sequence is given in Supplementary Table S1.

Wildtype Dpo4 contains only one cysteine. For the development of a valid synthesis strategy, we had to create a sufficient number of ligation sites. Therefore, we produced several mutants of Dpo4 and tested whether or not the respective amino acid substitutions have a deleterious effect on the enzyme's activity, and we eventually ended up with the mutant Dpo4 Ala71Cys Ala155Cys Val203Cys.

Mutations Ala71Cys Ala155Cys and Val203Cys were introduced by site-directed mutagenesis using the QuikChange Lightning Kit (Agilent) using primers given in Supplementary Table S2.

To test possible tolerance of ribonucleoside-triphosphates as substrates, mutations Tyr12Ala and Tyr12Ser were introduced by the same method, primer sequences are given in Supplementary Table S2.

Expression plasmids were transformed into *E. coli* strain NEB Express (New England Biolabs, Frankfurt, Germany) and 200 ml EnPresso-Medium (BioSilta, St. Ives, UK) with Ampicillin (Applichem, Darmstadt, Germany) was inoculated with the respective expression strain. Expression and purification over StrepTrapHP-columns using an ÄKTA-Express instrument (both GE Healthcare, Uppsala, Sweden) were conducted as described previously (25). Fractions were analyzed by SDS-PAGE and correct fractions were pooled, concentrated and re-buffered using VivaSpin 15R concentration devices with 10 000 molecular weight cut-off (MWCO) (Sartorius Stedim Biotech, Göttingen, Germany). Purified protein was stored at –20°C in a buffer consisting of 10 mM Tris–HCl pH 7.4, 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 50 % glycerol. Protein concentrations were determined as described previously (25).

Quantification of specific enzyme activity

Primer extension assays were assembled that contained 1x Thermopol Buffer (New England Biolabs, Frankfurt, Germany), 1 M betaine, 200 μM D-dNTPs (rovalab, Teltow, Germany) or L-dNTPs (GeneACT Inc., Shojima, Kurume, Fukuoka, Japan), 4 μM each of 'primer 19' (19-mer D- or L-DNA) and 'template 83' (83-mer D- or L-DNA), and known amounts of polymerase. Oligonucleotide sequences are given in Supplementary Table S3. Prior to the addition of polymerase, the mixture was heated to 95°C for three minutes and slowly cooled to room temperature for annealing. L- or D-Dpo4 was then added on ice. We used L-DNA oligonucleotides and L-dNTPs with D-Dpo4 and vice versa. Reactions were incubated for 10 min at 65°C and chilled on ice. To quantify the activity of the polymerases, the amount of inorganic pyrophosphate that is formed on each nucleotide incorporation event was determined using the PPiLight inorganic pyrophosphate detection kit (LONZA). Therefore, reactions were assembled in white-walled 96-well plates (twin.tec real time PCR plates, Eppendorf, Hamburg, Germany) that contained 36 μl H_2O, 4 μl sample (aqueous solutions of inorganic pyrophosphate or primer extension reaction) and 20 μl each of reconstituted conversion and detection reagent. We performed two pyrophosphate determinations from each primer elongation assay to rule out the influence, e.g. of air bubbles that inevitably form on assembly of the detection assay. Because the timing of the maximum chemiluminescence is dependent on the amount of pyrophosphate, the chemiluminescence was recorded every 90 s using a PolarStar optima plate reader (BMG) until the signal declined in all wells and the respective maximum values were used for all further calculations. After subtraction of the relevant negative control (water control for the standards, no enzyme control for primer extension assays), we calculated the absolute amount of pyrophosphate formed by a given amount of polymerase.

Polymerase chain reaction using mirror-image Dpo4-3C

Reactions with a final volume of 7.5 μl each were assembled that contained 1x Thermopol buffer, 0.2 mM D- or L-dNTPs, 1 M betaine, 1 mM DTT, 1 μM Sso7d D- or L-oligonucleotide A (21-mer) and C (81-mer) as forward and reverse primers and 10 nM Sso7d D- or L-oligonucleotide B (67-mer) as template and the amount of L- or D-Dpo4 that releases 350 pmol pyrophosphate within 10 minutes in our activity assay (2 μl D-Dpo4-3C or 4.3 ng L-Dpo4-3C or 3.4 ng wildtype L-Dpo4). For initial denaturation and annealing prior to the addition of Dpo4 and DTT, the mastermix was heated to 95°C for 5 min and slowly (0.2°C/s) cooled to room temperature. The reactions went through 30 cycles of 1 min 56°C, 30 min 60°C, 20 s 83°C. PCR products were analyzed by denaturing TBE-Urea PAGE. 0.5 volumes of loading buffer consisting of 95% formamide, 18 mM EDTA and 0.025% SDS was added to the samples and they were heated to 95°C for 5 min for full denaturation. Samples were applied to denaturing gels consisting of 1× TBE buffer (Fisher Scientific, Schwerte, Germany), 10% acrylamide:bisacrylamide 4K 19:1 and 7.2 M Urea (both from Applichem, Darmstadt, Germany). Following electrophoresis (20 min at 12 W per gel for 8 × 8 cm gels; 45 min at 600 V for 18 × 18 cm gels), gels were stained using ethidium bromide in 1× TBE buffer. The gel shown in Figure 3 was scanned with a MolecularImager FX (Bio-Rad, Munich, Germany) and densitometric analysis was performed using the ImageLab software (Bio-Rad, Munich, Germany).

For gene assembly, we prepared 30 μl reactions containing 1× Thermopol buffer, 0.2 mM D- or L-dNTPs, 1 M betaine, 1 mM DTT, 2 μM Sso7d D- or L-oligonucleotides A and F (23-mer), 200 nM Sso7d D- or L-oligonucleotides B and E (67-mer), 20 nM Sso7d D- or L-oligonucleotides C and D (67-mer), and the amount of L- or D-Dpo4 that releases 1350 pmol pyrophosphate within 10 min in our activity assay. Initial denaturation and cycling were done as described above. The oligonucleotide sequences are given in Supplementary Table S3.

DNase I digest

PCR assays or 100 pmol each of Sso7d D- or L-oligonucleotides A-F in 1x DNase I buffer were incubated with 2 units DNase I (New England Biolabs, Frankfurt, Germany) at 37°C for 1 h and analysed by denaturing PAGE next to undigested controls.

Primer extension assay for ribonucleotide incorporation

10 μl reactions were assembled that contained 1× Thermopol buffer, 200 μM D-dNTPs or D-NTPs, 1 M betaine, 0.4 μM each D-template and radiolabeled D-primer, and 10 ng L-Dpo4-3C or mutants thereof (Tyr12Ala, Tyr12Ser). Prior to the addition of polymerase, the reactions were heated to 95°C for 5 min and slowly cooled to room temperature for annealing, then primer extension was allowed to proceed at 65°C for 30 min. The template was Sso7d oligonucleotide C, the sequences of 25-mer D-DNA and RNA primer ('Primer 25') are given in Supplementary Table S3. Radiolabeling was performed using T4 polynucleotide kinase (Invitrogen, Karlsruhe, Germany), and [γ-^{32}P]adenosine 5'-triphosphate (Hartmann Analytic, Braunschweig, Germany). For analysis, the samples were applied to a denaturing sequencing gel (24% acrylamide:bisacrylamide 19:1, 7.2 M urea, 1× TBE buffer) and run at 50 W constant power for 4.5 h. A K-Screen (Kodak; obtained from Bio-Rad, Munich, Germany) was exposed at –80°C overnight and scanned with a MolecularImager FX (Bio-Rad, Munich, Germany).

RESULTS AND DISCUSSION

To yield the full-length D-enzyme, we devised a convergent synthesis strategy with five fragments joined by native chemical ligation (NCL) (26) and segment condensation (Figure 1). NCL requires a C-terminal thioester function and an N-terminal cysteine at the designated junction site. Since the native protein sequence contains only one cysteine, three further cysteines were introduced in place of Ala71, Ala155 and Val203 to enable ligation at these positions. The corresponding mutant, dubbed L-Dpo4-3C, was produced by recombinant expression. Since we found the artificially introduced cysteines to be only minimally disruptive (Supplementary Figure S1), we chose not to use ligation-desulfurization chemistry (27,28) to avoid further reaction and purification steps, which could however be used to produce an exact mirror image of the wildtype enzyme. On this basis, the fragments of D-Dpo4-3C were synthesized with appropriate N- and C-terminal functionalities and assembled as follows: fully protected fragments 4 (Boc-D-Cys^{203}–D-Pro^{281}-OH) and 5 (H-D-Lys^{282}–D-Thr^{352}-NH_2) were joined by segment condensation. After deprotection, fragment 3 (Z-D-Cys^{155}–D-Leu^{202}-*para*-acetamidothiophenylester (SPh4aa)) was linked by NCL; the Z-protection group prevents oligomerization or circularization of fragment 3. Fragments 1 (Ac-D-Met^{1}–D-Asn^{70}-SPh4aa) and 2 (H-D-Cys^{71}–D-Asn^{154}-hydrazide (-$NHNH_2$)) were fused by NCL. To avoid issues such as circularization, oligomerization or premature thioester hydrolysis during the first NCL, fragment 2 was augmented with a *C*-terminal hydrazide function instead of a thioester function. Hydrazides are inert under NCL conditions and prior to the following NCL, they can selectively be converted into a reactive thioester by oxidation to an azide (-N_3) and subsequent thiolysis with a thiol (12,19). The full-length D-enzyme was finally obtained by NCL of the joint fragments 1•2 and 3•4•5 following thioester conversion and Z-deprotection, respectively. All NCL reactions were carried out under denaturing conditions in the presence of 6 M guanidine–HCl (GuaHCl). All precursors (Supplementary Figures S2–S9) and the final product (Figure 2) were analysed by UPLC and LC–MS. The final product was folded into its active conformation by slow removal of GuaHCl using a stepwise dialysis protocol. We note that the yield of this protocol still poses a bottleneck and we continue to work on alternatives such as on-column folding of immobilized material. We tested the activity and applicability of D-Dpo4-3C in a number of biochemical assays. At first, we determined the specific activity by quantification of inorganic, achiral pyrophosphate that is released on the incorporation of dNTPs in a primer extension assay. Using primer, template and dNTPs of the respective handedness, we applied this assay to compare the activity of recombinant wildtype L-Dpo4, L-Dpo4-3C and synthetic D-Dpo4-3C (Supplementary Figure S1) in order to normalize the catalytic activities for all downstream assays.

Using equal catalytic activities of D-Dpo4-3C and L-Dpo4 (wildtype and -3C) with the appropriate substrates in PCR, we found strong bands of comparable intensity on an ethidium bromide-stained gel representing the expected full-length product already after 10 cycles (Figure 3; Supplementary Figure S10). To confirm that the product of the mirror-image PCR actually is L-DNA, we ran another PCR, split the PCR products in halves and one aliquot was treated with DNase I. The product of the mirror-image PCR was fully resistant to nucleolytic digest, whereas the product from the natural handedness PCR was fully digested (Supplementary Figure S11A). Analogous to the Stemmer assembly (29), we then aimed to assemble a first mirror-image gene as a proof of concept for the generation of longer stretches of mirror-image dsDNA; as a model, we chose the coding sequence for Sso7d, one of the most abundant proteins in *S. solfataricus* P2 (207 bp, GenBank AE006641.1). We reassured the chirality of the oligonucleotides used by DNase I treatment and, as expected, we found full digestion of the D-DNA oligonucleotides, whereas the L-DNA oligonucleotides were fully resistant to nucleolytic digest (Supplementary Figure S11B). Cross-chiral activity of Dpo4 can be excluded (Supplementary

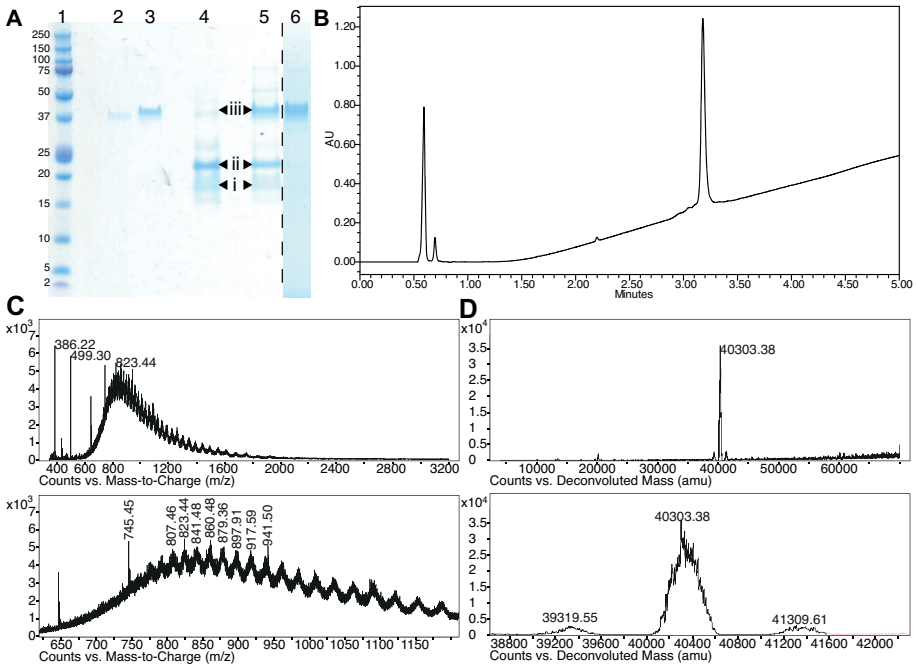

Figure 2. Analysis of the final ligation step to yield D-Dpo4-3C. (**A**) SDS-PAGE analysis. Lane 1, protein ladder. Lanes 2 and 3, 50 and 200 ng, respectively, of recombinant L-Dpo4-3C. Lanes 4 and 5, NCL reactions just after assembly (≈ 1 min) and after 24 h, respectively. Lane 6, purified D-Dpo4-3C (from a different gel, scaled to size). Band i, fragment 1•2. Band ii, fragment 3•4•5. Band iii, D-Dpo4-3C. (**B**) UPLC profile of purified D-Dpo4-3C. Column: BEH 300 C8 1.7 μm 2.1 × 100 mm, gradient 5–95% acetonitrile in H_2O with 0.1 % TFA (**C**) raw ion spectrum of purified D-Dpo4-3C (**D**) deconvoluted LC–MS analysis of the purified D-Dpo4-3C. $m_{calcd} = 40302$ Da, $m_{found} = 40\,303$ Da.

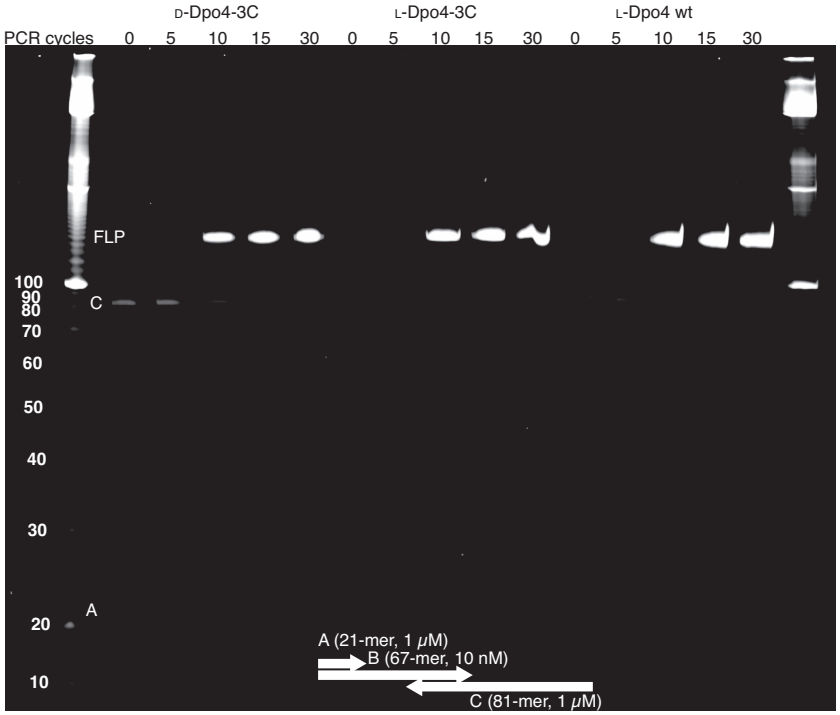

Figure 3. PCR using D-Dpo4-3C, L-Dpo4-3C and L-Dpo4 wildtype. 7.5 μl PCRs were assembled that contained 2 μl D-Dpo4-3C or equal catalytic activities (according to the data shown in Supplementary Figure S1) of L-Dpo4-3C (4.3 ng) or wildtype L-Dpo4 (3.4 ng). The oligonucleotide setup is shown as an inlay, bands of the forward primer (**A**, 21 nt), the reverse primer (**C**, 81 nt) and the full-length product (FLP, 118 nt) are indicated. For FLP formation, the forward primer has to be elongated by 97 nucleotides and the reverse primer by 37 nucleotides, thus 137 of 236 nucleotides of the double strand FLP (58%) are enzymatically synthesized. In all cases, strong bands of the FLP can be observed already after 10 cycles. For a densitometric evaluation, see Supplementary Figure S10.

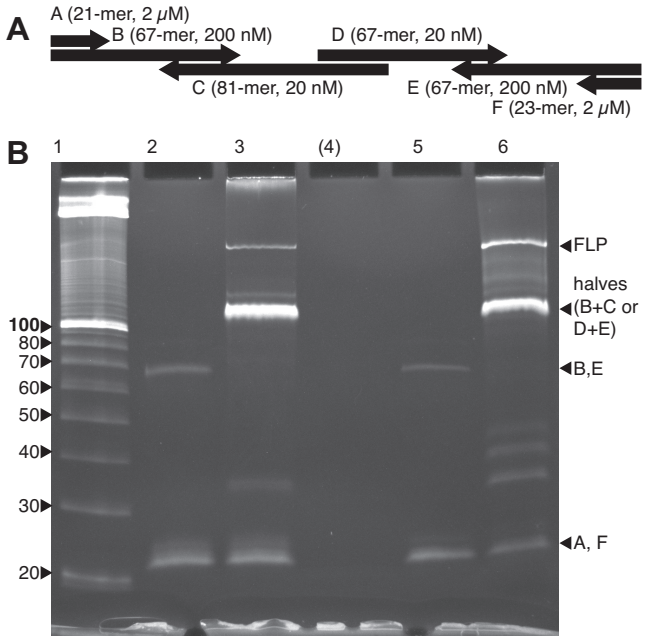

Figure 4. Assembly of a mirror-image gene. (**A**) schematic of the oligonucleotide setup. (**B**) lane 1, 3 μl of 10 bp DNA ladder. Lane 2, mirror-image no-enzyme control. Lane 3, mirror-image gene assembly. Lane 4, empty. Lane 5, natural handedness no enzyme control. Lane 6, natural handedness gene assembly.

Figure S11c). Subsequently, we used D-Dpo4-3C to assemble the mirror-image gene from four L-oligonucleotides (Figure 4). This data confirms that D-Dpo4-3C is generally applicable for gene assembly and replication, which is a cornerstone on the way toward creating mirror life.

Another cornerstone would be the transcription of DNA into RNA. It is already known that residue tyrosine 12 (Tyr12) of Dpo4 is the key amino acid facilitating the discrimination between NTPs and dNTPs by (i) stabilizing the binding of an incoming dNTP by a stacking interaction between the Tyr12 side chain and the deoxy-ribose ring and (*ii*) steric exclusion of an NTP's 2'-OH. Mutation of Tyr12 into Ala12 removes the steric hindrance of NTP binding, which increases the tolerance for NTPs (30,31). However, the activity of the Dpo4 Tyr12Ala mutant is described to be impaired due to the lost stacking interaction between the (deoxy)-ribose ring and the Tyr12 side chain (30,31) (Supplementary Figure S12a-c). We produced an L-Dpo4-3C Tyr12Ala mutant and also a Tyr12Ser mutant, which we hoped might compensate for the lost stacking interaction by offering a new hydrogen bond between the Ser12 side-chain-OH and the NTPs 2'- and/or 3'-OH (Supplementary Figure S12D). Both mutants were able to elongate a 25-mer D-DNA or D-RNA primer hybridized to an 81-mer D-DNA template to full length using either dNTPs or NTPs as a substrate, whereby the Tyr12Ser mutant appears to be slightly faster in RNA synthesis (Supplementary Figure S13).

Due to the modular synthesis strategy for D-Dpo4-3C, the Tyr12Ala or Ser-mutation could rather easily be introduced into fragment 1 to obtain a mirror-image polymerase being able to transcribe L-RNA from L-DNA templates. Such an enzyme might be used to obtain (unmodified) L-ribosomal and L-messenger RNA to establish a self-replicating mirror-image version of a coupled transcription-translation system like the PURE system (32) as suggested earlier (11); modified nucleotides might potentially be introduced by a recently reported method (33). tRNA and tRNA-acylating ribozymes (Flexizymes (34)) can easily be synthesized (25). With the exception of ribosomal protein S1, which is expendable for the translation of leaderless mRNA (35), all ribosomal proteins are smaller than Dpo4-3C and judging only by their length, we believe that they should be accessible to chemical synthesis. A subset of translational factors (elongation and termination/recycling) would also be needed to get started with translation and to produce the remaining factors along with aminoacyl-tRNA synthetases, RNA and DNA polymerases.

In summary, we have demonstrated that D-Dpo4-3C can be exploited to amplify mirror-image DNA and we provide evidence that respective mutants could make mirror-image RNA transcripts; both are essential for a mirror-image translation system as an intermediate step towards mirror life.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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