Peptide rescue of an N-terminal truncation of the Stoffel fragment of *Taq* DNA polymerase

INNA VAINSHTEIN,^{1,2} ALEXEY ATRAZHEV,² SOO HYUN EOM,³ JOHN F. ELLIOTT,²
DAVID S. WISHART,⁴ AND BRUCE A. MALCOLM¹

¹ Protein Engineering Network of Centers of Excellence and Department of Biochemistry, University of Alberta, Edmonton, Alberta T6G 2H7, Canada

² Department of Medical Microbiology and Immunology, Glaxo Heritage Research Institute, University of Alberta, Edmonton, Alberta T6G 2H7, Canada

³ Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut

⁴ Protein Engineering Network of Centers of Excellence and Faculty of Pharmacy, University of Alberta, Edmonton, Alberta T6G 2H7, Canada

(RECEIVED May 5, 1996; ACCEPTED June 25, 1996)

Abstract

Deletion of the first 289 amino acids of the DNA polymerase from *Thermus aquaticus* (*Taq* polymerase) removes the 5' to 3' exonuclease domain to yield the thermostable Stoffel polymerase fragment (Lawyer et al., 1989). Preliminary N-terminal truncation studies of the Stoffel fragment suggested that removal of an additional 12 amino acids (the Stof Δ 12 mutant) had no significant effect on activity or stability, but that the further truncation of the protein (the Stof Δ 47, in which 47 amino acids were deleted), resulted in a significant loss of both activity and thermostability. A 33-amino acid synthetic peptide, based on this critical region (i.e., residues 303–335 inclusive), was able to restore 85% of the Stof Δ 12 activity when added back to the truncated Stof Δ 47 protein as well as return the temperature optimum to that of the Stof Δ 12 and Stoffel proteins. Examination of the crystal structure of *Taq* polymerase (Kim et al., 1995) shows that residues 302–336 of the enzyme form a three-stranded β -sheet structure that interacts with the remainder of the protein. CD analysis of the 33-amino acid peptide indicates that the free peptide also adopts an ordered structure in solution with more than 50% β -sheet content. These data suggest that this 33-amino acid peptide constitutes a stable β -sheet structure capable of rescuing the truncated polymerase in a fashion analogous to the well-documented complementation of Ribonuclease S protein by the 15-residue, α -helical, S peptide.

Keywords: deletion; peptide rescue; Stoffel fragment; *Taq* polymerase; thermostability

How organisms modify their biochemical processes to adjust to extremes of temperature is a question that has intrigued biochemists since the discovery of the first thermophiles. Modification of essential proteins is one of the fundamental requirements for survival of cells subjected to elevated environmental temperatures. Despite a number of investigations on thermal stability of certain proteins, the precise molecular basis for the extreme stabilization of proteins in thermophiles remains unknown. Studies have demonstrated that single amino acid substitutions can affect protein stability (Nicholson et al., 1988; Mrabet et al., 1992), whereas others have shown that the addition of certain structures like β -hairpins into a protein (Eijsink et al., 1992) or construction of

chimeric proteins from thermophile and mesophile (Numata et al., 1995) can result in an increase in protein stability. However, simple genetic screens for favorable mutations or rational modification of proteins have not succeeded in transforming mesophilic proteins into ones that can cope with the extremes encountered by thermophilic organisms.

Protein engineering studies on the thermophilic DNA polymerase from *Thermus aquaticus* (*Taq* polymerase), aimed at eliminating the 5'-3' exonuclease domain, demonstrated that 289 amino acids could be removed from the amino terminus of the protein without a significant loss of thermostability or polymerase activity (Erich et al., 1991; Barnes, 1992). In an effort to understand how this protein maintains its stability at elevated temperatures, additional N-terminal truncations were generated. Activity and stability were eventually compromised by the progressive deletions. To test whether the deleted sections were capable of restoring thermostability and activity, a peptide rescue experiment was designed. The recently published crystal structure of *Taq* polymerase (Kim et al., 1995) provides a foundation for interpretation of the results presented.

Reprint requests to: John F. Elliott, Department of Medical Microbiology and Immunology, University of Alberta, Edmonton, Alberta, T6G 2H7, Canada; phone: (403)492-0895; FAX: (403)492-7521; e-mail: john.elliott@ualberta.ca.

Abbreviations: dNTPs, deoxy nucleotide-5-triphosphates; ddNTPs, di-deoxy nucleotide-5'-triphosphates; TCA, trichloroacetic acid; MOI, multiplicity of infection; PMSF, phenylmethylsulfonyl fluoride.

Results

Construction and expression of *Taq* polymerase truncations

The shortest enzymatically active truncated form of *Taq* polymerase reported, the Stoffel fragment (Lawyer et al., 1989), lacks 289 amino acid residues from the N-terminus. The N-terminal deletions of the Stoffel fragment used in this study are shown in Figure 1. The N-terminus of the Stof Δ 12 mutant (residue 302) was positioned immediately after a run of prolines at positions 298–302. The amino-terminus of Stof Δ 47 was designed to fall into what was predicted to be the next unstructured region, 334–339, based on Chou–Fasman/Robson–Garnier prediction methods (Wishart et al., 1994).

The gene fragments for Stof Δ 12 and Stof Δ 47 were generated by PCR (Table 1) and subcloned into M13mp18 (see the Materials and methods). The deleted genes were expressed using the lac promoter of the M13mp18 vector. Because the DNA constructs were cloned into the *Eco*R I–*Xba* I sites of M13mp18, the expressed proteins all contained an identical 7-amino acid sequence (M-N-M-I-T-N-S) at their N-termini derived from the β -galactosidase fragment directly upstream of the insert. The molecular weights of the protein bands corresponded to the predicted sizes of the truncated derivatives (data not shown).

Temperature-activity profiles of truncated polymerases

In order to make an accurate comparison between the different truncated polymerases, steady-state kinetic experiments were conducted. Figure 2 shows activities of the Stoffel fragment and the Stof Δ 12 and Stof Δ 47 proteins over a range of temperatures from 37 to 84 °C. Polymerization time courses were taken using activated calf thymus DNA (see Materials and methods). The Stoffel fragment and Stof Δ 12 exhibited virtually identical profiles with temperature optima of 76 °C. The profile of Stof Δ 47 shows a lowering of the temperature optimum to 65 °C coupled with a threefold decrease in activity.

Peptide rescue experiments

Temperature-activity profiles (Fig. 2) strongly suggested that the deletion of the additional 35 residues from the Stoffel fragment (i.e., from residue 302 to 337) was responsible for the loss of activity and stability of the protein. A 33-residue synthetic peptide (Pep 33) identical in sequence to the putative stabilizing region (sequence 1 below), containing amino acids 303–335 inclusive, was prepared. Both the N- and C-terminal prolines of the original deleted sequence were not included. As a negative control, a pep-

Fig. 1. Schematic representation of the N-terminal deletions of *Taq* polymerase (numbering according to *Taq* polymerase).

Fig. 2. Polymerase activity of the Stoffel fragment and the truncated mutants at various temperatures. Reactions were performed as described in the Materials and methods using activated calf thymus DNA. Reaction mixes were prewarmed to indicated temperatures and reactions were initiated by adding enzyme (final concentration of 0.1 μ g/mL). Activity units are defined in Materials and methods.

tide of the same length and amino acid content (sequence 2) was synthesized in which the amino acids were redistributed (scrambled) to break up the two hydrophobic stretches 305–311 and 321–327 (underlined sequences) and flanking groups of charged and polar amino acid residues (shown in bold). Both peptides were purified by HPLC and confirmed by mass spectrometry.

- 310 320 330
 | | |
- (1) **EGAFVGFVLSRKEPMWADLLALAAARGGRVHRA** Pep 33
- (2) **FVGALEGSFVHARLVALDAERPAMLGKARWGRA** Scram

The Stof Δ 47 protein was mixed with Pep 33 in varying molar ratios, incubated at 30 °C for 30 min to allow the peptide and protein to associate and tested for polymerase activity. The Scram peptide was tested in parallel. Assays were performed at 65 °C, the temperature optimum for the Stof Δ 47 protein by itself. Augmentation of Stof Δ 47 specific activity was noted upon addition of Pep 33 but no change was observed when an equivalent amount of Scram peptide was used. The maximum effect was seen at a peptide to protein molar ratio of 10:1. The activity of the Stof Δ 47–Pep 33 complex approaches 82% of the activity of Stof Δ 12 enzyme (Fig. 3). In parallel assays, no statistically significant improvement was seen using the Scram peptide at similar molar ratios. This would suggest that the Pep 33 effect is sequence specific. It is interesting to note that molar ratios of peptide to protein higher than 10:1 led to significant inhibition of activity with both peptides (data not shown).

Temperature-activity profile of the Stof Δ 47–Pep 33 complex

As noted above, the Stof Δ 47 truncation appears to lower the temperature optimum from 76 °C (Stoffel fragment/Stof Δ 12) to 65 °C

Fig. 3. Comparison of polymerization rates for StofΔ47, StofΔ47-Pep 33, and StofΔ47-Scram at various temperatures. Assays were performed as described in Materials and methods. Initial polymerization rates are expressed as a percent of polymerization rate for the StofΔ12 mutant at the temperature indicated.

(Fig. 2). To determine whether rescue by Pep 33 increases activity as well as stability, the StofΔ47-Pep 33 complex was assayed at several temperatures between 65–80 °C. Figure 3 shows that the activity of the StofΔ47 mutant alone, or in combination with the Scram peptide, decreased at temperatures above 65 °C relative to StofΔ12. As expected, the StofΔ47-Pep 33 complex showed significantly increased rates over StofΔ47 at all temperatures assayed, and in contrast to the truncated polymerase alone, showed increasing polymerization rates between 65 and 70 °C, approaching 85% of the values seen for StofΔ12 at 69 °C.

Between 70 and 80 °C, polymerization rates for the StofΔ47-Pep 33 complex begin to fall off relative to those of StofΔ12. These rates, however, are still significantly higher than those for StofΔ47 alone or with the Scram peptide. At 80 °C, the StofΔ47-Pep 33 complex still shows residual activity that is eight times higher than that of StofΔ47 alone or together with the Scram peptide (Fig. 3). Other experiments demonstrated that maximum activity for the StofΔ47-Pep 33 complex was obtained at 73 °C (data not shown). Increased polymerization rates at high temperatures and a shift in the temperature optimum from 65 to 73 °C for the StofΔ47-Pep 33 complex suggest a restoration of activity and stability of StofΔ47 by Pep 33.

Kinetic constants for the StofΔ47-Pep 33 complex

To investigate quantitatively the restoration of StofΔ47 enzymatic function, kinetic constants for the polymerization reaction were determined. Polymerization assays were performed at 55 and 65 °C. A homopolymeric DNA template-primer system-poly(dA)-oligo(dT) was employed for this set of experiments. An oligo(dT)-35-mer was used to provide a more stable DNA-primer complex at elevated temperatures.

At 55 °C, StofΔ47 alone or together with the Scram peptide showed comparable K_{mDNA} values, whereas the StofΔ47-Pep 33 complex showed a lower K_{mDNA} value, nearly equal to that of StofΔ12 (Table 2). The k_{cat} values for the StofΔ12 mutant, StofΔ47 alone, and both StofΔ47-peptide complexes were nearly the same at 55 °C. The specificity constant (k_{cat}/K_m) for the StofΔ47-Pep 33 complex, therefore, was fourfold higher than for StofΔ47 alone or in combination with the Scram peptide; roughly 70% of the value for StofΔ12.

At 65 °C, polymerization rates measured over a range of DNA concentrations from 15 nM to 1 μM (concentration of 3'-ends) demonstrated that the less active StofΔ47 and StofΔ47-Scram peptide control had increased K_{mDNA} values (Table 2). K_{mDNA} for the StofΔ47-Pep 33 complex was 10 times lower, comparable to that of StofΔ12. The k_{cat} value for StofΔ12 increased almost fivefold as the temperature was raised from 55 to 65 °C whereas k_{cat} for the StofΔ47-Pep 33 complex increased only threefold. Comparison of k_{cat}/K_m at 65 °C shows that the StofΔ47-Pep 33 complex has 10-fold higher specific activity than StofΔ47; with or without the Scram peptide and roughly 60% of the value for StofΔ12.

The mutants and complexes were subsequently studied for their K_{mNTP} values at 55 °C. Polymerase activity assays were conducted using the poly(dA)-oligo(dT) system and K_m 's for dTTP were determined for both mutants and both protein-peptide combinations. No significant differences in K_{mTTP} values between any of the complexes or mutants were apparent (data not shown).

CD analysis of Pep 33

In order to ascertain whether Pep 33 had any significant degree of structure in solution, a CD spectra of Pep 33 and the Scram peptide were obtained at 25 °C in unbuffered H₂O (pH ~ 5.0). Figure 4 shows the spectra of both Pep 33 and the Scram peptide. The spectra of the two peptides are significantly different. As evidenced by the minimum at 200 nm (Johnson, 1990), both appear to contain some degree of random coil; however, the Scram peptide appears to be significantly more disordered. The positive molar ellipticity at 190 nm of Pep 33 suggests that some of the random coil has been replaced by β-sheet (Johnson, 1990). The deconvolution algorithm of Provencher and Glockner (1981) suggests that

Table 1. Oligonucleotide primers for PCR of Taq polymerase gene

Oligonucleotide	Function	Mutant	Location
TTCTGGAATTCTAGCCCAAGGCCCT ^a	5'-PCR	Stoffel	289
GTTGAATTCGCCGGAAGGGGCTTC	5'-PCR	StofΔ12	302
GTTGAATTCGGAGCCTTATAAAGCCCTC	5'-PCR	StofΔ47	337
GAGTATCTAGACTATCACTCCTTGCGG	3'-PCR		832

^a Restriction sites are shown in bold; indicated location is underlined.

Table 2. Kinetic parameters for *Stof*Δ12, *Stof*Δ47, and the *Stof*Δ47–Pep 33 and *Stof*Δ47–Scram complexes

Protein or complex	k_{cat} (s ⁻¹)	K_{mDNA}	k_{cat}/K_m
		μM 3' ends	s ⁻¹ μM ⁻¹ 3' ends
55 °C			
StofΔ12	96.0 ± 5.8	0.20 ± 0.03	480
StofΔ47	70.0 ± 15.0	0.77 ± 0.04	91
StofΔ47–Pep 33	82.0 ± 7.0	0.24 ± 0.05	342
StofΔ47–Scram	85.0 ± 6.0	1.00 ± 0.10	85
65 °C			
StofΔ12	480 ± 30	0.24 ± 0.04	2000
StofΔ47	280 ± 10	2.60 ± 0.80	108
StofΔ47–Pep 33	240 ± 60	0.20 ± 0.01	1200
StofΔ47–Scram	210 ± 70	2.00 ± 0.90	105

Pep 33 is composed of approximately 54% β-sheet, 41% random coil and 5% α-helix. Spectra of Pep 33 obtained at temperatures up to 65 °C (data not shown) were virtually identical to those obtained at 25 °C (Fig. 4), suggesting that the structure in solution is quite stable.

Discussion

In an effort to delineate the minimal thermostable domain of the Stoffel fragment of *Taq* polymerase, the amino terminus was progressively truncated. In the absence of a crystallographic structure, predictive algorithms were used to suggest logical points of truncation. Temperature-activity profiles and kinetic parameters (Table 2) showed that removal of 12 amino acids from the

N-terminus of Stoffel fragment (the *Stof*Δ12 mutant) had no significant effect on polymerase function or stability, but that removal of an additional 35 amino acids—predicted to form a stable secondary structure—resulted in an enzyme (the *Stof*Δ47 protein) with reduced stability and activity.

These data suggested that *Stof*Δ12 represented the minimal thermostable polymerase core of *Taq* and that part of that thermostability might be due in part to a structural element. Previous studies had demonstrated that peptide complementation can restore activity for a number of enzymes, including ribonuclease (Richards & Vithayathil, 1959), β-galactosidase (Langley et al., 1975), aspartate transcarbamoylase (Yang & Schachman, 1993), the β-subunit of F₁-ATPase (Tozawa et al., 1992) and SH2 domains of cytosolic proteins (Williams & Shoelson, 1993). Inspired by these precedents and in light of the results presented above, a peptide complementation/rescue experiment was designed. The deleted portion of *Stof*Δ47 (residues 303–335, Pep 33) was synthesized chemically and added back to *Stof*Δ47. Temperature activity profiles (Fig. 3) and kinetic parameters (Table 2) suggested that a significant portion of the activity and stability had been restored by the addition of an excess of Pep 33. No evidence of polymerase rescue was found with a Scram peptide containing the same amino acids (but in random order), confirming that the effect of Pep 33 was sequence specific.

Peptide to protein molar ratios of 10 to 1 led to optimal polymerase activity. Although roughly equimolar ratios of peptide and protein might on first thought be expected to provide optimal complementation as initially seen in the ribonuclease system (Richards & Vithayathil, 1959), higher concentrations of free peptide may be necessary to form the *Stof*Δ47–Pep 33 complex due to simple equilibrium/binding considerations as noted subsequently for the ribonuclease system using synthetic peptide sequences (Finn & Hofmann, 1965). Furthermore, the presence of the artifactual amino acids (from the β-galactosidase sequences) on the N terminus of *Stof*Δ47 (see Materials and methods) might partially interfere with binding by Pep 33, requiring higher concentrations to maintain complex formation. Alternatively, the lack of the flanking prolines (301 and 336) may slightly compromise the effect of the peptide on the truncated protein. Experiments are in progress to explore both of these possibilities.

Inhibition of polymerase activity was observed when the peptide to protein molar ratios were further increased. As a highly hydrophobic peptide, high concentrations of Pep 33 or the Scram peptide may disrupt the protein in a detergent-like manner (Schafmeister et al., 1993). Alternatively, high concentrations of these peptides may simply interfere with the polymerase function or assay.

Measurements of K_{mDNA} for *Stof*Δ12, *Stof*Δ47, and the peptide–protein complexes revealed significant improvement in the values for *Stof*Δ47–Pep 33 relative to *Stof*Δ47 alone. The *Stof*Δ47 mutant when complemented with Pep 33 recovered its ability to bind the DNA substrate with an affinity approaching that of *Stof*Δ12. No effect on affinity for the nucleotide triphosphate substrate was apparent, suggesting that the dNTP binding sites had not been disrupted in either the *Stof*Δ12 or the *Stof*Δ47 proteins.

Examination of the structure of *Taq* polymerase recently solved by Kim et al. (1995) provided a structural framework for the interpretation of these results. In the structure, it appears that residues 305–312, 319–328, and 331–335 form a triple stranded, antiparallel β-sheet structure that fits nicely into a hydrophobic slot defined by the adjacent remainder of the *Taq* polymerase (Fig. 5). A rationale for the impact of Pep 33 on *Stof*Δ47 activity and stability now becomes evident. Methionine 317 and W318 in the loop of Pep 33,

Fig. 4. CD spectra of the Pep 33 (—) and the Scram (----) peptides. For measurement conditions, see Materials and methods.

Fig. 5. A ribbon representation of the Stof Δ 47-Pep 33 complex based on the crystal structure of *Taq* polymerase (Kim et al., 1995). The Stof Δ 47 protein is shown in grey, Pep 33 in red, and interacting side chains at the base of the thumb domain in green (*Taq* polymerase numbering).

and H554 and H561 in the bottom of the thumb domain, interact to form a hydrophobic core (Fig. 5). These interactions clearly play a role in orienting and/or immobilizing the thumb domain. The crystal structure of the *Taq*-DNA complex (Eom et al., 1996) shows that the thumb domain interacts with the DNA backbone across the minor groove of the template and primer strands. Disruption of these interactions at the base of this domain, as in Stof Δ 47, would result in repositioning and/or increasing the mobility of the thumb which in turn could reduce activity and stability of the protein and in particular lower its K_m for DNA (Table 2).

In Figure 6, a close up of the Stof Δ 47-Pep 33 region (residues 303–424) of *Taq* polymerase is shown to indicate how the Pep 33 portion (residues 303–334) interacts with the remainder of the domain. The right half of the figure illustrates the contact regions (shaded in green) where the most significant burial of accessible surface area occurs. These contact areas correspond closely to the most hydrophobic regions of both the peptide and protein components (data not shown). Evaluation of the extent of burial/exposure of amino acid residues was made by the program VADAR (D.S. Wishart, L. Willard, & B.D. Sykes, in prep.) using coordinates of the 303–424 domain, kindly provided by Dr. T. Steitz. This domain defines all relevant contacts of Stof Δ 47 with Pep 33. Structures corresponding to (1) the peptide (Pep 33) alone; (2) the complete *Taq* polymerase domain (residues 303–424); and (3) the *Taq* domain without the Pep 33 region were evaluated separately. Accessible surface areas (probe radius = 1.4 Å) were determined for all residues in all three cases. Those exhibiting a 10-fold (i.e., significant) difference in fractional accessible surface area upon removal of Pep 33 were identified and highlighted using the pro-

gram GRASP (Nicholls et al., 1991) (Fig. 6). Among the residues in Pep 33 that experience the greatest change in exposure are F306, V307, F309, P316, L321, L324, A325, A326, and A327. Among the residues in Stof Δ 47 that experience the greatest change in exposure are: A341, L356, L359, A402, and A406. All 11 of these residues are quite hydrophobic.

It appears that Pep 33 may act as a hydrophobic “plug” that buries a number of hydrophobic residues that might otherwise be exposed in the N-terminal “hand-like” domain of *Taq*. The burying of exposed hydrophobic residues is a common theme underlying the results of several peptide rescue experiments (Richards & Vithayathil, 1959; Tozawa et al., 1992; Williams & Shoelson, 1993). What is particularly unique about the current result is that the rescuing peptide, although still quite small (33 residues), is the only such peptide that exhibits a primarily β -sheet structure. Evidence gathered from CD spectroscopy at elevated temperatures indicates that this β -sheet structure is quite stable even in the absence of the complementing Stof Δ 47. Although characterization of Pep 33 is far from complete, the identification of such a small peptide with greater than 50% β -sheet content could open the door to a better understanding of β -sheet formation, much the same way that the S-peptide of ribonuclease has opened the door to our understanding of α -helix formation.

Although the crystal structure provides a logical explanation of how Pep 33 might contribute to the stability of Stof Δ 47, whether this is actually the case is significantly harder to establish. In the case of the S-peptide, NMR studies have shown that this 15mer adopts a stable α -helical structure in solution that can interact effectively with the N-terminally truncated Ribonuclease S (Hearn et al., 1971; Connelly et al., 1990; Varadarajan & Richards, 1992). Preliminary CD studies of Pep 33 suggest that it adopts a stable β structure in solution. Whether this structure is identical to the “plug” or some other conformation (which could still potentially be induced by the protein into the appropriate structure) awaits further analysis. Further characterization of Pep 33 and its interaction with Stof Δ 47 is currently in progress.

Materials and methods

Restriction endonucleases, T4 polynucleotide kinase, and T4 DNA ligase were purchased from New England Biolabs (Beverly, Massachusetts) or Boehringer Mannheim (Laval, Quebec) and were used in supplied buffers according to manufacturer's instructions. Lysozyme, isopropyl- β -D-thio-galactopyranoside (IPTG), 5-bromo-4-chloro-3-indoxyl- β -D-galactopyranoside (XGal), molecular weight markers (prestained and nonprestained), calf thymus DNA, and PMSF were obtained from Sigma (St. Louis, Missouri). Poly(dA)_{350–450}, dNTPs, and ddNTPs were from Boehringer Mannheim Inc. DuPont NEN Research (Mississauga, Ontario) was the source of [α -³⁵S]dATP and [³H]dTTP (10 Ci/mmol). All the other chemicals were purchased from Bio-Rad, Inc. (Richmond, California) and Sigma Chemical Co. and were at least of analytical grade.

Escherichia coli strains TG-1 (Sambrook et al., 1989) and *E. coli* bacteriophage vector M13mp18 (Yanisch-Perron et al., 1985) were used for all DNA manipulations in cloning, mutagenesis, and expression of the Stoffel fragment and its truncated derivatives. PCR primers and oligonucleotides for sequencing were synthesized in-house using an Applied Biosystems Inc. model 391 DNA synthesizer (Table 1). Peptides were synthesized with f-moc chemistry on a Rainin PS-3 peptide synthesizer. Purification was done on a

Fig. 6. GRASP (Nicholls et al., 1991) representation of the amino terminal domain (residues 303–424, *Taq* polymerase numbering) of the StofΔ47–Pep 33 complex showing the predicted hydrophobic interactions. On the left, the speculated “complex structure” based on the crystal structure of the same region from the crystal structure of the entire *Taq* polymerase (Kim et al., 1995)—StofΔ47 (residues 336–424) in white, Pep 33 (residues 303–334) in grey. On the right, the complex has been “dissociated” to show the exposed hydrophobic side chains (green). The second representation of the Pep 33 fragment (on the far right) has been rotated 90° clockwise (viewed from the top) to show the exposed hydrophobic residues more clearly. Residue numbers are indicated in white: F306, V307, F309, P316, L324, A341, L356, L359, A402, and A406; the amino terminus of the complex and the StofΔ47 protein are indicated by N and N', respectively.

Vydac reverse phase column. Peptides were verified by mass spectrometry, amino acid analysis, and partial N-terminal sequence analysis.

Generation of N-terminal deletions

DNA polymerase from *T. aquaticus* was cloned as described in Engelke et al. (1990). The *Eco*R I–*Xba* I DNA fragment initially cloned in pTTQ-18 (Stark, 1987), containing the full-length coding sequence of *Taq* polymerase, was inserted into the *Eco*R I and *Xba* I sites of M13mp18. N-terminal deletions (the Stoffel fragment, StofΔ12, and StofΔ47) were generated using PCR with appropriate primers (Table 1) that incorporated *Eco*R I and *Xba* I sites to facilitate cloning of fragments. For all DNA manipulations, *E. coli* TG-1 was used as the host strain. PCR amplification products were subcloned into the *Eco*R I and *Xba* I sites of M13mp18. All constructs were verified by dideoxy sequencing (Sanger et al., 1977).

Overproduction and purification of mutant proteins

The truncated *Taq* polymerase genes were expressed in *E. coli* TG-1 using M13mp18. Cell lysates were prepared as described

in Engelke et al. (1990), with minor modifications for cells harboring a phage. Mid-log phase cultures were infected with M13mp18 bearing the corresponding *Taq* polymerase gene constructs at an MOI of 10 and, after 2 h of the growth at 37 °C, were induced with IPTG (0.4 mM final concentration). The cultures were incubating for an additional 3–4 h. Cells from 1-L cultures were harvested by centrifugation, resuspended in 200 mL of buffer A (50 mM Tris-HCl, pH 7.9, 50 mM dextrose, 1 mM EDTA), collected by centrifugation, resuspended in 40 mL buffer A with 4 mg/mL lysozyme, and incubated 15–30 min at room temperature. Forty milliliters of buffer B (10 mM Tris-HCl, pH 7.9, 1 mM EDTA, 1 mM PMSF, 0.5% Tween-20, 0.5% Nonidet-P40) were added and the mixture was incubated in a 75 °C water bath for 1 h. For the thermosensitive mutant (StofΔ47), the heating step was omitted.

Cell debris and denatured proteins were removed by centrifugation at $118,000 \times g$, 4 °C for 1 h and the cleared supernatants were subjected to anion-exchange chromatography using DEAE-cellulose. Ten-milliliter DEAE-columns were pre-equilibrated in 20 mM Tris-HCl, pH 8.0, and 10% glycerol (buffer C). Samples were applied to the columns and the columns were then washed with 10 volumes of buffer C containing 40 mM NaCl. Stepwise elution of polymerases was performed using 60 mM, 80 mM, and

finally 100 mM NaCl in the same buffer. Aliquots from the column fractions were evaluated by SDS/PAGE and pooled. The Stoffel fragment and StofΔ12 proteins were judged to be more than 90% pure and were simply dialyzed against storage buffer (20 mM Tris-HCl, pH 8.0, and 50% glycerol). StofΔ47 underwent further purification using FPLC with a MonoQ 5HR column using a linear 0–0.2 M NaCl gradient. Protein fractions were evaluated by SDS-PAGE, pooled, and dialyzed as before.

Polymerase activity assay

Purified proteins were assayed immediately after purification for polymerase activity (Polesky et al., 1990) at different temperatures using 60-μL reaction containing 10 mM Tris-HCl, pH 8.3, 4 mM MgCl₂, 10 mM KCl, 40 μg activated calf thymus DNA, 100 μM each dATP, dGTP, dCTP, and 100 μM [³H] TTP (50 cpm/pmol). Reaction mixes were prewarmed for 5 min and subsequently initiated by the addition of an appropriate dilution of enzyme. Aliquots (10 μL) were removed at appropriate time intervals and quenched by spotting onto DE-81 papers previously soaked in 25 mM EDTA. Unincorporated [³H] TTP was removed washing with 0.5 M Na₂HPO₄, pH 7.0. The dried DE-81 papers were counted using EcoLite™ (ICN Biomedicals, Costa Mesa, California). All assays were performed in duplicate.

The assay was calibrated by counting a known amount of the labeled TTP solution on a DE-81 paper (omitting the washes). One unit of DNA polymerase activity catalyzed the incorporation of 10 nmol nucleotide into a DE-81-bound form in 30 min. Specific activity of the mutants is expressed as units per picomole of enzyme as opposed to the traditional milligrams because the proteins differ in their molecular weights. The concentration of the polymerases was measured by Bradford colorimetric assay (Bradford, 1976) using commercial reagents (BioRad, Inc. Richmond, California). A homogenous preparation of the Stoffel fragment, with concentration determined by amino acid analysis, was used as a standard.

Determination of kinetic parameters

Kinetic experiments (Polesky et al., 1990) were performed on a homopolymeric substrate that was formed by mixing (dA_{350–450}) and (dT)₃₅ in a 3:1 molar ratio. Various concentrations of substrates were used for assessment of each enzyme or enzyme-peptide complex. Concentrations of substrates used range from 15 nM to 1 μM for poly(dA)-oligo(dT)—concentrations expressed in terms of 3' termini—and from 30 μM to 1 mM for TTP. Each reaction mixture (60 μL) contained the same reagents as described above for polymerase activity assays on activated DNA template, except that 200 μM dNTPs and 10 mM MgCl₂ were used. For each enzyme or enzyme-peptide complex, initial rates (*v*₀) were measured for a series of substrate concentrations under pseudo-first-order conditions of reactants. All determinations were performed in duplicate. Data were fitted using linear regression analysis. Kinetic parameters were estimated using the nonlinear regression analysis (Enzyme Kinetics v. 1.11, Trinity Software, 1992).

CD analysis of peptides

CD spectra were recorded on a Jasco J-500C spectropolarimeter (Jasco, Easton, Maryland) equipped with a Jasco DP-500N data

processor. The instrument was routinely calibrated with an aqueous solution of d-10-(+)-camphorsulfonic acid at 290.5 nm. Ellipticity is reported as mean residue ellipticity in deg cm²/dmol calculated using the following equation $[\theta] = [\theta]_{obs}(mrw)/10lc$ where $[\theta]$ is the ellipticity measured in degrees, *mrw* is the mean residue weight, *c* is the peptide concentration in grams/mL, and *l* is the optical path length of the cell in centimeters. The path length of the cells used in collecting these data was 0.05 cm. Spectra were recorded in unbuffered aqueous solutions at pH ~ 5.0 and were the average of four scans obtained by collecting data at 0.1-nm intervals between 255 and 190 nm. The scan rate was 20 nm/min. Peptide concentrations were determined by amino acid analysis.

Acknowledgments

We thank Robert Luty for his technical assistance obtaining CD spectra and Steven Ness for his able and artistic efforts generating the final figure for this publication. This work was supported by grants from the Alberta Heritage Foundation for Medical Research (AHFMR) and the Medical Research Council of Canada (MRC).

References

- Barnes WM. 1992. PCR amplification of up to 35-kb DNA with high fidelity and high yield from lambda bacteriophage templates. *Gene* 112:29–35.
- Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–54.
- Connelly PR, Varadarajan R, Sturtevant JM, Richards FM. 1990. Thermodynamics of protein-peptide interactions in the ribonuclease S system studied by titration calorimetry. *Biochemistry* 29:6108–6114.
- Eijssink VGH, Vriend G, van den Burg B, van der Zee GR, Veltmann OR, Stulp BK, Venema G. 1992. Introduction of a stabilizing 10 residue beta-hairpin in *Bacillus subtilis* neutral protease. *Protein Eng* 5:157–162.
- Engelke DR, Krikos A, Bruck ME, Ginsburg D. 1990. Purification of *Thermus aquaticus* DNA polymerase expressed in *Escherichia coli*. *Anal Biochem* 191:396–400.
- Eom SH, Wang J, Steitz TA. 1996. Structure of *Taq* polymerase with DNA at the polymerase active site. *Nature (London)* 382:278–281.
- Erllich HA, Gelfand D, Sninsky J. 1991. Recent advances in the polymerase chain reaction. *Science* 252:1643–1651.
- Finn FM, Hofmann K. 1965. Studies on polypeptides. XXXIII. Enzymatic properties of partially synthetic ribonucleases. *J Am Chem Soc* 87:645–650.
- Hearn RP, Richards FM, Sturtevant JM, Watt GD. 1971. Thermodynamics of the binding of S-peptide to S-protein to form ribonuclease S. *Biochemistry* 10:806–817.
- Johnson WC. 1990. Protein secondary structure and circular dichroism: A practical guide. *Proteins Struct Funct Genet* 7:205–214.
- Kim Y, Eom SH, Wang J, Lee D, Suh SW, Steitz TA. 1995. Crystal structure of *Thermus aquaticus* DNA polymerase. *Nature (Lond)* 376:612–616.
- Langley KE, Villarejo MR, Fowler AV, Zamenhof PJ, Zabin I. 1975. Molecular basis of beta-galactosidase alpha-complementation. *Proc Natl Acad Sci USA* 72:1254–1257.
- Lawyer FC, Stoffel S, Saiki RK, Myambo K, Drummond R, Gelfand DH. 1989. Isolation, characterization and expression in *Escherichia coli* of the DNA polymerase gene from *Thermus aquaticus*. *J Biol Chem* 264:6427–6437.
- Mrabet NT, van den Broeck A, van den Brande I, Stanssens P, Laroche Y, Lambeir AM, Matthijssens G, Jenkins J, Chiadmi M, van Tilbeurgh H, Rey F, Janin J, Quax WJ, Lasters I, de Maeyer M, Wodak SJ. 1992. Arginine residues as stabilizing elements in proteins. *Biochemistry* 31:2239–2253.
- Nicholls A, Sharp KA, Honig B. 1991. Protein folding and association: Insights from the interfacial and thermodynamic properties of hydrocarbons. *Proteins Struct Funct Genet* 11:281–296.
- Nicholson H, Becktel WJ, Matthews BW. 1988. Enhanced protein thermostability from designed mutations that interact with alpha-helix dipoles. *Nature (Lond)* 336:651–656.
- Numata K, Muro M, Akutsu N, Nosoh Y, Yamagishi A, Oshima T. 1995. Thermal stability of chimeric isopropylmalate dehydrogenase genes constructed from a thermophile and a mesophile. *Protein Eng* 8:39–43.

- Polesky AH, Steitz TA, Grindley NDF, Joyce CM. 1990. Identification of residues critical for the polymerase activity of the Klenow fragment of DNA polymerase I from *Escherichia coli*. *J Biol Chem* 265:14579–14591.
- Provencher SW, Glockner J. 1981. Estimation of globular protein secondary structure from circular dichroism. *Biochemistry* 20:33–37.
- Richards FM, Vithayathil PJ. 1959. The preparation of subtilisin-modified ribonuclease and the separation of the peptide and protein components. *J Biol Chem* 234:1459–1465.
- Sambrook J, Fritsch EF, Maniatis T. 1989. *Molecular cloning: A laboratory manual*, 2nd ed. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory.
- Sanger F, Nicklen S, Coulson AR. 1977. DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* 74:5463–5467.
- Schafmeister CE, Miercke LJW, Stroud RM. 1993. Structure at 2.5 Å of a designed peptide that maintains solubility of membrane proteins. *Science* 262:734–738.
- Stark MJ. 1987. Multicopy expression vectors carrying the lac repressor gene for regulated high-level expression of genes in *Escherichia coli*. *Gene* 51:255–67.
- Tozawa K, Odaka M, Date T, Yoshida M. 1992. Molecular dissection of the beta subunit of F1-ATPase into peptide fragments. *J Biol Chem* 267:16484–16490.
- Varadarajan R, Richards FM. 1992. Crystallographic structures of ribonuclease S variants with nonpolar substitutions at position 13: Packing and cavities. *Biochemistry* 31:12315–12327.
- Williams KP, Shoelson SE. 1993. Cooperative self-assembly of SH2 domain fragments restores phosphopeptide binding. *Biochemistry* 32:11279–11284.
- Wishart DS, Boyko RF, Willard L, Richards FM, Sykes BD. 1994. SEQSEE: A comprehensive program suite for protein sequence analysis. *Comput Appl Biosci* 10:121–132.
- Yang YR, Schachman HK. 1993. In vivo formation of active aspartate transcarbamoylase from complementing fragments of the catalytic polypeptide chains. *Protein Sci* 2:1013–1023.
- Yanisch-Perron C, Vieira J, Messing J. 1985. Improved M13 phage cloning vectors and host strains: Nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* 33:103–109.