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The PCR plateau phase – towards an understanding of its limitations

Peter Kainz *

University of Salzburg, Institute of Chemistry and Biochemistry, Hellbrunnerstrasse 34, A-5020 Salzburg, Austria

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Abstract

The DNA polymerases from *Thermus aquaticus* and *Thermus flavus* were recently found to bind to short double-stranded DNA fragments without sequence specificity [Kainz et al. (2000) Biotechniques 28, 278–82]. In the present study, it is shown that the accumulation of amplification products during later PCR cycles also exerts an inhibitory effect on several enzymes tested. To simulate later cycle conditions, a 1.7 kb sequence from phage λ DNA was amplified in the presence of various amounts of a 1 kb double-stranded DNA fragment. A 30-fold molar excess of fragments to polymerase molecules was found to be required for a complete inhibition of *Taq*, *Tfl* and *Pwo* DNA polymerase. This stoichiometric relation remained constant when PCR amplifications were performed using polymerase concentrations of 0.5, 1 or 1.5 U/50 μl reaction volume. The amount of 1 kb DNA fragments required for a complete inhibition was similar to the product yield of the controls (no fragment added), that were run to plateau phase levels. Additionally, PCR mixtures, that were subjected to different numbers of cycles, were compared in their ability to extend 3′-recessed ends by using a hairpin extension assay. The presence of endogenous amplicon DNA accumulated in later PCR cycles was found to inhibit completely the activity of DNA polymerase. PCR mixtures still in quasi-linear phase partially extended the hairpins. In both cases, a further addition of polymerase significantly improved their function. These results indicate that the main factor contributing to the plateau phase in PCR consists of binding of DNA polymerase to its amplification products.

Keywords: Polymerase chain reaction; *Taq* DNA polymerase; DNA binding

1. Introduction

Plateau in PCR can be defined as the attenuation in the rate of the exponential product accumulation, which is seen concomitantly in later PCR cycles. Typically the reaction will be first exponential, then will enter a quasi-linear phase, and finally reach a plateau. A number of factors have been presumed to contribute to this plateau: (1) utilization of substrates (dNTPs or primers); (2) thermal inactivation and limiting concentration of DNA polymerase; (3) inhibition of enzyme activity by increasing pyrophosphate concentration; (4) reannealing of specific product at concentrations above 10^{-8} M; (5) reduction in the denaturation efficiency per cycle and (6) destruction of product due to *Taq* DNA polymerase 5′–3′ exonuclease activity [1,2]. However, none of these 'conventional' factors provides sufficient explanation of the phenomenon [3].

A recent study presents a new method to produce hot start conditions in PCR [4]. In this work, the addition of short double-stranded DNA fragments was shown to inhibit the activity of DNA polymerases from *Thermus aquaticus* and *Thermus flavus*. It was found that the enzymes bind to the fragments without sequence specificity and are thereby prevented from extending misannealed primers. This provokes the question as to whether, under usual PCR conditions, the accumulation of product during later cycles might also exert an inhibitory effect on the enzyme before trivial factors such as exhausting primers or dNTPs stop the reaction. The experiments described in the present work aim to provide new information on this subject.

2. Materials and methods

2.1. DNA polymerases and DNA samples

DNA polymerases used in these experiments were: *Pwo*

* Fax: +43-662-8044-5751;
E-mail: peter.kainz@sbg.ac.at

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DNA polymerase (Roche Molecular Biochemicals, Mannheim, Germany), Biotherm *Taq* DNA polymerase (genXpress, Vienna, Austria), AmpliTaq DNA polymerase and AmpliTaq DNA polymerase, Stoffel Fragment (PE Biosystems, Vienna, Austria) and *Tfl* DNA polymerase (Epicentre Technologies, Madison, WI, USA).

λ DNA was purchased from Roche Molecular Biochemicals. The oligonucleotide primers were selected using OLIGO 6.0 primer analysis software (Medprobe A.S., St. Haunshagen, Norway) and were synthesized either by MWG (Ebersberg, Germany) or by genXpress. The sequences and DNA correspondences of the primer pairs were: (i) forward 20 393–20 417 and reverse 22 120–22 096 (target length: 1.7 kb); (ii) forward 10 443–10 467 and reverse 11 413–11 394 (target length: 1 kb).

2.2. Hairpin extension assay

To test the ability of PCR mixtures to extend 3′-recessed ends, a hairpin assay was performed as described recently [4,5] but modified as follows: 0.5 pmol of a ^{32}P-end-labeled DNA hairpin fragment was added to aliquots of PCR mixtures that had been subjected to a certain number of cycles (see legends to Fig. 2). After incubation for 1 min at 68°C in a total volume of 40 μl, the amplifications were stopped by the addition of 40 μl of 2× gel loading buffer. The extension products were resolved on 15% polyacrylamide (w/v) gels under denaturing conditions (7 M urea).

2.3. Amplification conditions

The 50 μl standard reaction mixture contained the following components: 0.4 μM of each primer, 0.2 mM of each dNTP, 1.5 mM MgCl ($MgSO_4$ for *Pwo* DNA polymerase, 4 mM $MgSO_4$ for Stoffel Fragment), 10 ng of λ DNA and 1 U (if not indicated otherwise) of *Taq*, *Tfl*, AmpliTaq-Stoffel or *Pwo* DNA polymerase using the standard buffer as supplied with the respective enzyme.

Cycling conditions: 2 min at 93°C, followed by 24 two-step cycles consisting of 1 min 30 s for annealing–extension at 68°C and 1 min for denaturation at 93°C followed by a final annealing–extension step for 3 min. All reactions were setup at room temperature and finally overlaid with 35 μl of light mineral oil (Sigma). The PCRs were carried out in 0.2 ml reaction tubes, using a 96-well Robocycler, both from Stratagene (La Jolla, CA, USA). The PCR products were analyzed by 1% agarose gel electrophoresis and ethidium bromide staining. The peqGOLD ladder-mix from peqLab Biotechnologie (Erlangen, Germany) was used as the molecular weight standard.

The 1 kb amplification products were pooled from several PCR reactions, purified using the high pure PCR product purification kit (La Roche Biochemicals) and quantified by spectrophotometry. The appropriate amounts of the 1 kb DNA fragment were added to the PCR aliquots during the reaction setup (see legends to Fig. 1).

3. Results and discussion

To simulate later cycle conditions, a 1.7 kb target sequence from phage λ DNA was amplified in the presence of various amounts of exogenous double-stranded DNA fragments of 1 kb length. The results of these experiments are summarized in Fig. 1. PCR amplifications using 0.5 U (Fig. 1a), 1 U (Fig. 1b) and 1.5 U (Fig. 1c) of *Taq* DNA polymerase were run to plateau levels without (lane 1) and in the presence of 250 ng to 2 μg of the 1 kb DNA fragment (lanes 2–7). The amount of the 1 kb fragment required for a complete inhibition of PCR was found to be related to the concentration of the polymerase as follows: calculating 1 U of *Taq* DNA polymerase to be 50 fmol [6] and 1 μg of a double-stranded DNA of 1 kb length cor-

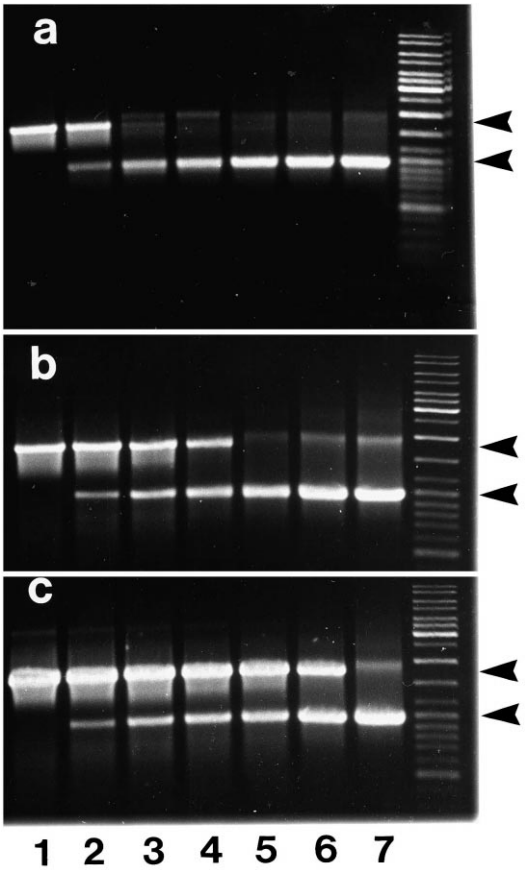

Fig. 1. The effect of different amounts of exogenous 1 kb double-stranded DNA fragments on the amplification of a 1.7 kb λ DNA sequence. 0.5 U (a), 1 U (b) and 1.5 U (c) of *Taq* DNA polymerase were used. Lanes 1, controls (no 1 kb fragment added); reactions shown in lanes 2–7 contained 250 ng, 500 ng, 750 ng, 1 μg, 1.5 μg and 2 μg of the 1 kb DNA fragment, respectively. Reaction parameters were as described in Section 2. The positions of the specific amplification product (upper arrow) and of the exogenous 1 kb DNA fragment added (lower arrow) are indicated.

responding to 1.5 pmol molecules, an at least 30-fold molar excess of DNA fragments was necessary to prevent the amplification of the 1.7 kb target sequence to detectable levels. This molar relationship remained constant at the three polymerase concentrations tested (Fig. 1a, lane 3; Fig. 1b, lane 5; Fig. 1c, lane 7).

Furthermore, by estimating visually the intensity of the ethidium bromide fluorescence, it was seen that the inhibiting amounts of the 1 kb DNA fragment were comparable to the product yields obtained in the controls (e.g. compare Fig. 1c, lane 7 to lane 1). Thus the inhibitory effect cannot be attributed to the addition of an excess of exogenous DNA but clearly reflects plateau phase conditions as they were seen in the controls.

By using a hairpin extension assay, the ability of PCR mixtures to extend 3′-recessed ends was tested by the addition of 0.5 pmol of a ^{32}P-labeled oligonucleotide to aliquots of PCR mixtures that had been subjected to a certain number of cycles. Each fifth cycle, starting with cycle 15 and ending with cycle 40, was measured regarding its status of polymerase activity. As shown in Fig. 2, the reactions having reached the plateau phase were unable to extend the 3′-recessed ends (panels 1 and 2, lanes g–i). Obviously the accumulation of the amplicon DNA by itself inhibited the enzyme activity. Partial inhibition was found to start already at the stage of quasi-linear extension (panels 1 and 2, lanes c and e). In this case, a further addition of 0.5 U of polymerase during reaction setup led to an improved ability in extending the hairpins as well as

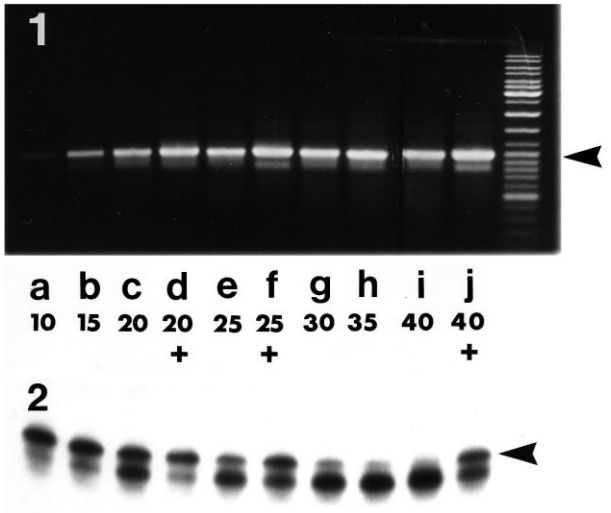

Fig. 2. Hairpin extension assay: the inhibitory effect of PCR mixtures to extend 3′-recessed ends as a function of cycle numbers. 1: A 10 μl aliquot of each amplification was separated electrophoretically. The numbers below lanes a–k indicate the number of cycles at which the amplifications were removed from the machine. + signifies PCR samples that were run using 2 U *Taq* DNA polymerase/50 μl. Reaction parameters were as described in Section 2. The position of the specific product (arrow) is indicated. 2: Hairpin extension assays (corresponding to lanes a–k in 1) were performed as described in Section 2. The arrow indicates the position of the extended hairpin.

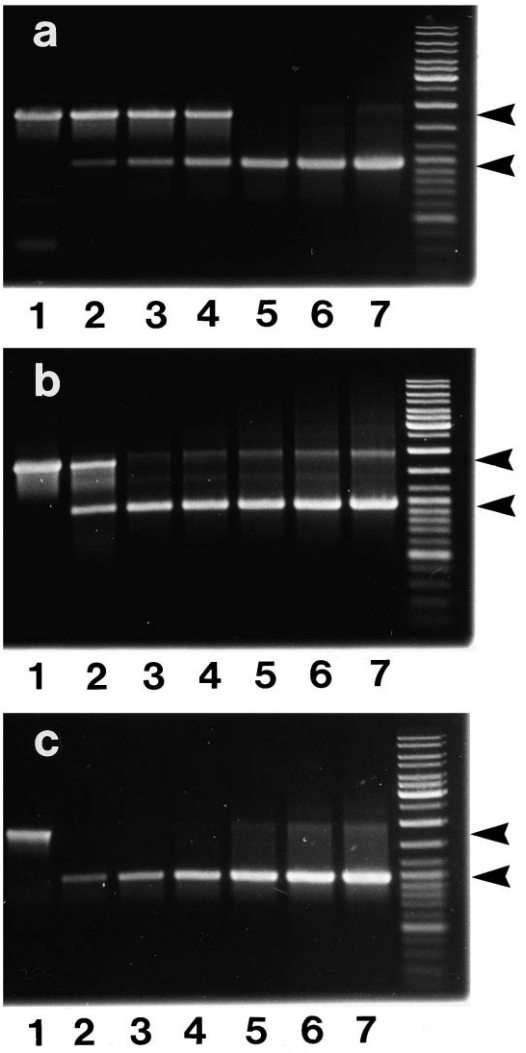

Fig. 3. The effect of different amounts of exogenous 1 kb double-stranded DNA fragments on the amplification of a 1.7 kb λ DNA target sequence. 1.5 U *Pwo* DNA polymerase (a), 0.5 U *Tfl* DNA polymerase (b) and 4 U AmpliTaq DNA polymerase, Stoffel Fragment (c) were used. Reactions correspond to those in Fig. 1.

to a slight increase in product yield in PCR (panels 1 and 2, lanes d and f). However, to overcome partially the inhibition as caused by PCR mixtures of later cycles, the addition of 1 U of polymerase immediately before the admixture of the hairpin was required (panel 2, lane j).

This result indicates PCR amplifications at plateau phase to be deficient in free polymerase molecules for the extension of the numerous starting sites. Shifting the plateau phase to some extent by using DNA polymerase concentrations being considerably higher than those usually used (1 U/50 μl) is not recommendable. This is because increased concentrations frequently induce the accumulation of non-specific products, especially if high levels of complex genomic DNA are present and/or target sequences of greater length are to be amplified [6,7].

Though *Taq* DNA polymerase is the enzyme by far the

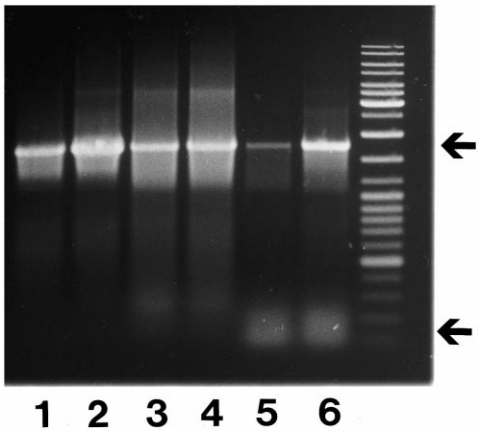

Fig. 4. The effect of the composition of PCR mixtures on a reamplification for a further 25 cycles. The cycling conditions for both amplifications were as described in Section 2. Lanes 1–6 show aliquots of the PCR mixtures after the completion of the second PCR. The composition of sample mixtures for the first PCR was as follows: lanes 1 and 2, blank-controls, containing dNTPs and *Taq* DNA polymerase in 1×buffer; lanes 3 and 4, containing the complete set of components; lanes 5 and 6, dNTPs, primers and enzyme but no template. Before restarting PCR for a further 25 cycles, the missing components were added: lanes 1 and 2, primers and template; lanes 3 and 4, none; lanes 5 and 6, template. To each of the PCR probes shown in lanes 2, 4 and 6, one further unit of *Taq* DNA polymerase was added before restarting the second PCR. The positions of the specific amplification product (upper arrow) as those of the primer artefacts (lower arrow) are indicated.

most frequently used for PCR, several other enzymes were tested using PCR conditions as described in Fig. 1. Representative results are summarized in Fig. 3. *Pwo* DNA polymerase (Fig. 3a) and *Tfl* DNA polymerase (Fig. 3b) were found to correspond to the results obtained with *Taq* DNA polymerase. AmpliTaq DNA polymerase, Stoffel Fragment, however, was shown to be completely inhibited even by a 2-fold molar excess of the 1 kb DNA fragment. Considering the approximately 10-fold lower processivity of the Stoffel Fragment compared to that of *Taq* DNA polymerase, its more sensitive response to possible adsorbers is obvious.

It is generally accepted that, above a certain low concentration of target DNA, amplification using PCR does not yield concentrations of product proportional to those of the starting levels of target. Instead a constant maximum level of product is obtained. On the other hand, coamplifications of different concentrations of different targets result in retention of the initial proportions. Beyond a certain ratio of the starting concentration of the two amplicons, the amplification to detectable levels of the target added at lower starting concentration does not occur. The results described in this study may explain this so far inherent contradiction: in either case the amplification products accumulate until their molar excess is sufficient to inhibit the enzyme. If different concentrations of different targets are coamplified, those initially present at a higher concentration contribute more to the molar excess, thereby preventing the lower copy number targets from being amplified to a similar level of product.

Morrison and Gannon [3], in their study on the impact of the PCR plateau phase on quantitative PCR, investigated possible inhibitors or features of PCR which lead to this plateau. One of their experiments consisted of the amplification of targets as well as of blank-controls (no template, no primers) over 25 cycles. After this reaction, a secondary amplicon was added to each PCR mixture, alone or together with one of the elements likely to be responsible for the attainment of plateau. These reaction mixtures were then subjected to a second PCR consisting of further 25 cycles. In no case did the re-addition of any of the missing (blank) or likely exhausted/denatured reagents permit the amplification of product to detectable levels.

The adverse effect of the products from the first PCR on the amplification of the secondary target sequence during the subsequent reaction can be explained from the results of the present study: the enzyme is prevented from amplifying the secondary target by its non-specific binding to double-stranded primary target DNA having been amplified during the first 25 PCR cycles.

However, the inhibitory effect of the blank-control reaction, containing only buffer, dNTPs and polymerase, on a subsequent PCR amplification was not reproducible. In contrast, no inhibitory effects of blanks on a second PCR cycling were noticeable, using amplification conditions as described in this study. These results are summarized in Fig. 4. Blank-control reactions after completion of the second PCR are shown in lanes 1 and 2. Before restarting for further 25 cycles, the missing primers and template were added (for details, see legends to Fig. 4). If the first PCR was performed in the presence of primers and template, the reamplification led, as expected, to an increase in unspecific products accompanied by a decrease in specific target (lanes 3 and 4). Interestingly, if only primers but no targets were present during the first PCR, low mass primer artefacts accumulated (lanes 5 and 6, lower arrow). Only a minor amount of specific product was visible after reamplification (lane 5), which might be the result of a low concentration of free polymerase. This is due to its affinity to these short fragments, an effect overcome by a further addition of enzyme (lane 6).

In summary, the inhibitory effect of exogenous double-stranded DNA as well as that of endogenous amplicon DNA on the activity of DNA polymerase, together with the findings of the previous study [4], make product inhibition as the main mechanism for the attainment of the PCR plateau phase highly probable.

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References

[1] A.D. Sardelli, Amplifications 9 (1993) 1–5.
[2] M.J. McPherson, B.D. Hames and G.R. Taylor, PCR 2 – A Practical Approach, IRL Press, Oxford, 1995.
[3] C. Morrison, F. Gannon, Biochim. Biophys. Acta 1219 (1994) 493–498.
[4] P. Kainz, A. Schmiedlechner, H.B. Strack, Biotechniques 28 (2000) 278–282.
[5] C. Dang, S.D. Jayasena, J. Mol. Biol. 264 (1996) 268–278.
[6] G. Ruano, D.E. Brash, K.K. Kidd, Amplifications 7 (1991) 1–4.
[7] P. Kainz, A. Schmiedlechner, H.B. Strack, Anal. Biochem. 202 (1992) 46–49.