

# Motions of the fingers subdomain of klentaq1 are fast and not rate limiting: implications for the molecular basis of fidelity in DNA polymerases.

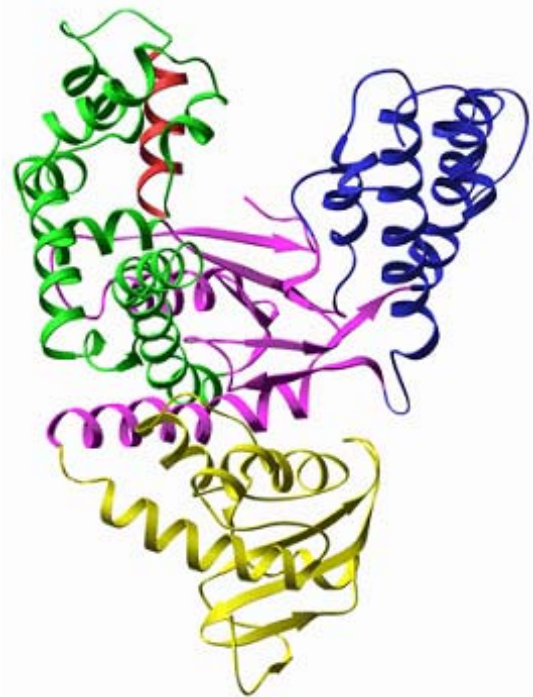
The following commentary was written by Dr Clare Sansom and Professor Gabriel Waksman. The original article was published in the August 2005 issue of *Molecular Cell*. Rothwell PJ, Mitaksov V, Waksman G. (2005) Motions of the fingers subdomain of klentaq1 are fast and not rate limiting: implications for the molecular basis of fidelity in DNA polymerases. *Mol Cell*. 2005 Aug 5;19(3):345-55.

Whenever a cell divides, its DNA must be replicated accurately to ensure that each of its daughter cells receives an exact copy of its genome. This complex process is exceptionally precise, with the wrong base being incorporated into a growing DNA strand once in about  $10^9$ - $10^{10}$  cases. It is dependent on an enzyme called DNA polymerase, which selects each nucleotide and catalyses its incorporation into the new strand. Exactly how this enzyme achieves its remarkable selectivity is not yet fully understood. Gabriel Waksman from the Institute of Structural Molecular Biology (ISMB) at UCL/Birkbeck, with colleagues Paul Rothwell from ISMB and Vesselin Mitaksov from Washington University School of Medicine, have now added another piece to this puzzle. They have established that the enzyme "closes" to grip the DNA molecule very fast - much faster than the complete reaction can occur - but that this only forms a complex that is stable enough for a new nucleotide to be incorporated if that nucleotide is the correct one. This work is published in the August 2005 issue of *Molecular Cell*<sup>1</sup>.

It is a truism in molecular biology that structure determines function. One of the first pieces in the puzzle of DNA replication fell into place when Tom Steitz and his colleagues at Yale University solved the structure of the DNA polymerase catalytic domain<sup>2</sup>. The shape of the DNA polymerase domain is shaped startlingly like a right hand, and its subdomains were immediately labelled fingers, palm and thumb accordingly (Figure 1).

The basic mechanism through which DNA polymerase catalyses the elongation of a new strand of DNA (the "primer"), using the original strand as a template, is now relatively well known. Firstly, the enzyme binds to the primer-template duplex. The next step is the binding of a nucleotide triphosphate to the enzyme-

DNA complex, which then undergoes a complex conformational change to activate the polymerase (step 3).



**Figure 1:** Structure of Klentaq1 polymerase. The thumb, palm, and fingers domains are colored in blue, magenta, and green, respectively. Together, they form the polymerase domain of Klentaq1. The N-terminal domain is colored in yellow and has no function in this polymerase. The helix in red (the O-helix) in the fingers domain is the site of nucleotide binding.

The enzyme then catalyses the formation of a phosphodiester bond between the nucleotide and the

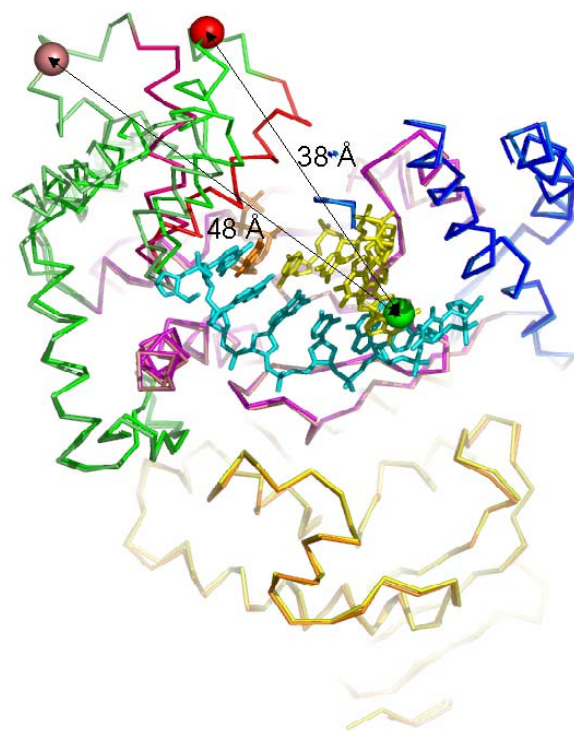
primer strand, to elongate that strand (step 4), and, fifthly, the enzyme either dissociates from the complex or moves along the template strand to incorporate the next nucleotide. The third and fourth steps are known to be crucial for the incorporation of the correct nucleotide into the primer strand, so adenine only pairs with thymine, guanine with cytosine, and *vice versa*; the third step is believed to be the slowest, "rate limiting" part of the reaction.

Structural studies have revealed that several of these steps are associated with distinct conformational changes. In the first step, the thumb domain moves to "grip" the DNA substrate. Nucleotide binding triggers a larger conformational change, mostly involving the fingers domain, which closes a cleft between that and the palm and thumb domains; this is described as a change from an "open" to a "closed" conformation. In family A DNA polymerases, including KlenTaq1 DNA polymerase I, this change involves a rotation of the helix in the fingers domain that binds the nucleotide. This is labelled the O-helix in Figure 1. A final conformational change reverses the second one, allowing the release of the DNA.

However, the exact correlation between the functional steps of the polymerisation reaction and the conformational changes observed with crystallography is not yet clear. It seemed logical to associate the gross conformational change between the open and closed forms with the rate-limiting step in the reaction that is crucial for accurate replication. However, site-directed mutagenesis has provided indirect evidence that this is not the crucial step, but rather, that that occurs in the closed conformation. Waksman and his collaborators, using the technique of fluorescence resonance energy transfer (FRET), have now proved this directly. They also suggest that the rate-determining step is a more subtle conformational change that only occurs in the closed conformation when this is stabilised by the presence of the correct nucleotide.

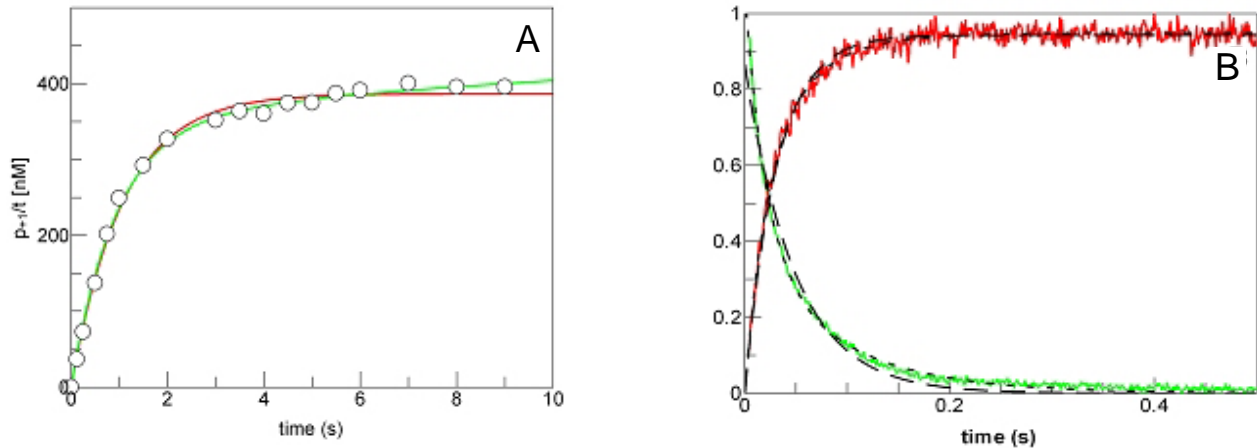
Fluorescence resonance energy transfer<sup>3</sup> is a method of measuring distances at atomic resolution, and monitoring intermolecular interactions and conformational changes in real time, using the phenomenon of energy transfer between fluorescent molecules. When two fluorescent molecules or groups - one a donor and the other an acceptor - are close together, energy will transfer from the donor to the acceptor, increasing acceptor fluorescence and decreasing donor fluorescence. As the amount of

energy transfer is strongly dependent on the distance between the donor and the acceptor, acceptor fluorescence will increase and donor fluorescence decrease as they come closer together. Distances of the order of a few tens of Ångströms ( $1\text{Å} = 10^{-10}\text{ m}$ ), which are typical of those observed between interacting proteins or between protein domains, can be readily monitored using this technique.



**Figure 2:** Positions of the donor and acceptor fluorescence probes for FRET measurement of fingers domain motions. The donor fluorescence probe is in green and is positioned on the DNA. The acceptor fluorescence probe is in pink (open form) and red (closed form) and is on the protein. The distance between probes is indicated and is in Å.

Waksman and colleagues used FRET to monitor changes in the conformation of the DNA-polymerase complex during the polymerisation reaction. The donor probe was attached to one of the DNA bases via an amino linker, and the acceptor probe to a cysteine residue in the fingers domain of KlenTaq1 DNA polymerase I. This cysteine was introduced into the protein by site-directed mutagenesis (the mutation being V649C) in order to anchor the probe in an optimum position to measure the change from the open to the closed conformation. The distance between the probes is approximately 48Å in the open conformation and 38Å in the closed conformation (Figure 2), which is well within the



**Figure 3.** Rate of step 3, the rate-limiting step of nucleotide incorporation (A) and Rate of fingers domain closure (B). In A, the rate of the rate-limiting step was measured using quench-flow and data was fitted using either a single exponential (red) or a double exponential (green). In B, the rate of fingers domain closure was measured using stopped-flow and the FRET system shown in Figure 2. As can be seen, the rate in B is much faster than in A.

range in which distances can be measured precisely using FRET.

The researchers monitored changes in fluorescence over time when the "correct" nucleotide, cytosine, was provided to the system (in the required form of the deoxynucleotide triphosphate). An increase in acceptor fluorescence was observed, corresponding to an overall decrease in the distance between the probes. The rate of this change increased with increasing temperature. At 60°C, it was too fast to be measured; the time-course at 40°C and 20°C could be fitted to double exponentials, indicating two processes. At the lower temperature, for example, the first step - the rate of initial FRET increase, corresponding to the closure of the fingers domain - was approximately six per second. This first step was about 3-5 times faster than the second (which appeared to slightly increase the probe distance), and both rates were about ten times faster at 40°C than at 20°C.

Rates alone, obviously, can mean very little. It was necessary to compare these rate constants with the enzyme's rate of reaction, which can be assumed to be equivalent to that of the slowest, rate-determining step. Brandis and co-workers<sup>4</sup> had already determined that the wild type enzyme incorporated nucleotides at a rate of 21s<sup>-1</sup> at 60°C. Using quench flow measurements, Waksman and co-workers repeated this for their V649C mutant over the whole temperature range they had studied using FRET. They measured the rate of nucleotide incorporation as 0.06 s<sup>-1</sup> at 20°C and 0.92 s<sup>-1</sup> at 40°C: at any temperature, the overall reaction was

obviously much slower than the closure of the fingers domain (Figure 3).

Neither the domain transition nor the nucleotide incorporation could be seen when the incorrect nucleotide was provided. However, this does not necessarily mean that the open form of the enzyme is able to discriminate between nucleotides. It is difficult to imagine how this might work, anyway, as the nucleotide binding site is far away from both the template strand and the enzyme active site in this conformation. It is more likely that the fingers domain closes whenever a nucleotide binds, but that the closed conformation is stabilised only in the presence of the correct nucleotide. Waksman's results are consistent with this theory, and suggest a mechanistic explanation. He proposes that, if the correct nucleotide is present, the closed conformation stabilises, allowing time for further conformational changes to occur in the active site to allow the nucleotide to be incorporated. If the incorrect nucleotide is present, however, the enzyme returns to the open position too rapidly for a FRET signal to be observed.

Waksman and his colleagues, therefore, have established that the rate-limiting step in DNA replication occurs after the closure of the fingers domain. Future research will no doubt focus on the mechanism of this crucial step in the reaction, which is responsible for the exquisite precision of this process, with its error rate of less than one in a billion base pairs.

## References

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