NUCLEASE ACTIVITY OF THE DNA POLYMERASES: AN EVASION OF MICROSCOPIC REVERSIBILITY LEADING TO FIDELITY IN EXCESS OF KINETIC CONTROL LEVELS

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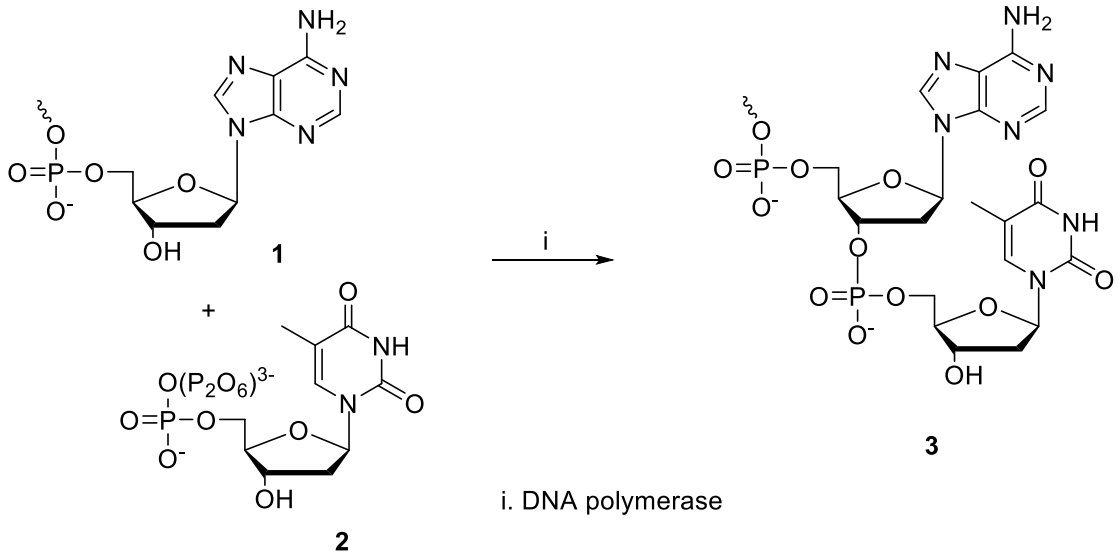
Abstract – The exonuclease and endonuclease activities of the DNA polymerases, which extend their proof-reading capabilities and maintain high fidelity in the replication process, apparently conflict with the principle of microscopic reversibility. However, deeper mechanistic analysis of the reaction leads to fascinating insights into the kinetic and thermodynamic effects controlling the replication of the primary genetic material. Thus, nucleotide addition and excision apparently involve different pathways, although violation of microscopic reversibility is avoided as the overall reaction does not reach equilibrium. Intriguingly, however, this allows the fidelity levels to exceed those expected from simple kinetic control.

Key words: DNA polymerase; DNA replication; fidelity; microscopic reversibility; nuclease.

Introduction

The replication of deoxyribonucleic acid (DNA), the primary genetic material of every living cell, is the key chemical event preceding cell division. In this sense, DNA replication is a defining characteristic of life itself. The replication process, which can also be carried out in vitro, is now fairly well understood in both overall scope and mechanistic detail.

Thus, DNA replication is catalyzed by the enzyme DNA polymerase, indeed a family of the polymerases being now known and characterized from various sources [1-5]. These enzymes



Scheme 1. DNA polymerase catalyzed growth of a DNA single strand, as exemplified by the reaction of a terminal deoxyadenosine 3'-OH group (**1**) with a deoxythymidine triphosphate (**2**), resulting in the incorporation of a deoxythymidine phosphate moiety (**3**)

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catalyze the addition of a deoxyribonucleotide phosphate moiety to the 3' end of a growing DNA oligomer (Scheme 1), although this process needs to be primed by a complementary strand (which can even be a short DNA or RNA oligomer, not shown). The need for a complementary "template" strand is the basis of the famed fidelity of DNA replication (typically < 10^{-6} [1-3]), as it ensures that DNA cannot replicate randomly on its own, even in the presence of DNA polymerase. Clearly, DNA polymerase also ensures the integrity of the genome in terms of the base sequences of the constituent DNA!

It is currently believed that the fidelity of DNA replication is achieved by two modes: firstly, a high level of specificity in the addition step of the deoxyribonucleotide phosphate moiety, ensured by Watson-Crick base pairing between the growing daughter and template strands at the active site of DNA polymerase; and secondly, subsequent proof-reading and error-correction via the excision of a mis-paired nucleotide that has been incorporated inadvertently. (The mis-incorporation is a consequence of the limits on the specificity of the

base pairing and the low but finite probability that a higher energy pathway would be traversed.)

The evolution in DNA polymerase of the above dual mode for ensuring fidelity is indeed intriguing. In fact, the stability of the DNA double helix in the solution phase remains unclear, as evidence apparently indicates that a substantial fraction of single strands (partial and full) may exist in equilibrium with the double stranded form [6]. On this basis, the kinetic fidelity in the replication process – rather than the thermodynamic stability of the double helix – would determine the integrity of the genome. Clearly, the evolution of DNA polymerase to include all possible modes for ensuring fidelity is unsurprising!

The excision capabilities of certain DNA polymerases, however, come into conflict with the principle of microscopic reversibility, which requires that the forward and reverse reactions follow the same mechanism (although in reverse). In fact, excision apparently occurs at a different active site than the incorporation site, hence the conflict with microscopic reversibility. Two modes of excision are also known: exonuclease action, which involves immediate deletion of the mis-incoporated nucleotide at the growing terminus; and endonuclease action, which involves deletion from an elongated strand at an internal mis-incorporation position, upon subsequent proof reading.

The basis and consequences of these intriguing characteristics of DNA polymerase, in purely physico-chemical terms, are analyzed below. No attempt is made to review the molecular biological and other aspects of DNA polymerase, which have been the subject of numerous authoritative and masterly treatments elsewhere [1-3]. (No distinction is made between endonuclease action and exonuclease action below, as the mechanistic conclusions are similar.)

Results and discussion

Certain of the DNA polymerases (although apparently not all) have evolved nuclease activity, and at a site distinct from the polymerase active site. Insofar as fidelity is of supreme importance in DNA replication, these features must have evolved to increase fidelity levels beyond what can be achieved by normal kinetic control [7, 8]. The key to understanding how this can be brought about lies in the principle of microscopic reversibility (PMR) [7-9].

According to the PMR, the forward and reverse reactions comprising a chemical equilibrium must occur by the same mechanism in reverse. The most meaningful interpretation of this is that the same pathways and transition states must be traversed to equal extents in both the forward and reverse directions, at equilibrium. In other words, the reaction flux of a particular path will be the same in forward and reverse directions at equilibrium.

The fact that in DNA polymerase catalysis, nucleotide addition and excision occur at different active sites – hence defining different pathways – clearly poses a challenge to the PMR. (The addition and excision are overall reverse processes and thus expected to occur via the same pathway.) Also, the PMR is a cornerstone of both kinetics and thermodynamics and its violation would pose a serious challenge to current theory.

A way around the conundrum, however, would invoke a key requirement for applying the PMR – the attainment of equilibrium. The qualitative energy profile diagrams for the nucleotide addition and excision processes are shown in Figure 1. Note that mis-incorporation can occur via two pathways – 2 and 3 – bearing in mind that these occur at distinct active sites.

The PMR can be applied only when the overall process has attained equilibrium along all pathways. Interestingly, the mis-incorporation step via the polymerase site (profile 2), with a high transition state energy, would attain equilibrium relatively slowly. Pathway 3, however,

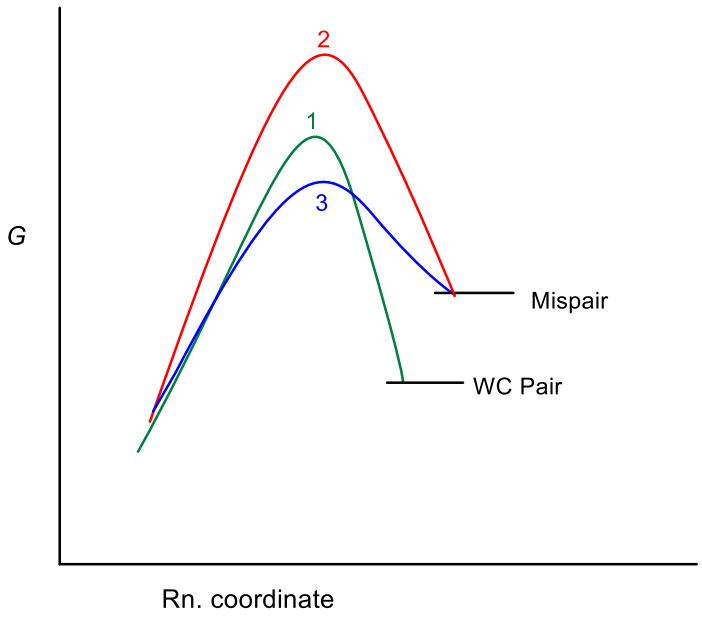

Figure 1. Gibbs free energy (*G*) profiles for nucleotide incorporation and excision under DNA polymerase catalysis. Profile 1 (green) represents incorporation of a Watson-Crick (WC) base pair and profile 2 (red) mis-incorporation, both at the polymerase active site; profile 3 (blue) represents mis-incorporation (forward reaction) and excision (reverse reaction) at a nuclease site

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being of lower energy would attain equilibrium relatively rapidly. The overall consequence would be that the mis-incorporation of a nucleotide at the polymerase active site (path 2) would be rapidly reversed at the nuclease active site (reverse of path 3). Thus, the forward (addition) and reverse processes (deletion) occur via different mechanisms.

However, this would not violate the PMR as long as the reaction along path 2 has not attained equilibrium, i.e., the reverse reaction flux has not been established to a significant extent. This is unlikely to occur for at least two reasons. Firstly, the high activation energy for path 2 precludes the rapid attainment of equilibrium along that path; secondly, rapid deletion via path 3 implies that deletion cannot occur significantly along path 2. (Also, further incorporation and chain elongation would be faster than reversal of path 2.)

It is noteworthy in the above analysis that mis-incorporation into the growing chain also occurs at the nuclease sites. This is because the PMR requires that the nuclease sites operate in both forward and reverse directions. In fact, the mis-incorporation at the nuclease sites would be faster than at the polymerase site, as the former is a lower energy pathway (profile 3). This implies that mis-incorporation is likely thermodynamically controlled: intriguingly, this indicates – given the high fidelity of the overall replication process – that the Watson-Crick pairs are of overwhelming thermodynamic stability relative to the mis-pairs.

The above analysis also indicates that the specificity obtaining at the polymerase site for base incorporation is much lower than that derived from thermodynamic considerations, i.e., based on the stability of the double helix. This is likely because the base pairs are not fully formed at the polymerase active site, so the thermodynamic stabilities are not fully manifested. The thermodynamic control imposed via the nuclease site apparently enhances the specificity for the aforementioned reasons. Thus, the fidelity in the overall replication process exceeds that obtained from the kinetic control at the polymerase site, and approaches that expected from the stability of the double helix.

It is noteworthy that thermodynamic control implies reversibility and hence is meaningfully imposed at the nuclease site. This is because it makes little sense to reverse the incorporation of a Watson-Crick pair, so this occurs under kinetic control of the specificity. The excision process, however, can occur under thermodynamic control as mis-incorporation needs to be reversed for higher fidelity. (Some DNA polymerases do not possess nuclease activity, in which case a distinct nuclease enzyme performs the above mentioned excision function; however, the mechanistic arguments and conclusions remain the same as above.)

It is also noteworthy that DNA replication involves incorporation of either Watson-Crick pairs or mis-pairs, with the latter being a lower energy pathway (profile 3 above)! This may appear paradoxical but is, in fact, an unavoidable consequence of the PMR. All the same, the

thermodynamic stability of the Watson-Crick pairs – manifested in a mechanistically convoluted manner as described above – ultimately determines the overall fidelity.

Conclusions

The evolution of nuclease activity as an important part of the process of DNA replication implies that it is critical to ensuring high fidelity. However, the excision of mis-pairs via nuclease action poses particular challenges to the principle of microscopic reversibility (PMR), as the excision process occurs by a different pathway than the original incorporation route. However, a formal violation of the PMR is avoided as the mis-incorporation step at the polymerase site does not reach equilibrium. Intriguingly, the reaction at the nuclease site is relatively rapid and likely attains thermodynamic equilibrium. This appears to be the key to enhancing fidelity, as the thermodynamic stability of the Watson-Crick pairs then plays a role in the overall specificity. These arguments imply that the specificity of base incorporation at the polymerase site is relatively low, as the thermodynamic stability of the Watson-Crick pairing is not fully manifested therein. Thus, the high fidelity in the replication of DNA – critical to ensuring the integrity of the genome – is apparently achieved by a complex mechanistic scheme which barely skirts round a violation of the PMR, a cornerstone of chemical theory!

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