

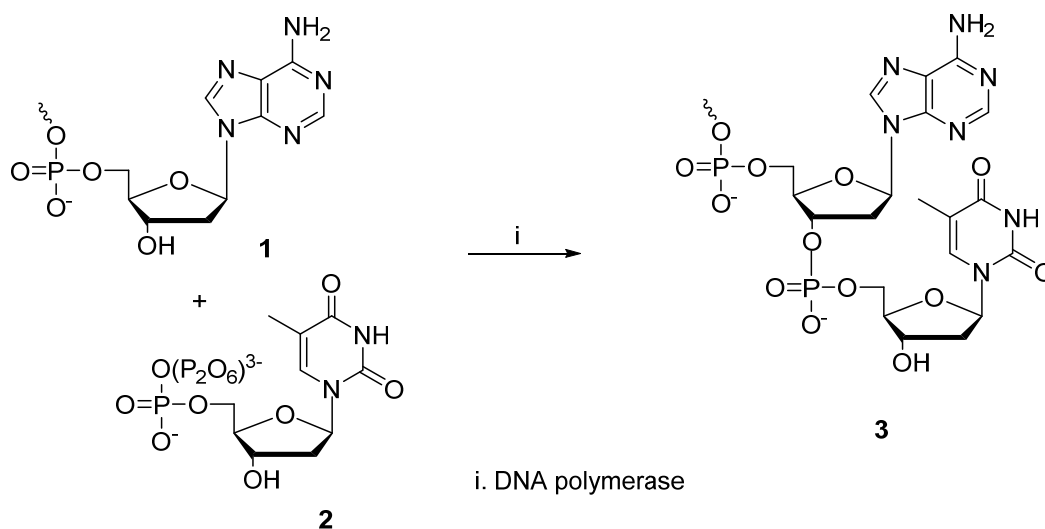
**NUCLEASE ACTIVITY OF THE DNA POLYMERASES: AN EVASION OF
MICROSCOPIC REVERSIBILITY LEADING TO FIDELITY more than KINETIC
CONTROL LEVELS**

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, more in-depth mechanistic analysis of the reactions leads to fascinating insights into the kinetic and thermodynamic effects controlling the replication of the primary genetic material. Thus, nucleotide addition and excision involve different pathways, although a 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.

Keywords: DNA polymerase; DNA replication; fidelity; microscopic reversibility; nuclease.

1. INTRODUCTION

The replication of deoxyribonucleic acid (DNA), the primary genetic material of every living cell, is the critical chemical event preceding cell division. In this sense, DNA replication is a defining characteristic of life itself [1]. The replication process, which can also be carried out in vitro, is now relatively well understood in both overall scope and mechanistic detail. Thus, DNA replication is catalysed by the enzyme DNA polymerase [2-8], indeed a family of the



22

23 **Scheme 1.** DNA polymerase-catalysed growth of a DNA single strand, as exemplified by the
 24 reaction of a terminal deoxyadenosine 3'-OH group (1) with a deoxythymidine triphosphate
 25 (2), resulting in the incorporation of a deoxythymidine phosphate moiety (3); the released
 26 pyrophosphate is not shown)

27 Polymerases being now known and characterised from various sources [4-6]. These enzymes
 28 catalyse the addition of a deoxyribonucleotide phosphate moiety to the 3' end of a growing
 29 DNA oligomer (Scheme 1), although this process needs to be primed by a complementary
 30 "template" strand (which can even be a short DNA or RNA oligomer, not shown). Thus, the
 31 "daughter" strand grows in the 5'-3' direction, even as the template strand is read in the anti-
 32 parallel 3'-5' direction. (These events would have been preceded by the partial unwinding of
 33 the DNA double helix, catalysed by certain helicase and topoisomerase enzymes, with the
 34 DNA synthesis following the "unzipping" process.)

35 The need for a complimentary "template" strand is the basis of the famed fidelity of DNA
 36 replication (typically $< 10^{-6}$ [2-4]), as it ensures that DNA cannot grow randomly on its own,
 37 even in the presence of DNA polymerase. DNA polymerase also provides the integrity of the
 38 genome regarding the base sequences of the constituent DNA!

39 It is currently believed that two modes achieve the fidelity of DNA replication: firstly, a high
40 level of specificity in the addition step of the deoxyribonucleotide phosphate moiety, ensured
41 by Watson-Crick base pairing between the growing daughter and template strands at the
42 active site of DNA polymerase; and secondly, subsequent proof-reading and error-correction
43 via the excision of a mispaired nucleotide that has been incorporated inadvertently. (The
44 misincorporation is a consequence of the limits on the specificity of the base pairing and the
45 low but finite probability that a higher energy pathway would be traversed.)

46 The proof-reading capabilities of specific DNA polymerases likely derive from steric
47 deformations of the growing strand upon misincorporation, which would weaken binding at
48 the polymerase site and slow down further growth. This hiatus would invite the nuclease site
49 to bind and excise the misincorporated nucleotide. (Alternatively, a distinct nuclease could
50 perform this function.)

51 The evolution in DNA polymerase of the above dual mode for ensuring fidelity is indeed
52 intriguing. In fact, the stability of the DNA double helix in the solution phase remains
53 unclear, as evidence indicates that a substantial fraction of single strands (partial and full)
54 may exist in equilibrium with the double-stranded form [9]. On this basis, the kinetic fidelity
55 in the replication process – rather than the thermodynamic stability of the double helix –
56 would determine the integrity of the genome. (Thus, the complementary strands ensure
57 genome integrity via dynamic control and fidelity as above!) The evolution of DNA
58 polymerase to include all possible modes for providing accuracy is unsurprising!

59 The excision capabilities of specific DNA polymerases, however, come into conflict with the
60 principle of microscopic reversibility (PMR), which requires that the forward and reverse
61 reactions follow the same mechanism (although in reverse) [10, 11]. In fact, excision occurs
62 at a different active site than the incorporation site, hence the conflict with microscopic
63 reversibility. Two modes of removal are also known: exonuclease action, which involves

64 immediate deletion of the misincorporated nucleotide at the growing terminus; and
65 endonuclease action, which requires suppression from an elongated strand at an internal
66 misincorporation position, upon subsequent proofreading.

67 The basis and consequences of these intriguing characteristics of DNA polymerase, in purely
68 physicochemical terms, are analysed below. No attempt is made to review the molecular
69 biological and other aspects of DNA polymerase, which have been the subject of numerous
70 authoritative and masterly treatments elsewhere [2-8]. No distinction is made between
71 endonuclease action and exonuclease action below, as the mechanistic conclusions are
72 similar. Also, in some cases, the DNA polymerase is bereft of a nuclease site, proof-reading
73 then being performed by a distinct nuclease enzyme. Again the mechanistic arguments
74 concerning the PMR remain the same, so these cases are not dealt with separately.

75 **2. DISCUSSION**

76 **2.1 General Considerations**

77 Sure of the DNA polymerases (although not all) have evolved nuclease activity and at a site
78 distinct from the polymerase active site. Insofar as fidelity is of supreme importance in DNA
79 replication, these features must have changed to increase fidelity levels beyond what can be
80 achieved by standard kinetic control [12, 13]. The key to how this can be brought about lies
81 in the principle of microscopic reversibility (PMR) [10, 11], as discussed in detail below.

82 Indeed, the importance of fidelity in the DNA replication process cannot be overemphasised:
83 the incorporation of incorrect bases would ultimately lead to faulty and malfunctioning
84 proteins (whether structural or functional, e.g., enzymes), via transcription to RNA and
85 translation thence. The resulting pathological conditions would hardly be conducive to the
86 survival of the whole organism [14]. Thus, fidelity in replication, genetic integrity and the

87 health of the body – not to mention that of the species as a whole – are most intimately
88 connected!

89 Hence, unravelling the physicochemical basis of the fidelity of the replication of the primary
90 genetic material (DNA) would lead to an understanding of one of the foundational principles
91 of life itself. And, intriguingly, the essential basis of this critical phenomenon appears to
92 define a grey area despite immense progress in molecular biology over several decades.

93 As noted above, misincorporation at the polymerase site is a consequence of the limited
94 possibility that a higher energy pathway would occasionally be traversed. This is
95 quantitatively defined by the principles of dynamic control [12, 13] regarding the difference
96 in the free energies of activation, which would determine the ratio of products via the two
97 routes. The evolution of the proof-reading capabilities and the nuclease site in the DNA
98 polymerases implies that simple kinetic control at the polymerase site leads to insufficient
99 levels of fidelity and maintenance of genomic integrity.

100 **2.2 The Principle of Microscopic Reversibility (PMR)**

101 The PMR is a fundamental principle that serves as the basis of – and a connecting link
102 between – chemical thermodynamics and kinetics. All reversible processes, in fact, must
103 submit to the PMR and accord with its requirements [10, 11].

104 According to the PMR, the forward and reverse reactions comprising a chemical equilibrium
105 must occur by the same mechanism in reverse. The most meaningful interpretation of this is
106 that the same pathways and transition states must be traversed to equal extents in both the
107 forward and reverse directions, at equilibrium. In other words, the reaction flux of a particular
108 path will be the same in forward and reverse directions at equilibrium. (The PMR plays a
109 much broader role in chemical biology and may be of importance to the mechanism of ATP
110 synthesis [15].)

2.3 DNA Polymerase, Proofreading and the PMR

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 removal are overall reverse processes and thus expected to occur via the same path.) 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 problem, however, would invoke an essential 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 misincorporation 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 misincorporation step via the polymerase site (profile 2), with a high transition state energy, would achieve stability relatively slowly. Channel 3, however, being of lower power would attain equilibrium relatively rapidly. The overall consequence would be that the misincorporation of a nucleotide at the polymerase active site (path 2) would be reversed quickly at the active nuclease site (reverse of route 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 two 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, speedy deletion via path 3 implies that removal cannot occur significantly along path 2. (Also, further incorporation and chain elongation would be faster than the reversal of track 2.)

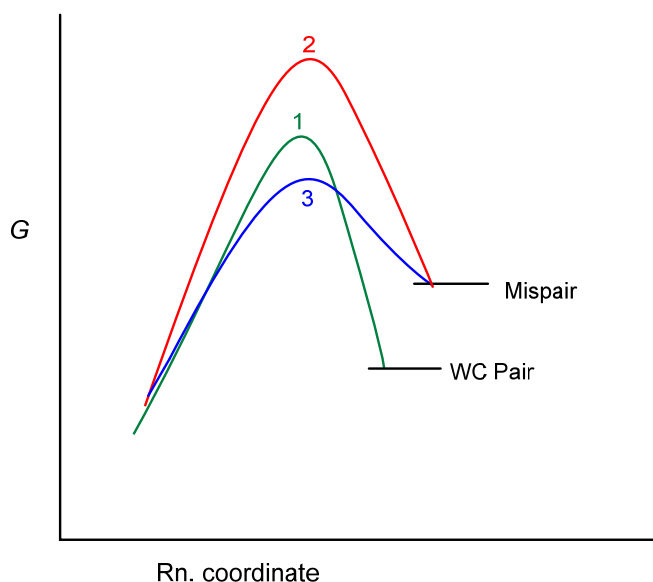


Figure 1. Gibbs free energy (G) profiles for nucleotide incorporation and excision under DNA polymerase catalysis. Pattern 1 (green) represents incorporation of a Watson-Crick (WC) base pair and model 2 (red) misincorporation, both at the polymerase active site; profile 3 (blue) represents misincorporation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

It is noteworthy in the above analysis that misincorporation 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 misincorporation 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 misincorporation 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 mispairs.

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 enhances the specificity for the reasons as mentioned earlier. Thus, the fidelity in the overall replication process exceeds that obtained from the active 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 active control of the specificity. The excision process, however, can happen under thermodynamic control as misincorporation needs to be reversed for higher fidelity. (As noted earlier, some DNA polymerases do not possess nuclease activity, in which case a distinct nuclease enzyme performs the excision as mentioned earlier function; however, the mechanistic arguments and conclusions remain the same as above.)

2.4 The Problem of Thermodynamic Control

The limitations concerning specificity at the polymerase site are unavoidable given that replication is enzyme catalysed! However, the idea of thermodynamic control based on the stability of the double helix is problematical, as this is controversial in the solution state as noted above [9]. All the same, there is no doubt the double helix possesses inherent stability and would be one of the species in solution (in equilibrium with others). Thus, at the hydrophobic polymerase site – in the absence of other solvated species – Watson-Crick base pairing would be the sole means of stabilising the growing chain.

Thus, the above arguments are predicated on two reasonable assumptions: firstly, that the thermodynamic stability of the Watson-Crick pairs offers the maximum specificity; and secondly, that equilibrium (between pairs and mispairs) is not attained via the polymerase site. Otherwise, indeed, there would be no need for the nuclease site!

Also, DNA replication involves incorporation of either Watson-Crick pairs or mispairs, with the latter being a lower energy pathway (profile three above)! Thus, it is easier to stabilise the mispairing transition state (as it is less constrained by the base pairing requirements)! 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.

3. CONCLUSIONS

Fidelity in DNA replication is critical for the integrity of the genome. The evolution of nuclease activity as an essential part of the process of DNA replication implies that it is essential to ensuring high fidelity. However, the excision of mispairs 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. Intriguingly, a formal violation of the PMR is avoided as the misincorporation step at the polymerase site does not reach equilibrium. Also, 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 uniqueness 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 achieved by a complicated mechanistic scheme which barely skirts round a violation of the PMR, a cornerstone of chemical theory!

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