

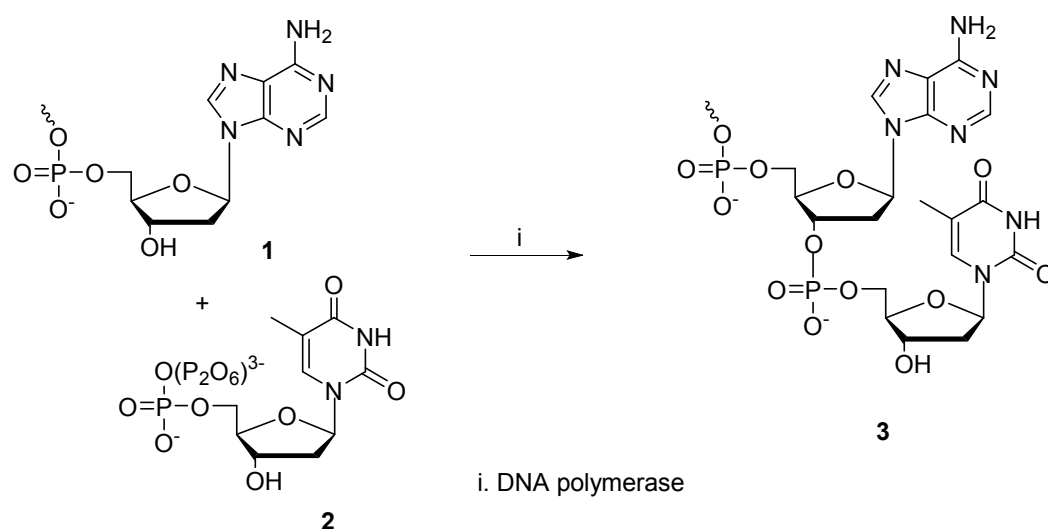
**NUCLEASE ACTIVITY OF THE DNA POLYMERASES: AN EVASION OF
MICROSCOPIC REVERSIBILITY LEADING TO FIDELITY IN EXCESS OF
KINETIC CONTROL LEVELS**

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, deeper 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 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.

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 key 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 fairly well understood in both overall scope and mechanistic detail. Thus, DNA replication is catalyzed by the enzyme DNA polymerase [2-8], indeed a family of the



22

23 **Scheme 1.** DNA polymerase catalyzed 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 characterized from various sources [4-6]. These enzymes
 28 catalyze 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, catalyzed by certain helicase and topoisomerase enzymes, with the
 34 DNA synthesis following the "unzipping" process.)

35 The need for a complementary "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. Clearly, DNA polymerase also ensures the
 38 integrity of the genome in terms of the base sequences of the constituent DNA!

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

46 The proof-reading capabilities of certain 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, apparently, would invite the
49 nuclease site to bind and excise the misincorporated nucleotide. (Alternatively, a distinct
50 nuclease could 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 apparently indicates that a substantial fraction of single strands (partial
54 and full) may exist in equilibrium with the double stranded form [9]. On this basis, the kinetic
55 fidelity in the replication process – rather than the thermodynamic stability of the double
56 helix – would determine the integrity of the genome. (Thus, the complementary strands
57 ensure genome integrity via kinetic control and fidelity as above!) Clearly, the evolution of
58 DNA polymerase to include all possible modes for ensuring fidelity is unsurprising!

59 The excision capabilities of certain 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
62 apparently occurs at a different active site than the incorporation site, hence the conflict with
63 microscopic reversibility. Two modes of excision are also known: exonuclease action, which

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

67 The basis and consequences of these intriguing characteristics of DNA polymerase, in purely
68 physico-chemical terms, are analyzed 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 Certain of the DNA polymerases (although apparently not all) have evolved nuclease activity,
78 and at a site distinct from the polymerase active site. Insofar as fidelity is of supreme
79 importance in DNA replication, these features must have evolved to increase fidelity levels
80 beyond what can be achieved by normal kinetic control [12, 13]. The key to how this can be
81 brought about apparently lies in the principle of microscopic reversibility (PMR) [10, 11], as
82 discussed in detail below.

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

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

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

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

101 **2.2 The Principle of Microscopic Reversibility (PMR)**

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

105 According to the PMR, the forward and reverse reactions comprising a chemical equilibrium
106 must occur by the same mechanism in reverse. The most meaningful interpretation of this is
107 that the same pathways and transition states must be traversed to equal extents in both the
108 forward and reverse directions, at equilibrium. In other words, the reaction flux of a particular
109 path will be the same in forward and reverse directions at equilibrium. (The PMR apparently
110 plays a much broader role in chemical biology, and may be of importance to the mechanism
111 of ATP 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 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 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 attain equilibrium relatively slowly. Pathway 3, however, being of lower energy 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 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.)

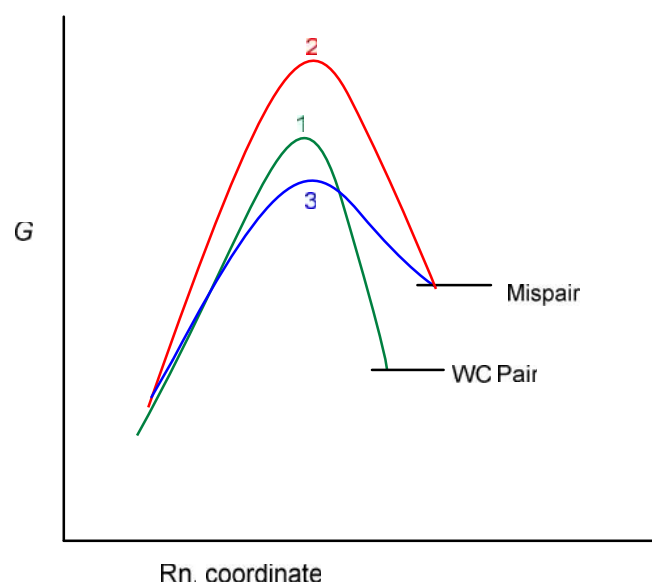


Figure 1. Gibbs free energy (G) profiles for nucleotide incorporation and excision under DNA polymerase catalysis. Profile 1 (green) represents incorporation of a Watson-Crick (WC) base pair and profile 2 (red) misincorporation, both at the polymerase active site; profile 3 (blue) represents misincorporation (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 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 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 above mentioned excision 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 apparently controversial in the solution state as noted above [9]. All the same, there is no doubt the double helix possesses an 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 3 above)! Thus, it is easier to stabilize 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 important part of the process of DNA replication implies that it is critical 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 apparently 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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