Enzymology of Replication

- DNA helicase:

➢ SSB (single stranded binding protein)

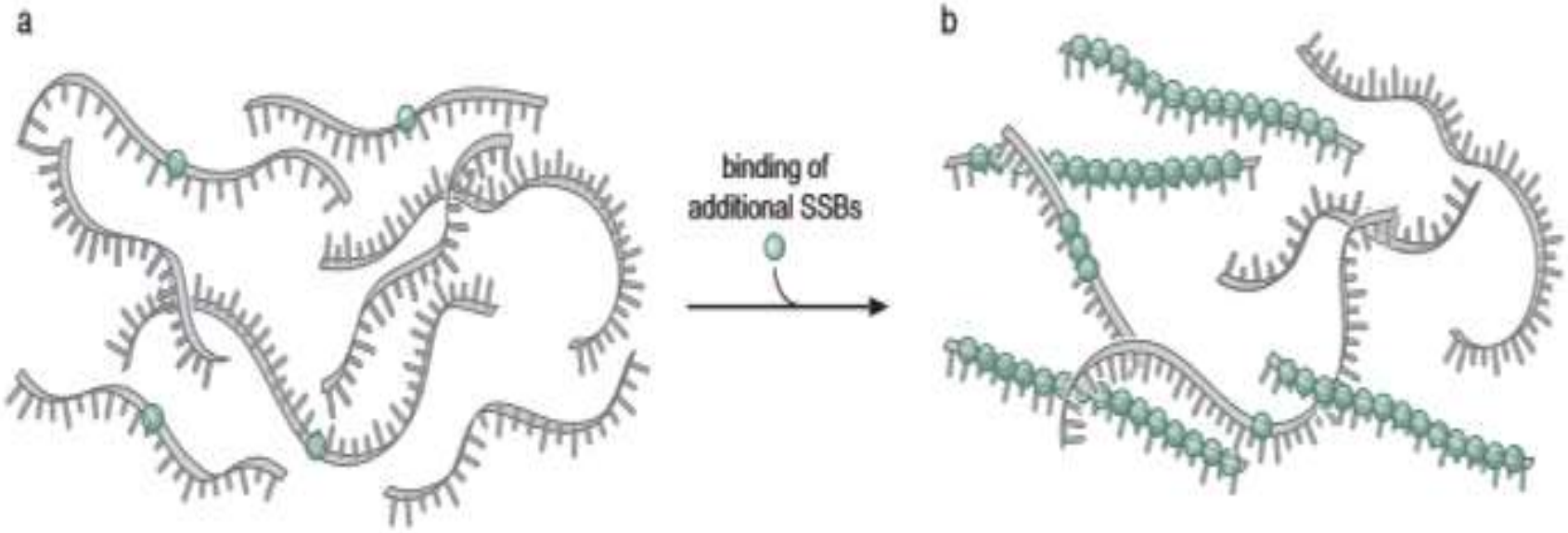


FIGURE 9-16 Binding of single-stranded binding protein (SSB) to DNA. (a) A limiting amount of SSBs is bound to four of the nine ssDNA molecules shown. (b) As more SSBs bind to DNA, they preferentially bind adjacent to previously bound SSB molecules. Only after SSBs have completely coated the initially bound ssDNA molecules does binding occur on other molecules. Note that when ssDNA is coated with SSBs, it assumes a more extended conformation that inhibits the formation of intramolecular base pairs.

➢ Topoisomerases Remove Supercoils Produced by DNA Unwinding at the Replication Fork

• As the strands of DNA are separated at the replication fork, the ds DNA in front of the fork becomes increasingly positively supercoiled.
• This accumulation of supercoils is the result of DNA helicase eliminating the base pairs between the two strands.
• If the DNA strands remain unbroken, there can be no reduction in linking number (the number of times the two DNA strands are intertwined) to accommodate this unwinding of the DNA duplex.

• Thus, as the DNA helicase proceeds, the DNA must accommodate the same linking number within a smaller and smaller number of base pairs.
• Indeed, for the DNA in front of the replication fork to remain relaxed, one DNA link must be removed for every approx 10 bp of DNA unwound.
• If there were no mechanism to relieve the accumulation of these supercoils, the replication machinery would grind to a halt in the face of mounting strain placed on the DNA in front of the replication fork.

• The supercoils introduced by the action of the DNA helicase are removed by topoisomerases that act on the unreplicated ds DNA in front of the replication fork.
• These enzymes do this by breaking either one or both strands of the DNA without letting go of the DNA and passing the same number of DNA strands through the break.
• This action relieves the accumulation of supercoils.
• In this way, topoisomerases act as a “swivelase” that prevents the accumulation of positive supercoils ahead of the replication fork.

❖ Type I isomerases:

• They are nicking-closing enzymes that break and reseal one strand of DNA at a time, changing the linking number in steps of one.

• When one DNA strand is cleaved transiently, it allows the passage of another strand.

❖ Type II isomerases:

• It makes a transient double-stranded break in the helix and form a covalent linkage to both strands of the DNA helix at the same time.

• Once a type II topoisomerase molecule binds to such a crossing site, the protein uses ATP hydrolysis to perform the following set of reactions efficiently:
a) Breaks one double helix reversibly to create a DNA gate
b) Causes the second, nearby double helix to pass through this break.
c) Reseals the break and dissociates from the DNA.

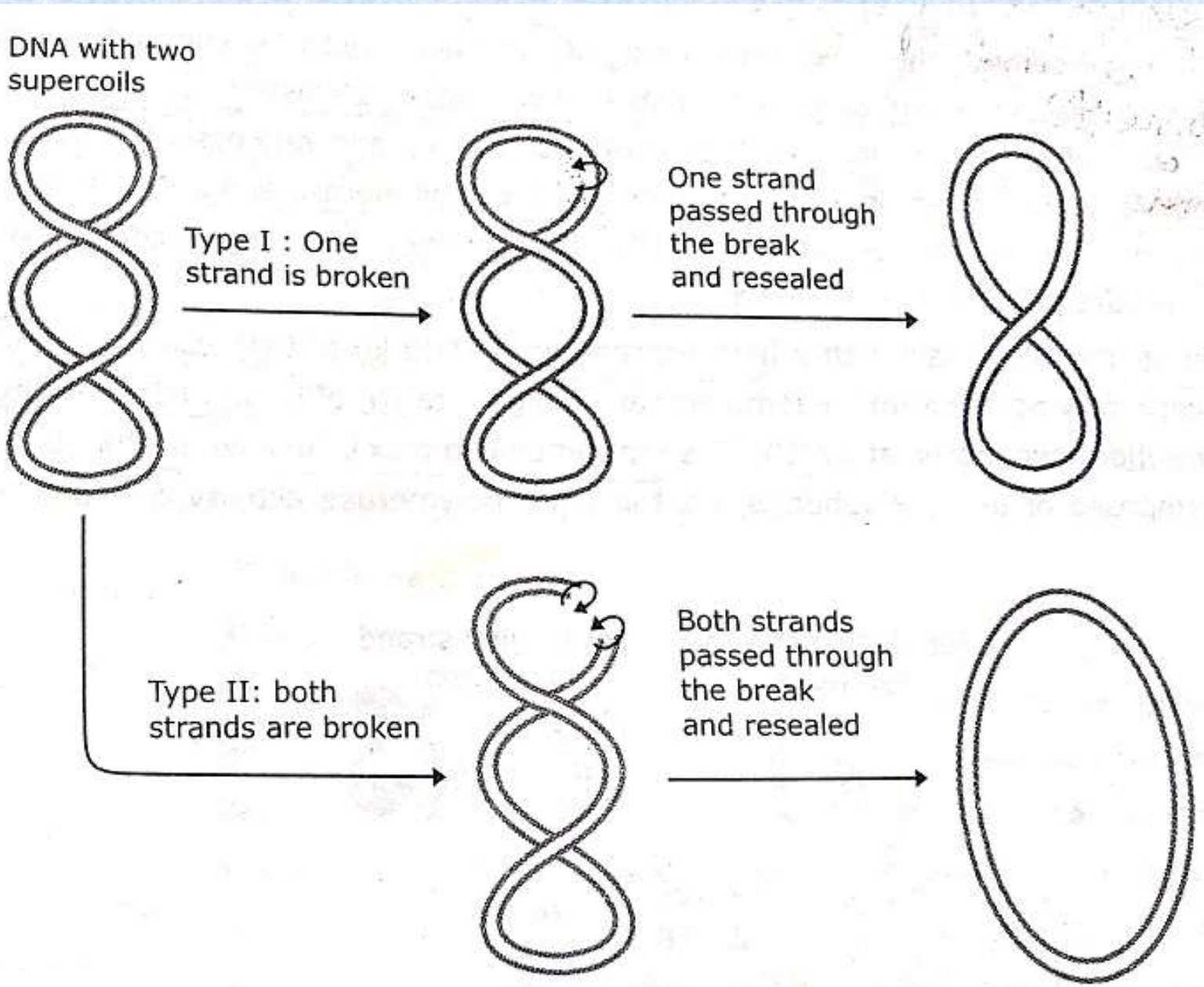
• DNA gyrase (the first type II topoisomerase discovered from *E.coli*) has the ability to introduce supercoils into DNA.

- It can introduce negative supercoils into DNA using the free energy from ATP hydrolysis.

- DNA gyrase is a tetramer of two different subunits.

- The Gyr A subunit cuts and rejoins the DNA and the Gyr B subunit is responsible for providing energy by ATP hydrolysis.

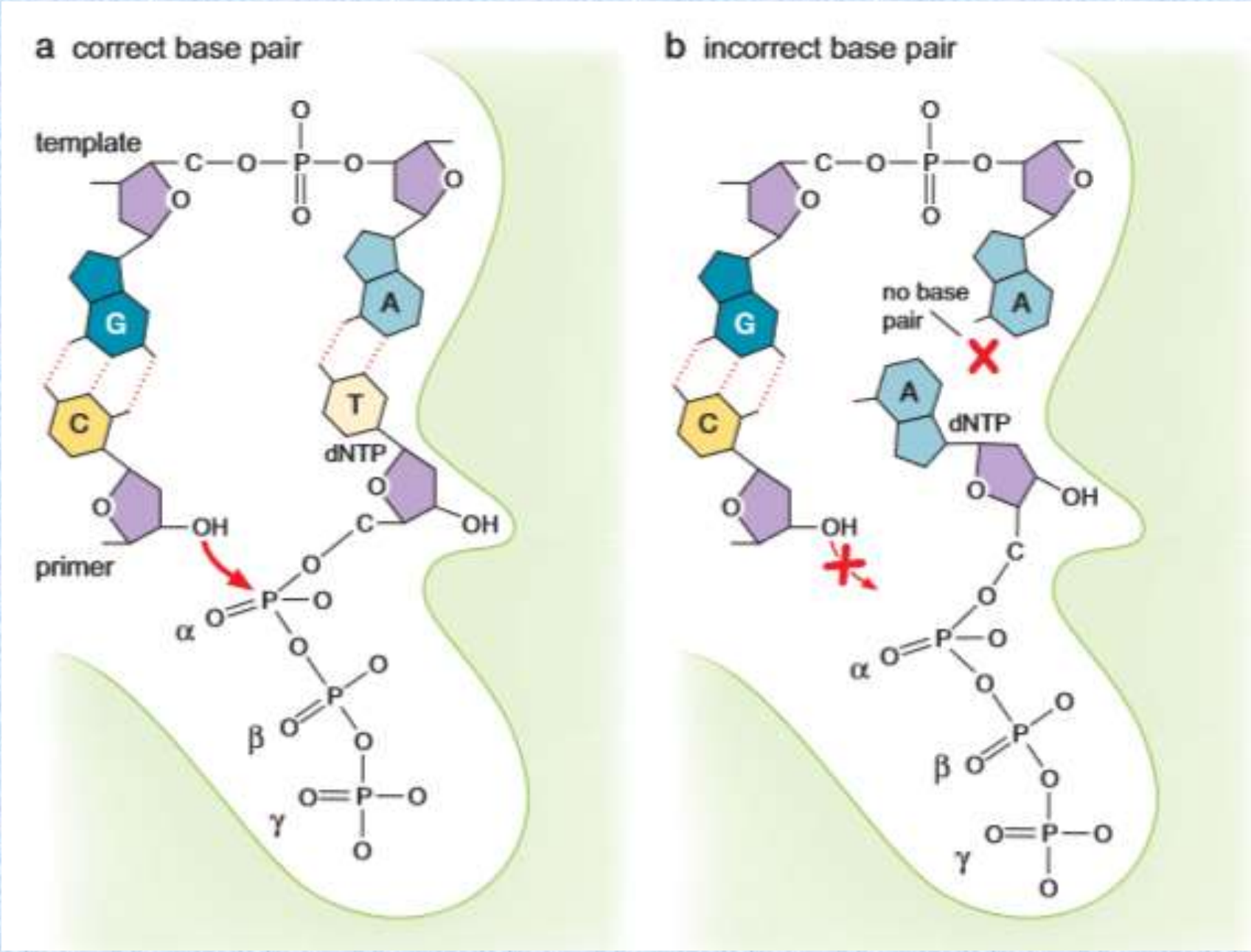
- DNA gyrase is inhibited by quinolone antibiotics such as nalidixic acid and their functional derivatives.


DNA with two supercoils
Type I : One strand is broken
One strand passed through the break and resealed
Type II: both strands are broken
Both strands passed through the break and resealed

➢ DNA polymerase

• The synthesis of DNA is catalyzed by a class of enzymes called DNA polymerases.
• Unlike most enzymes, which have one active site that catalyzes one reaction, DNA polymerase uses a single active site to catalyze the addition of any of the four deoxynucleoside triphosphates.
• Only when a correct base pair is formed are the 3′-OH of the primer and the α –phosphate of the incoming nucleoside triphosphate in the optimum position for catalysis to occur.

• Incorrect base pairing leads to dramatically lower rates of nucleotide addition as a result of a catalytically unfavorable alignment of these substrates.
• DNA polymerases show an impressive ability to distinguish between ribonucleoside and deoxyribonucleoside triphosphates (rNTPs and dNTPs).
• This discrimination is mediated by steric exclusion of rNTPs from the DNA polymerase active site. In DNA polymerase, the nucleotide-binding pocket cannot accommodate a 2′-OH on the incoming nucleotide.


a correct base pair
template
G
C
A
T
dNTP
OH
primer
α
β
γ
b incorrect base pair
no base
pair
G
C
A
dNTP
OH
α
β
γ

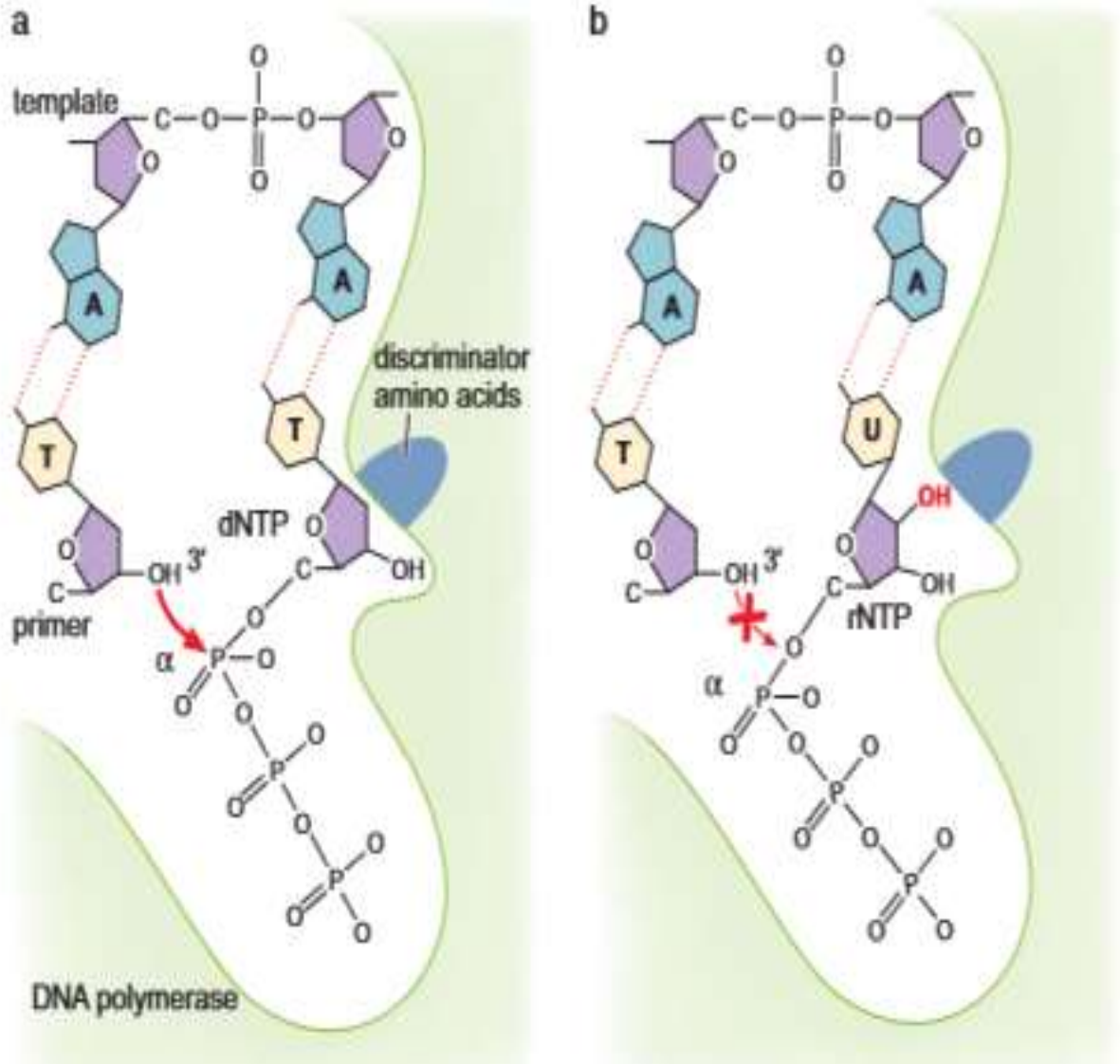
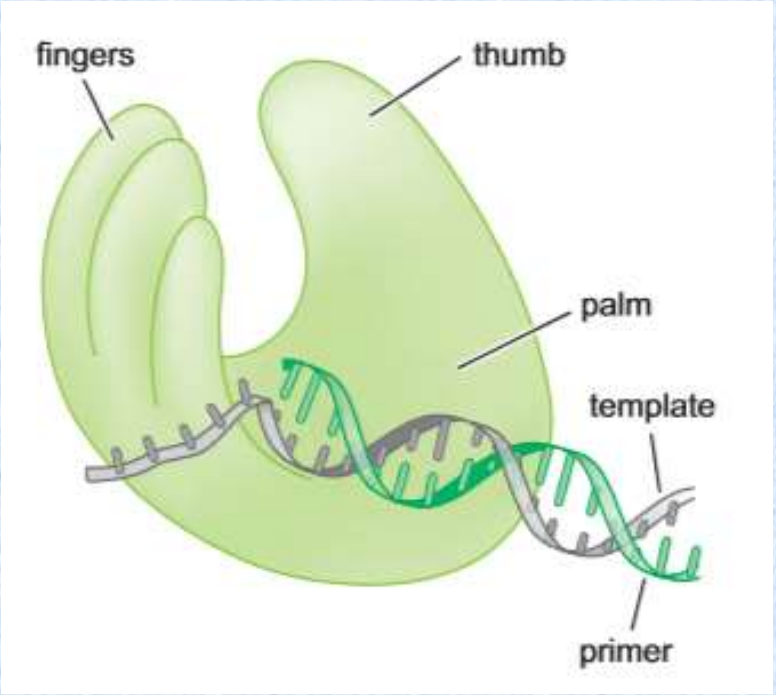


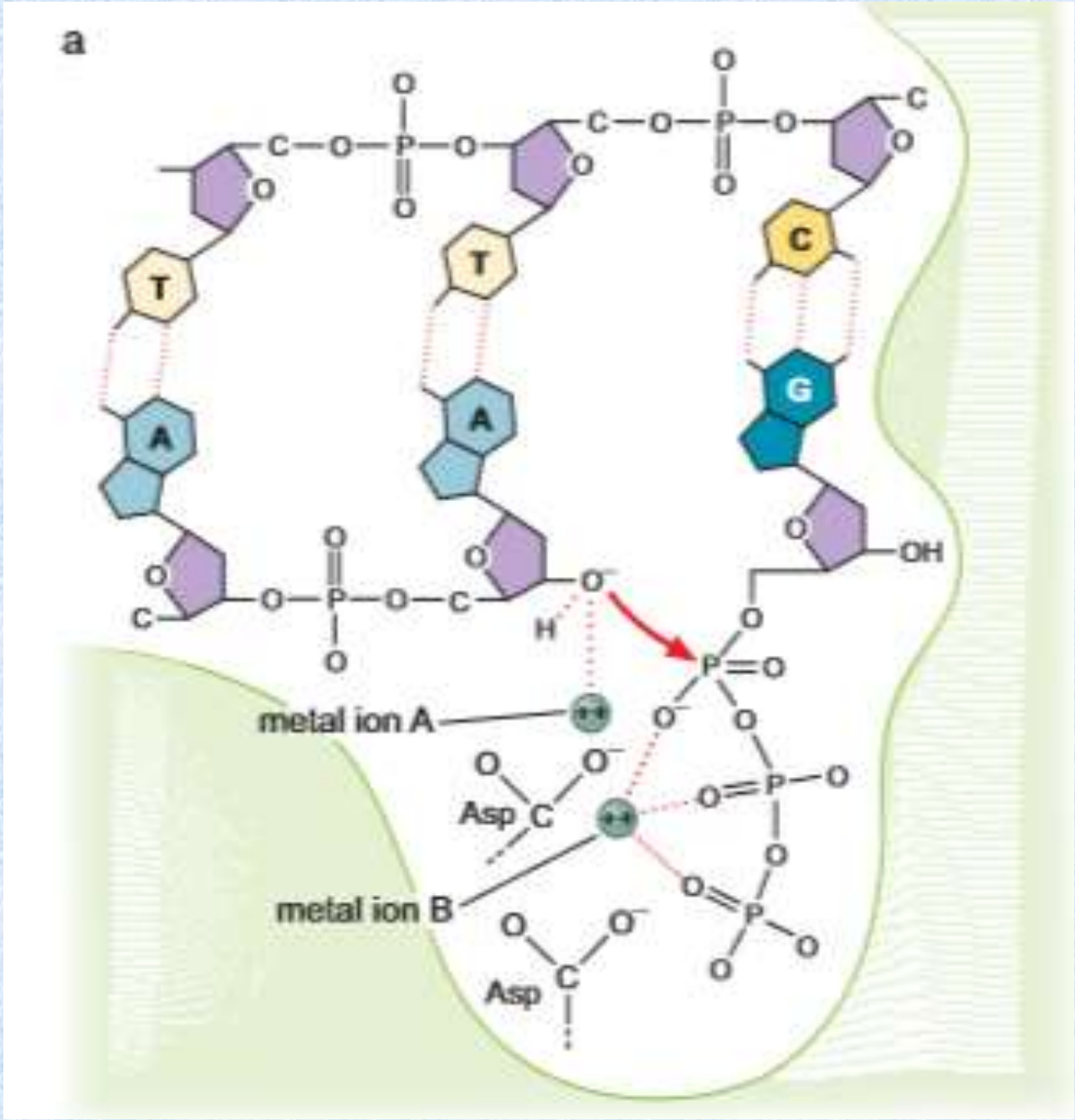
FIGURE 9-4 **Schematic illustration of the steric constraints preventing DNA polymerase from using rNTP precursors.** (a) Binding of a correctly base-paired dNTP to the DNA polymerase. Under these conditions, the 3′-OH of the primer and the α-phosphate of the dNTP are in close proximity. (b) Addition of a 2′-OH results in a steric clash with amino acids (the discriminator amino acids) in the nucleotide-binding pocket. This results in the α-phosphate of the dNTP being displaced. In this state, the α-phosphate is incorrectly aligned with the 3′-OH of the primer, dramatically reducing the rate of catalysis.

❖ DNA Polymerases Resemble a Hand That Grips the Primer : Template Junction

• Structures reveal that the DNA substrate sits in a large cleft that resembles a partially closed right hand. Based on the hand analogy, the three domains of the polymerase are called the thumb, fingers, and palm.



• The palm domain is composed of a β sheet and contains the primary elements of the catalytic site.
• In particular, this region of DNA polymerase binds two divalent metal ions (typically Mg^{2+} or Zn^{2+}) that alter the chemical environment around the correctly base-paired dNTP and the 3′-OH of the primer.
• One metal ion reduces the affinity of the 3’-OH for its hydrogen.
• This generates a 3′O that is primed for the nucleophilic attack of the α-phosphate of the incoming dNTP.


a
T
T
C
A
A
G
OH
H
metal ion A
Asp
metal ion B
Asp

• The second metal ion coordinates the negative charges of the β-phosphate and γ-phosphate of the dNTP and stabilizes the pyrophosphate produced by joining the primer and the incoming nucleotide.
• The fingers are also important for catalysis. Several residues located within the fingers bind to the incoming dNTP.
• More importantly, once a correct base pair is formed between the incoming dNTP and the template, the finger domain moves to enclose the dNTP.

• The thumb interacts with the DNA that has been most recently synthesized. This serves two purposes.
• First, it maintains the correct position of the primer and the active site.
• Second, the thumb helps to maintain a strong association between the DNA polymerase and its substrate.
• This association contributes to the ability of the DNA polymerase to add many dNTPs each time it binds a primer : template junction

❖ DNA Polymerases Are Processive Enzymes :

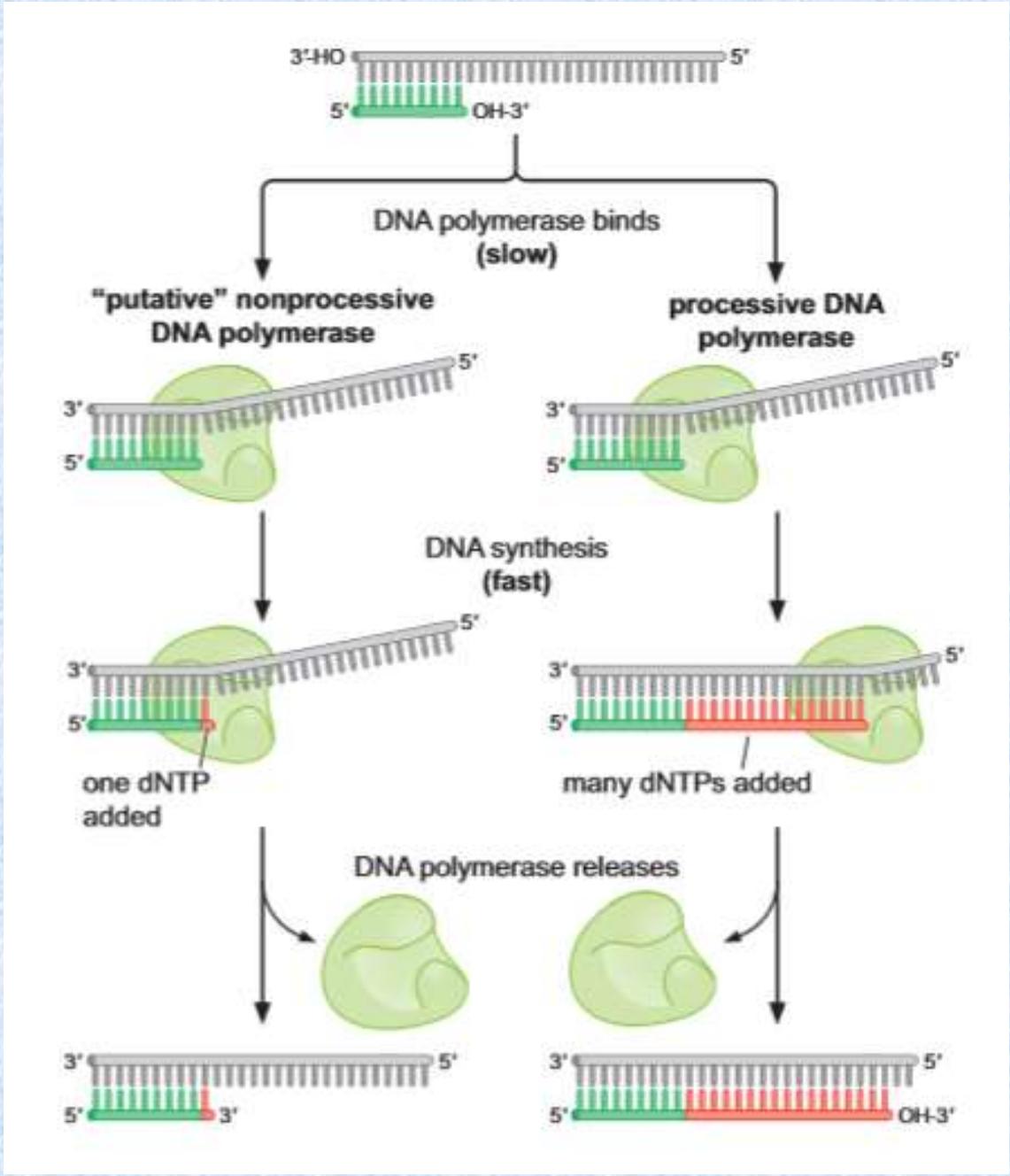
• Catalysis by DNA polymerase is rapid. DNA polymerases are capable of adding as many as 1000 nucleotides/sec to a primer strand.

• The speed of DNA synthesis is largely due to the processive nature of DNA polymerase.

• Processivity is a characteristic of enzymes that operate on polymeric substrates.

• In the case of DNA polymerases, the degree of processivity is defined as the average number of nucleotides added each time the enzyme binds a primer : template junction.

• Each DNA polymerase has a characteristic processivity that can range from only a few nucleotides to more than 50,000 bases added per binding event.
• A completely nonprocessive DNA polymerase would add approx.1 bp /sec. In contrast, the fastest DNA polymerases add as many as 1000 nucleotides/sec by remaining associated with the template for thousands of rounds of dNTP addition.
• Consequently, a highly processive polymerase increases the overall rate of DNA synthesis by as much as 1000-fold compared with a nonprocessive enzyme.


3'-HO
5'
5'
OH-3'
DNA polymerase binds
(slow)
"putative" nonprocessive
DNA polymerase
processive DNA
polymerase
DNA synthesis
(fast)
one dNTP
added
many dNTPs added
DNA polymerase releases
OH-3'

• DNAP III has a high processivity and can extend a chain for several thousand nucleotides without dissociation.

• DNAP I has low processivity and dissociates rapidly from the template.

• It extends the primer by only 10-50 nucleotides for each template-binding event.

• DNAP II has a processivity of approx. 50 nucleotides.

❖ Fidelity:

• Fidelity of polymerases refers to the low frequency of errors in replication.

• Replication by DNA polymerases show very high fidelity.

• the overall fidelity of DNA replication is the result of three distinct reactions.

a) Polymerization reaction per se in which there is nucleotide selection.

b) The 3′- 5 ′ exonuclease proofreading activity for removing incorrectly inserted nucleotides before the DNA is elongated.

c) The post replication repair system which removes incorrectly inserted nucleotides after the polymerase has extended the DNA chain (mismatch repair).

- As a result of these reactions the overall frequency of error is only 10^{-9} to 10^{-10} .

- Such precise genome duplication is essential for survival.

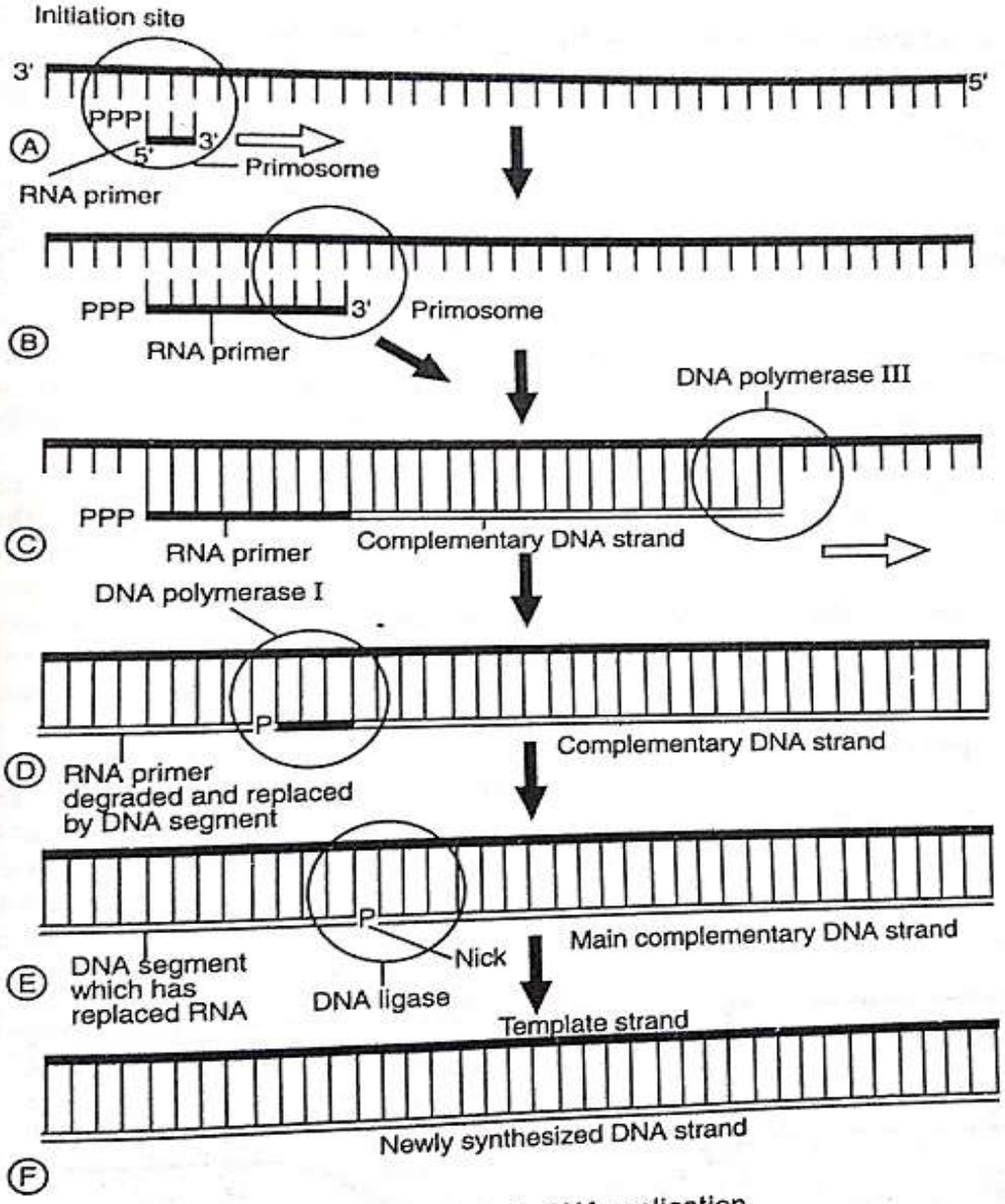
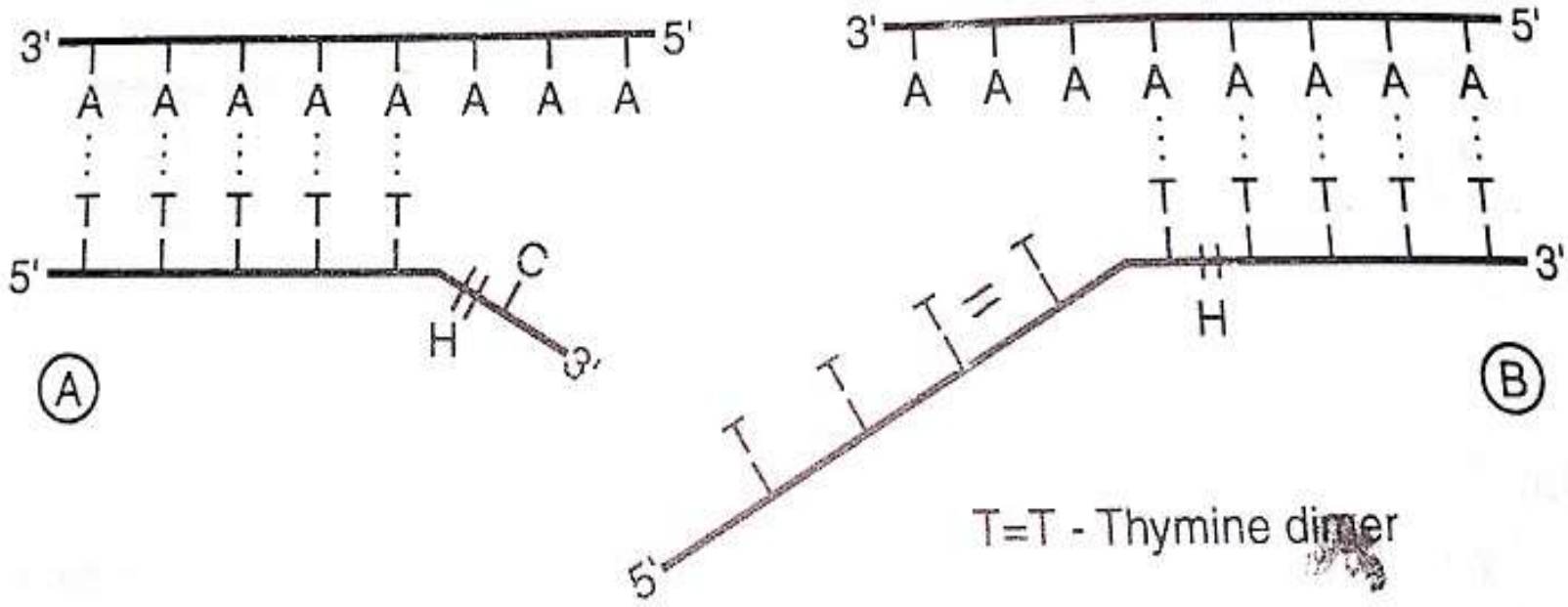


Fig. 14.21. The role of polmerases in DNA replication.

❖ DNA polymerase I :

• DNA pol I is the first DNA synthesizing enzyme discovered by Arthur Kornberg.
• DNA repair and discontinuous DNA synthesis.
▪ DNA pol I activities:

• 5′ to 3′ polymerization activity
• 5′ to 3 ′ exonuclease activity
• 3′ to 5 ′ exonuclease activity



A. 3' → 5' exonuclease activity. B. 5' → 3' endonuclease activity.

Fig. 14.22. Nuclease activities of DNA polymerase I.

(A) 3′ → 5′ exonuclease activity.
(B) 5′ → 3′ exonucelase activity. H-Hydrolysis site.

▪ Structure of DNA pol I:

• Single polypeptide chain of molecular mass 109 kDa.

• It is a metalloenzyme with one atom of Zn present per chain.

• It contains a number of sites which have functional importance.

a) Template site: attaches to the DNA template and holds a section of DNA in place.

b) Primer site: contains the primer , a short (approx 100 nucleotides) complementary segment of RNA on which the newly synthesized DNA strand grows.

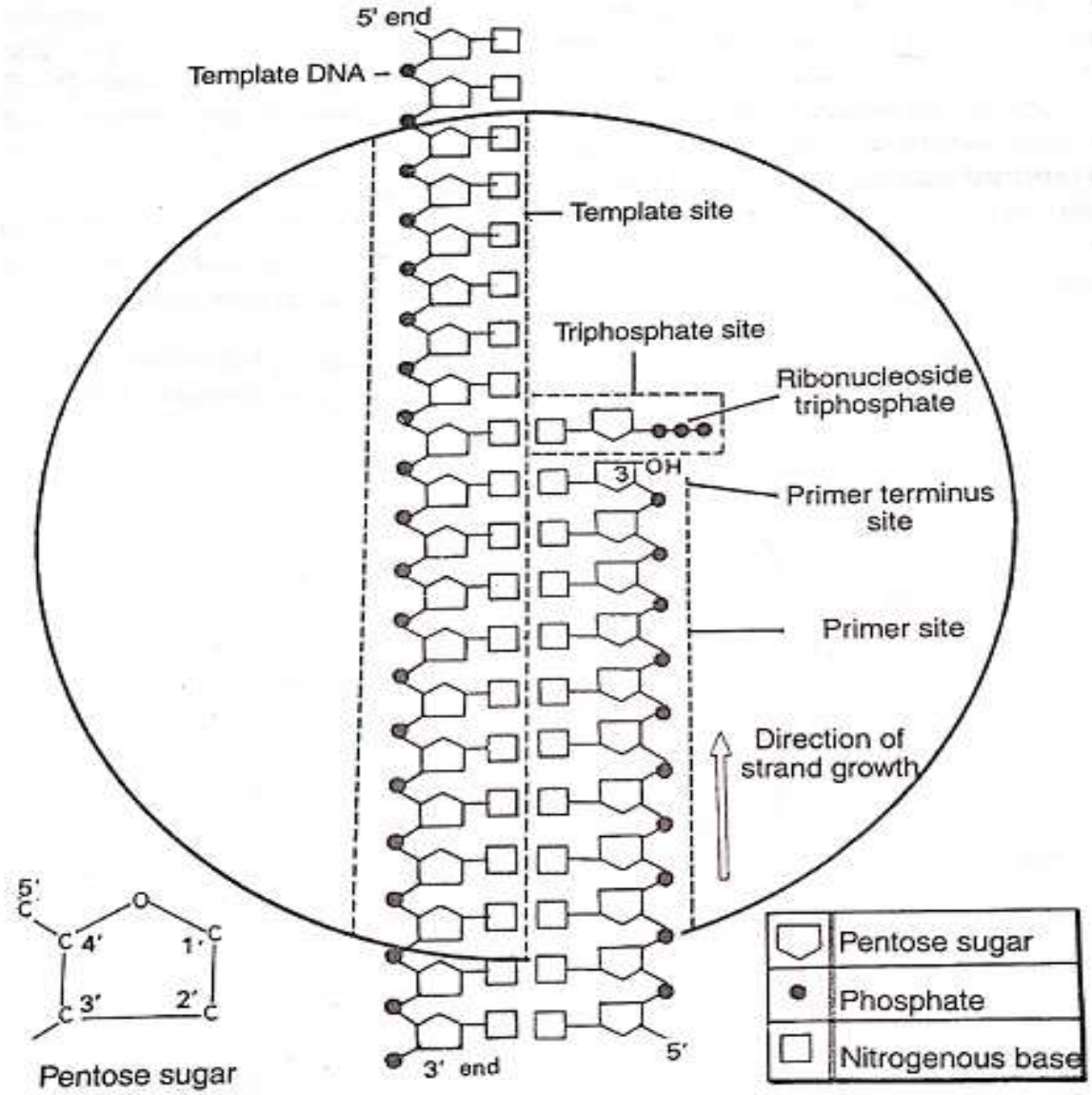


Fig. 14.23. DNA polymerase I.

c) Primer terminus site: at the tip of the primer, which has a terminal 3 ´-OH group.
d) Triphosphate site: an incoming nucleoside triphosphate matches a complementary nucleotide on the DNA template and is bound to the 3´-OH group.

- The Klenow fragment:
- The polypeptide chain of bacterial DNAP I is cleaved by proteases such as trypsin and subtilisn into a large (68kDa) fragment called the Klenow fragment and a small (36kDa) fragment.

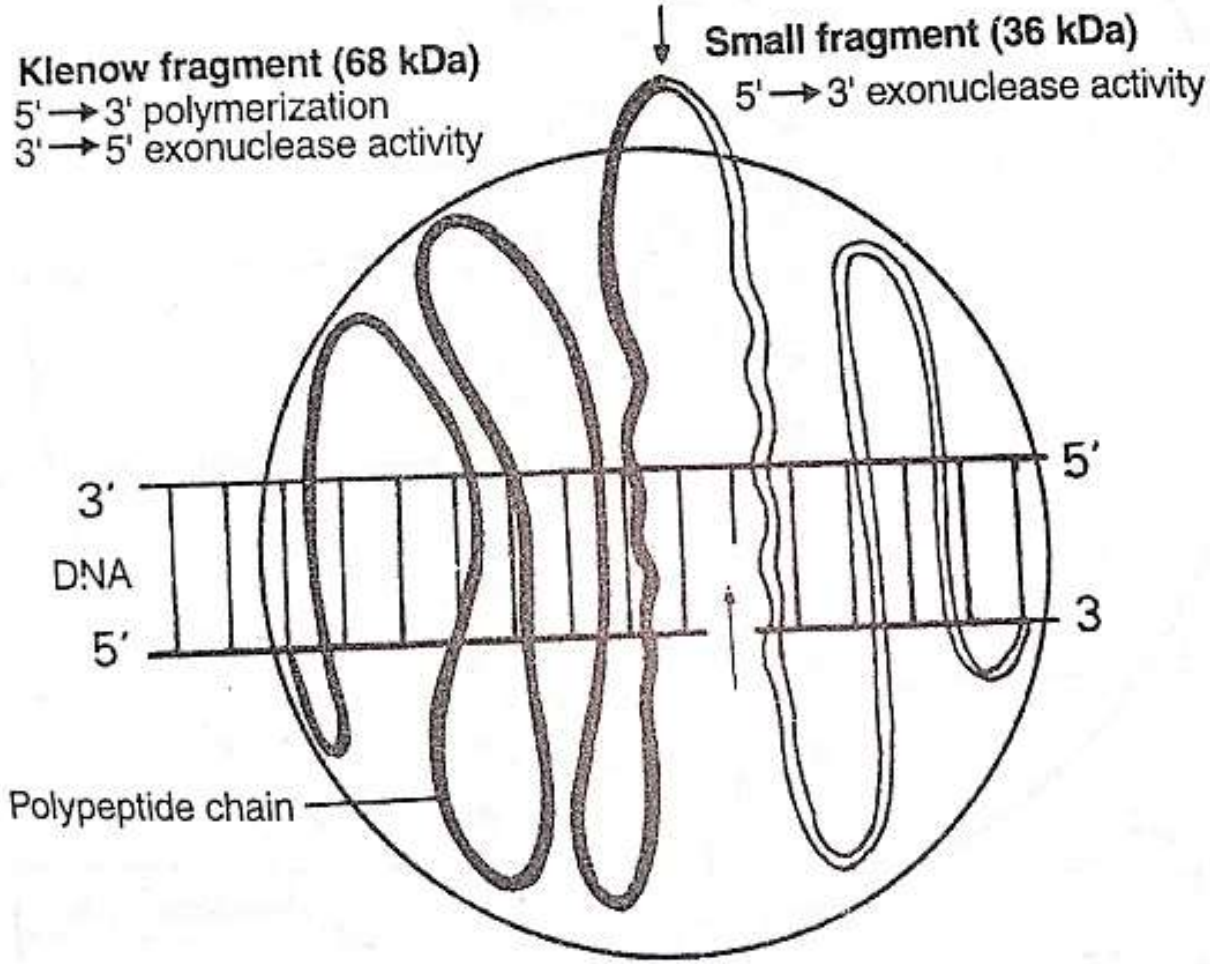


Fig. 14.24.Diagram of DNA polymerase I showing its activities. The enzyme trypsin cuts (arrow pointing downwards) the polypeptide chain into a large (black) and a small (white) fragment. The large fragment (MW 75 kDa) has 3′→5′ exnuclease activity and 5′→3′ polymerase activity, while the small fragment (MW 36 kDa) has 5′ → 3′ exonuclease activity.

- The Klenow fragment has two domains:

a) The larger C-terminal domain has a very prominent cleft in which it is located in polymerase active site.

b) The smaller N-terminal domain contains the 3´ to 5 ´ exonuclease active site. The two active sites are located about 3 nm apart. The DNA binding functions are shared between the polymerase and exonuclease domains.

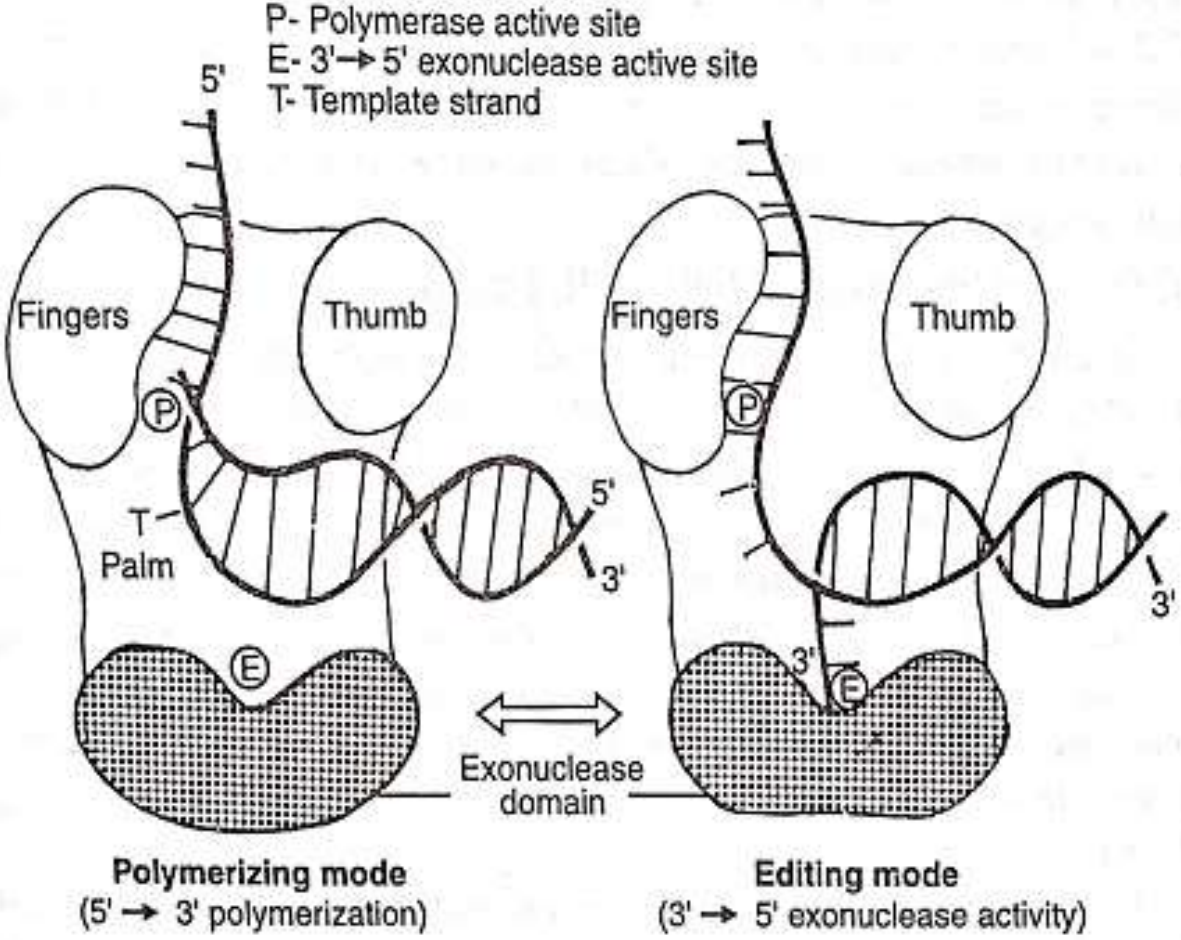


Fig. 14.25. Proofreading function and direction of synthesis in Klenow fragment.

4b. DNA polymerase II (DNAP II, Pol II)

DNAP II is a single polypeptide chain with a molecular weight of about 90 kDa. There are about 40 molecules of DNAP II present in each *E.coli* cell. DNAP II shows ***5′→3′ polymerization activity***, and also contains an associated ***nuclease*** digesting in the 5′→ 3′ direction. Unlike polymerase I, however, ***it does not show 5′→ 3′ exonuclease activity***. The ***polymerization activity*** of DNAP II is much less than that of DNAP I. Only about 60 nucleotides are polymerized per minute in *E.coli*. Moreover, DNAP II can synthesize not more than 50 nucleotides of template DNA. The ***3′→5′ exonuclease activity*** of DNAP II indicates that it may have an '*editing*' role in *repair replication* of ultraviolet-induced DNA damage.

It has been found that DNAP II can ***elongate Okazaki fragments*** in the absence of polymerase I. It may therefore provide an alternate pathway for joining Okazaki fragments into longer DNA segments during replication.

❖ DNA Pol III HE:

• It consists of a multisubunit particle made up of 10 types of subunits.

• Its main function is chromosomal duplication, i.e it is a chromosomal replicase.

Table 14.4. Subunits and subassembles of DNA polymerase III holoenzyme.

Subunit	Gene	Mass (kDa)	Function	Assembly
Theta (θ)	*holE*	8.6	Stimulates ε exonuclease.	CORE, Pol III', Pol III*, Pol III holoenzyme (DNAP III HE)
Epsilon (ε)	*dnaQ*	27.5	Proof reading 3′→5′ exonuclease	
Alpha (α)	*dnaE*	129.9	DNA polymerase	
Tau (τ)	*dnaX*	71.1	DNA-dep. ATPase, dimerizes core	
Gamma (γ)	*dnaX*	47.5	Binds ATP	γ-complex
Delta (δ)	*holA*	38.7	Binds to β	
Delta' (δ')	*holB*	36.9	Stimulates clamp loading	
Chi (χ)	*holC*	16.6	Binds SSB	
Psi (ψ)	*holD*	15.2	Bridge between χ and γ	
Beta (β)	*dnaN*	40.6	Sliding clamp on DNA	

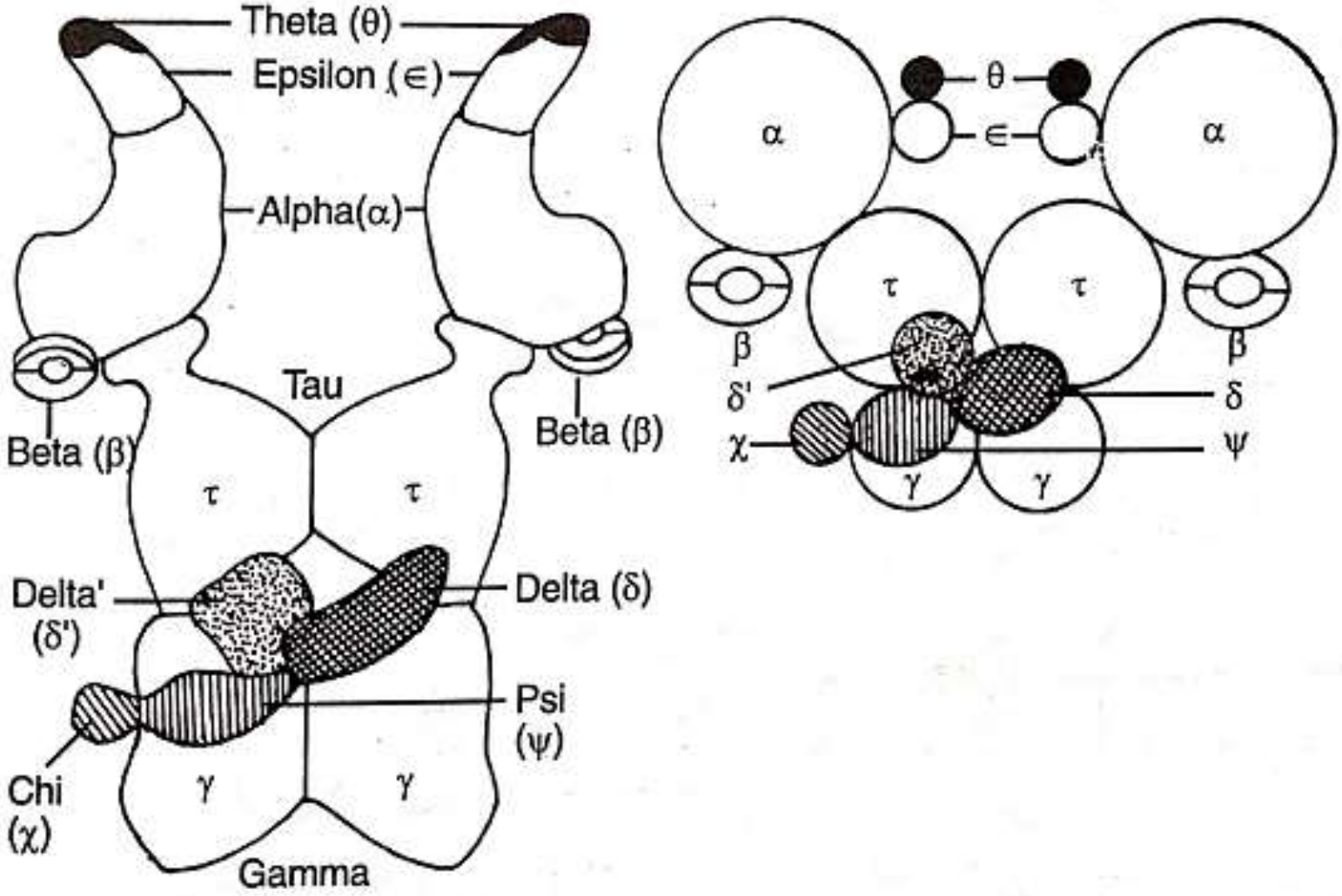


Fig. 14.26. DNA polymerase III holoenzyme (DNAP III HE) subunits.

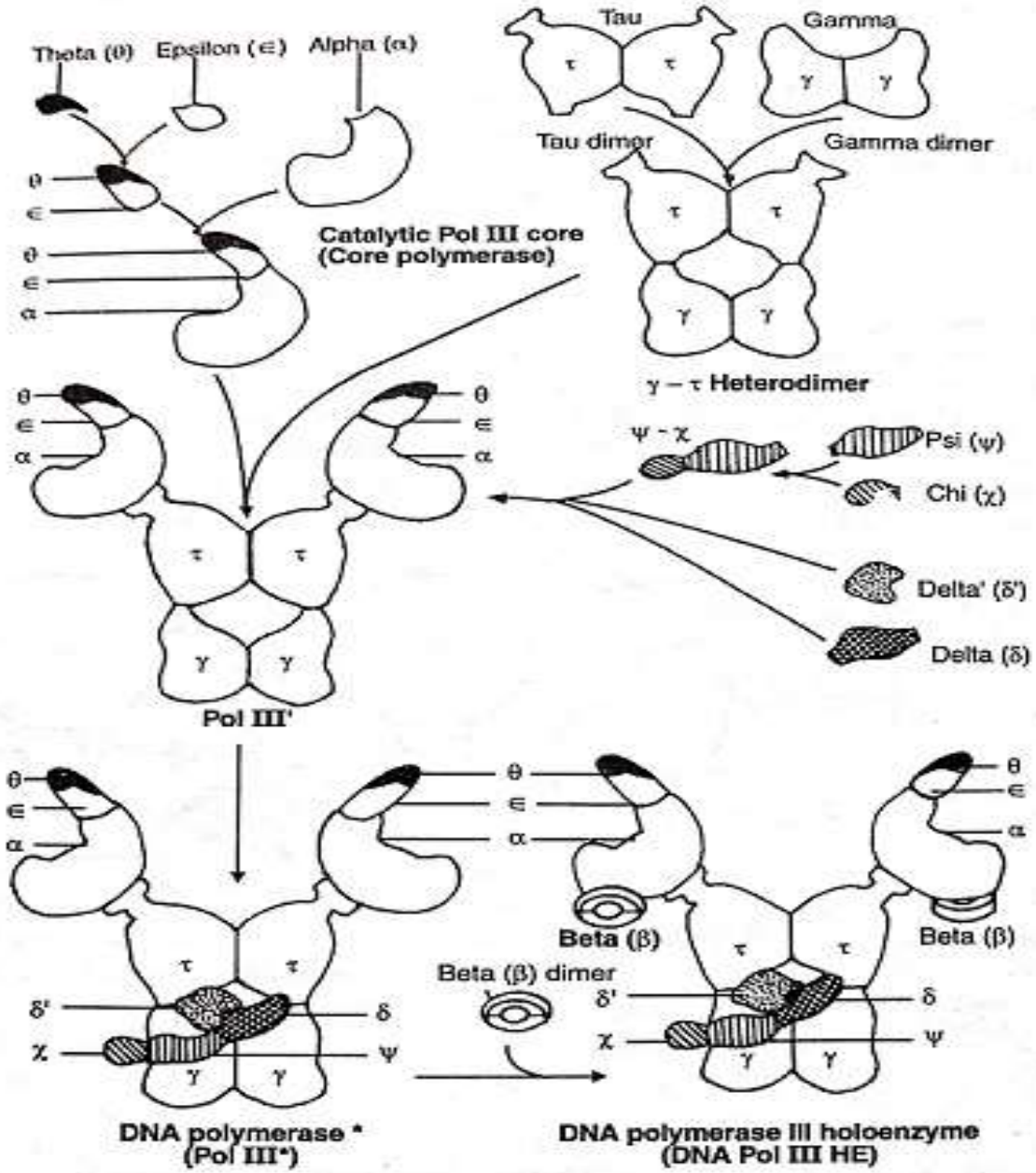


Fig. 14.27. Assembly of DNA polymerase III holoenzyme (DNAP III HE).

- β subunit:

• One key to the high processivity of the DNA polymerases that act at replication forks is their association with proteins called sliding DNA clamps.

• These proteins are composed of multiple identical subunits that assemble in the shape of a “doughnut.”

• The hole in the center of the clamp is large enough to encircle the DNA double helix and leave room for a layer of one or two water molecules between the DNA and the protein.

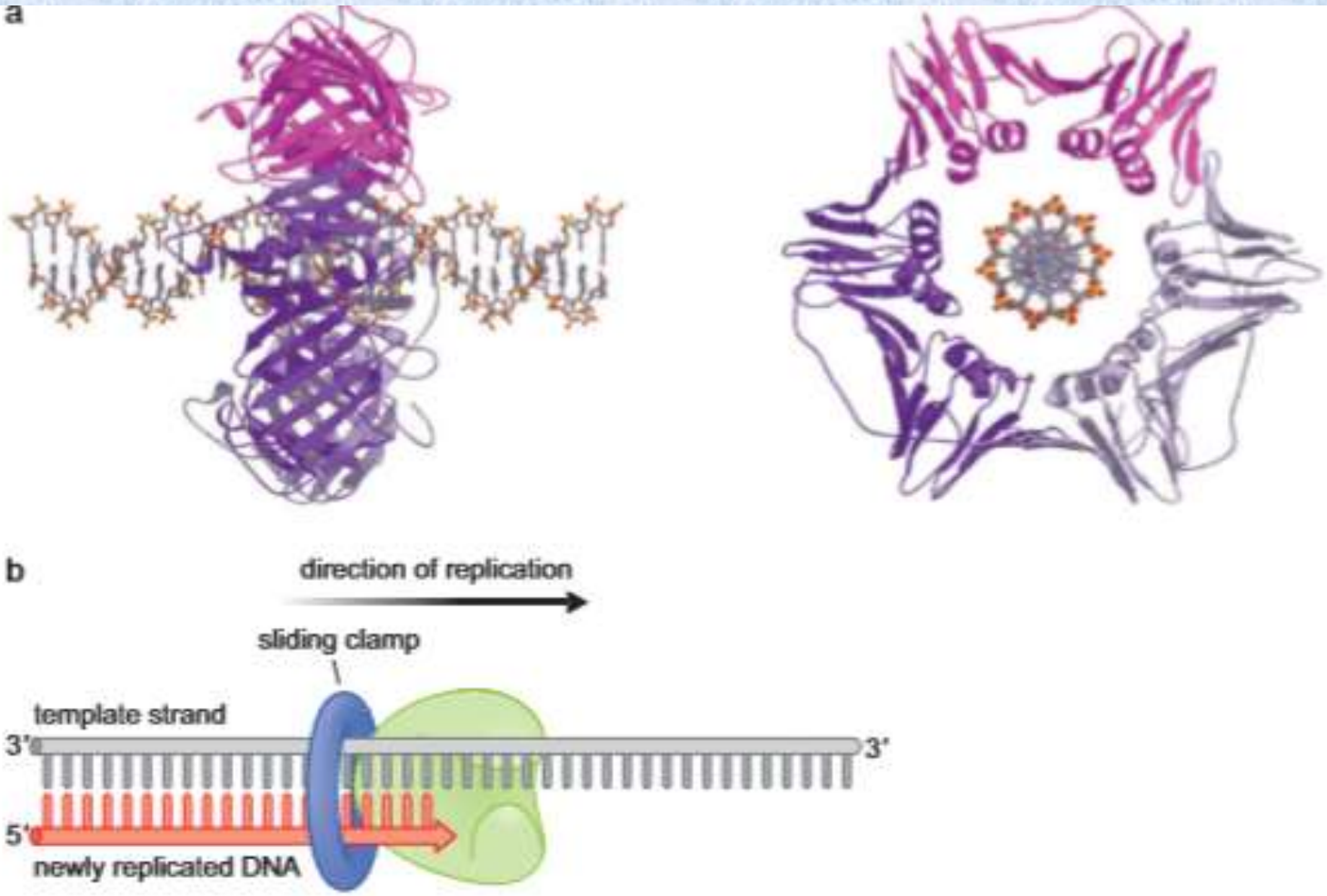


FIGURE 9-19 Structure of a sliding DNA clamp. (a) 3D structure of a sliding DNA clamp associated with DNA. The opening through the center of the sliding clamp is ~35 Å, and the width of the DNA helix is ~20 Å. This provides enough space to allow a thin layer of one or two water molecules between the sliding clamp and the DNA. This is thought to allow the clamp to slide along the DNA easily. (Adapted from Krishna T.S. et al. 1994. *Cell* **79**: 1233–1243. Image prepared with MolScript, BobScript, and Raster3D. DNA modeled by Leemor Joshua-Tor.) (b) Sliding DNA clamps encircle the newly replicated DNA produced by an associated DNA polymerase. The sliding clamp interacts with the part of the DNA polymerase that is closest to the newly synthesized DNA as it emerges from the DNA polymerase.

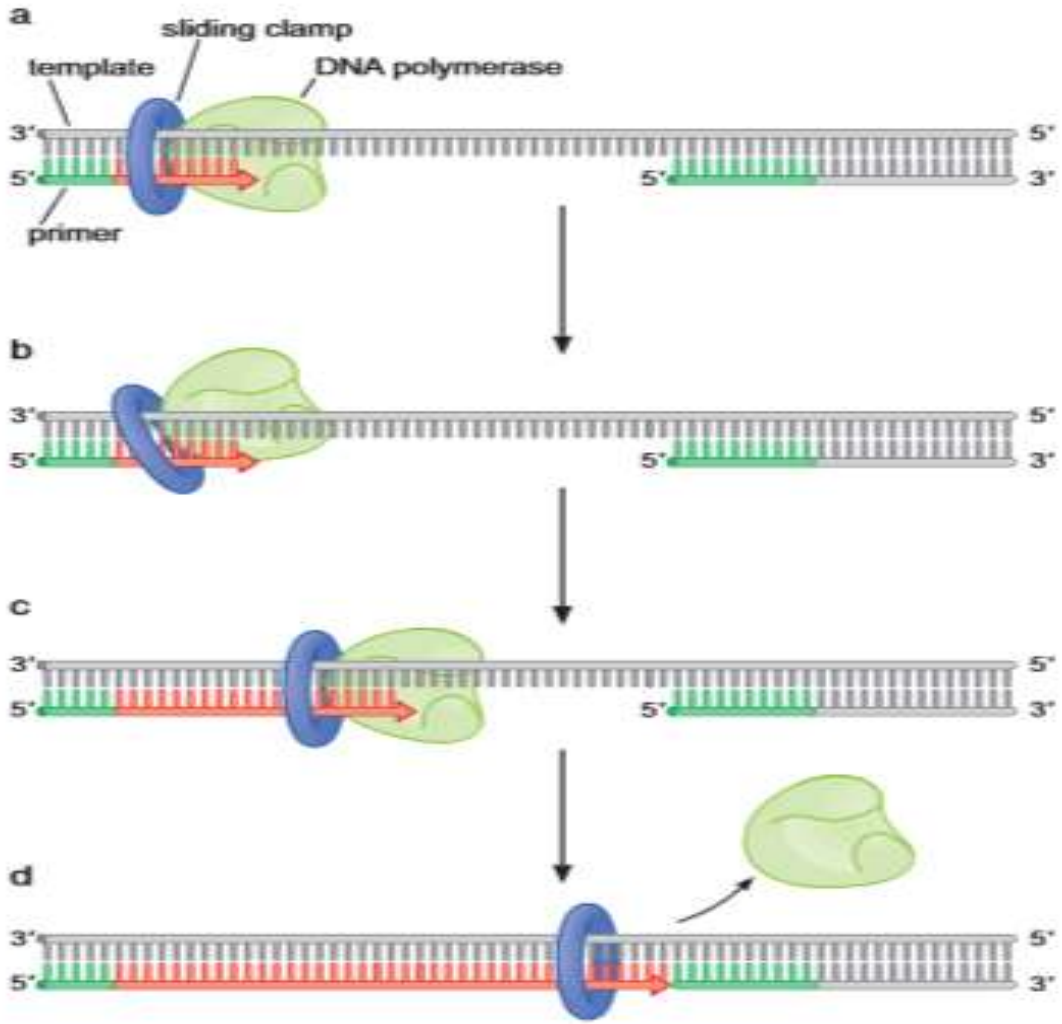
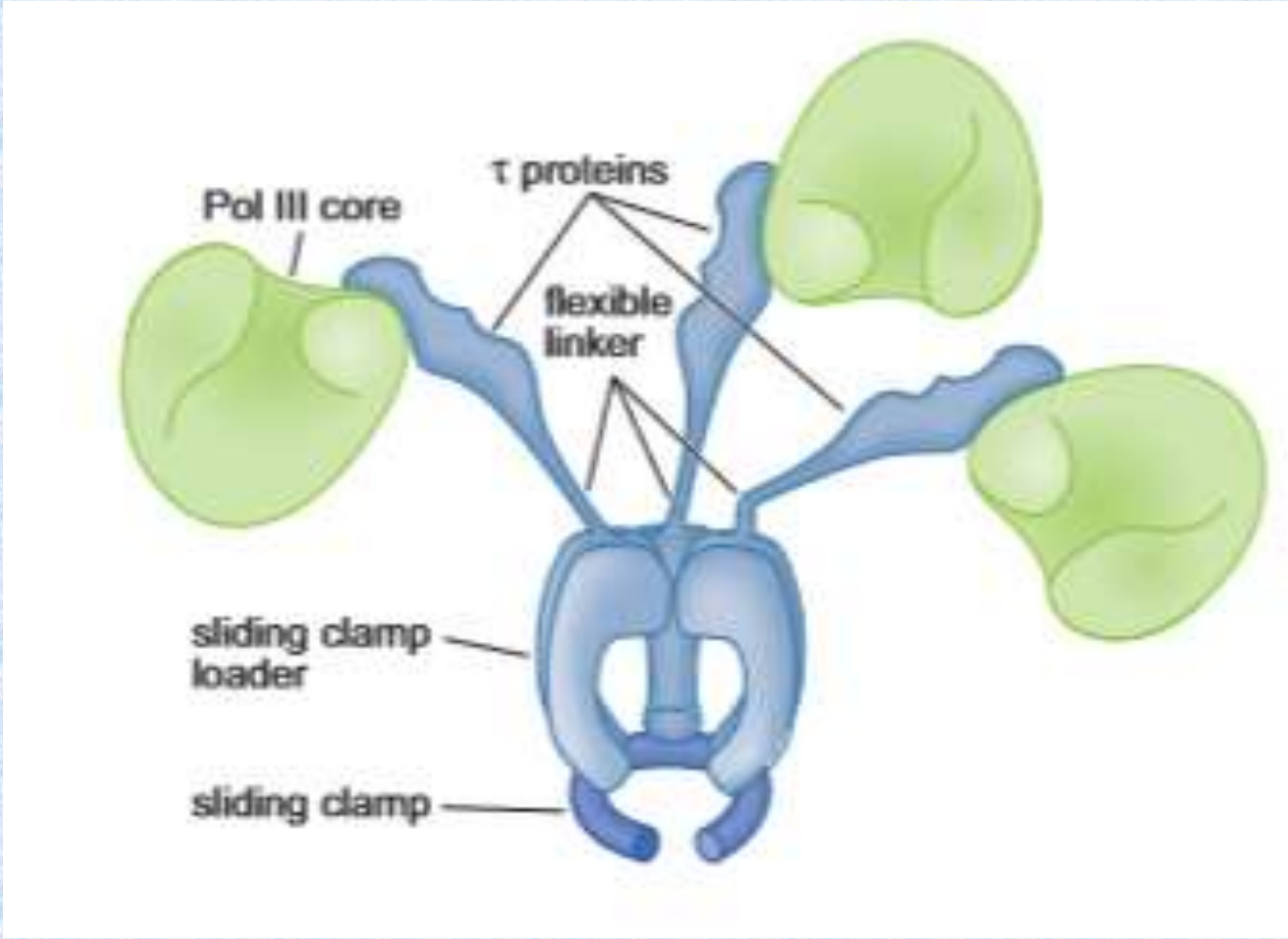


FIGURE 9-20 **Sliding DNA clamps increase the processivity of associated DNA polymerases.** (a) The sliding DNA clamp encircles the DNA and simultaneously binds the DNA polymerase. (b) The relatively low processivity of DNA polymerases leads to frequent release from the primer:template junction, but the association of the polymerase with the sliding clamp prevents diffusion away from the DNA. (c) The association of DNA polymerase with the sliding clamp ensures that the DNA polymerase rebinds the same primer:template junction and resumes DNA synthesis. (d) After DNA polymerase has completed synthesis of the template, the absence of a primer:template junction causes a change in the DNA polymerase that releases it from the sliding clamp.


τ proteins
Pol III core
flexible linker
sliding clamp loader
sliding clamp