Topic Introduction

Ligation and Ligases

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DNA ligases are used chiefly to create novel combinations of nucleic acid molecules and to attach them to vectors before molecular cloning. They are either of bacterial origin or bacteriophage encoded and have different properties, as discussed here.

DNA LIGASES

The discovery of DNA ligases in the mid 1960s was critical to the development of molecular cloning in the mid 1970s. It had been obvious for some time that enzymes capable of sealing breaks in double-stranded DNA should exist so that nicks introduced during replication, recombination, and repair of DNA could be sealed. By 1965, the search for an enzyme that could join DNA molecules had begun in earnest. The breakthrough came at the beginning of 1967, when Marty Gellert at the NIH showed that extracts of *Escherichia coli* could convert hydrogen-bonded circles of bacteriophage λ DNA into a covalently closed, circular form. Within a few months, Gellert's and several others groups had independently purified an enzymatic activity that catalyzed the formation of phosphodiester bonds between apposing 3'-hydroxyl and 5'-phosphoryl termini in a strand of DNA held in a double-stranded configuration by Watson-Crick pairing (for review, see Lehman 1974).

Ligation of DNA is a three-step reaction: (i) formation of a covalent ligase-AMP intermediate, (ii) transfer of the AMP to the 5'-phosphoryl terminus of DNA, and (iii) attack on the AMP-DNA bond by the apposing 3'-hydroxyl group. The nick in the DNA is sealed and AMP is liberated (for review, see Pascal 2008). These three sequential steps are carried out by three different ligase domains that form a catalytic region encircling the DNA substrate with each domain in contact with the DNA duplex.

DNA ligases used in molecular cloning are either of bacterial origin or bacteriophage encoded. All eubacteria, whether thermophilic or mesophilic, contain a single ligase gene that encodes an NAD^+ -dependent enzyme (Olivera and Lehman 1967; Takahashi et al. 1984). During the first step of a ligation reaction, the diphosphate linkage of NAD^+ is used as a phosphoanhydride and the adenyl group is transferred to the ϵ -amino group of a lysine residue in DNA ligase. The adenyl residue is then transferred to the 5'-phosphoryl terminus of the DNA substrate, which becomes vulnerable to nucleophilic attack by the apposing 3'-hydroxyl group. This results in the formation of a phosphodiester bond, elimination of AMP, and covalent joining of the DNA strands (for reviews, see Pascal 2008; Shuman 2009).

The DNA ligases used in molecular cloning differ in their abilities to ligate noncanonical substrates, such as blunt-ended duplexes, DNA-RNA hybrids, or single-stranded DNAs. These and other properties are summarized in Table 1. The enzyme most commonly used to catalyze in vitro ligations in molecular cloning the DNA ligase encoded by bacteriophage T4.

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TABLE 1. DNA ligases

Ligase	Substrates ^a				Cofactors and activators	Temperature	Sulphydryl reagents
	Cohesive termini	Blunt ends	DNA–RNA hybrids	RNA–RNA hybrids			
<i>E. coli</i> ligase	Yes	Yes ^b	No	No	DPN ⁺ Mg ²⁺ (1–3 mM)	10°C–15°C for cohesive termini; 37°C for closing nicks ^c	Not required ^d
T4 ligase	Yes	Yes ^b	Yes ^e	Yes ^e	ATP Mg ²⁺ (10 mM)	4°C for cohesive termini ^f ; 15°C–25°C for blunt ends ^h ; 37°C for closing nicks ⁱ	Dithiothreitol required ^g
Ligases of thermophilic bacteria	Yes	No	No	No	DPN ⁺ Mg ²⁺ (10 mM)	24°C–37°C for cohesive termini; 65°C–72°C for closing nicks ^{k,l,m}	Required ^{k,m}

^aDNA ligases will not join pairs of DNAs whose termini carry the following groups at the point of ligation:

- 5'-hydroxyl and 3'-hydroxyl
- 5'-hydroxyl and 3'-phosphate
- 5'-phosphate and 3'-dideoxynucleoside
- 5'-triphosphate and 3'-hydroxyl

^b*E. coli* DNA ligase was originally reported (Sugino et al. 1977) to be incompetent at joining of blunt-ended DNA molecules except in the presence of condensing agents such as PEG or Ficoll (Zimmerman and Pfeiffer 1983) and with monovalent cations such as Na⁺ (Hayashi et al. 1985a,b). More recently, however, Barringer et al. (1990) have shown that *E. coli* DNA ligase is capable of joining blunt-ended and some single-stranded nucleic acids with kinetics that are dependent on enzyme and substrate concentration. However, for routine ligation of blunt-ended DNAs, bacteriophage T4 ligase is the enzyme of choice. T4 DNA ligase will ligate blunt-ended molecules (Ehrlich et al. 1977; Sgarrella and Ehrlich 1978), but the rate of reaction is not linearly dependent on enzyme concentration and works efficiently only in high concentrations of DNA and enzyme. In addition, condensing agents such as PEG, Ficoll, and hexaminecobalt chloride accelerate the rate of blunt-end ligation by T4 DNA ligases by a factor of 1000 and permit ligation at lower enzyme, ATP, and DNA concentrations (Zimmerman and Pfeiffer 1983; Rusche and Howard-Flanders 1985). Blunt-end ligation is inhibited by high concentrations of Na⁺ (≥50 mM) and phosphate (≥25 mM) (Raaij et al. 1975).

^cDugaiczky et al. (1975).

^dWeiss and Richardson (1967).

^eT4 DNA ligase can join RNA molecules annealed to either complementary DNA or RNA templates, albeit with low efficiency (Kleppe et al. 1970).

^fFerretti and Sgarrella (1981).

^gWeiss et al. (1968a,b).

^hSgarrella and Ehrlich (1978).

ⁱPohl et al. (1982).

^jAlmost all thermophilic ligases, like mesophilic eubacterial ligases, use DPN⁺ as a cofactor. However, one thermostable ligase that requires ATP as a cofactor has been cloned and sequenced (Kletzin 1992). The properties of this enzyme have not been investigated in detail.

^kTakahashi et al. (1984).

^lTakahashi and Uchida (1986).

^mBarany (1991a,b).

BACTERIOPHAGE T4 DNA LIGASE

- T4 DNA ligase, which is encoded by gene 30 of bacteriophage T4 (Wilson and Murray 1979), is a monomeric protein of 487 amino acids (calculated $M_r = 55,230$) (Weiss et al. 1968b; Armstrong et al. 1983).
- T4 DNA ligase, which is purified commercially from overproducing strains of *E. coli* (Tait et al. 1980), has a K_m of 6×10^{-7} M for cohesive termini (Sugino et al. 1977), 5×10^{-5} M for blunt ends, and 1.9×10^{-9} for nicks. The K_m of the enzyme for ATP is $\sim 5 \times 10^{-5}$ M (Weiss et al. 1968b).
- T4 RNA ligase has been reported to stimulate the activity of T4 DNA ligase (Sugino et al. 1977). However, agents such as polyethylene glycol (Pheiffer and Zimmerman 1983) and hexaminecobalt chloride (Rusche and Howard-Flanders 1985), which increase macromolecular crowding and increase the rate of ligation by three orders of magnitude, are less expensive (see Box 1).

PROPERTIES OF DNA LIGASES COMMONLY USED IN CLONING

- As shown in Table 1, T4 DNA ligase can catalyze the ligation of cohesive termini (Hedgpeth et al. 1972; Mertz and Davis 1972), oligodeoxynucleotides, or oligoribonucleotides in RNA–DNA hybrids (Olivera and Lehman 1968; Kleppe et al. 1970; Fareed et al. 1971). In addition, the enzyme can efficiently promote the end-to-end joining of two duplex molecules with fully base-paired termini (Sgaramella et al. 1970; Ehrlich et al. 1977).
- T4 DNA ligase shows a strong (Wiaderkiewicz and Ruiz-Carillo 1987; Landegren et al. 1988; Wu and Wallace 1989), but not absolute, aversion (Goffin et al. 1987) to joining oligonucleotides that are hybridized to a complementary template and contain a mispaired base at either the 3' or 5' junction of the two strands. This ability to discriminate between perfectly and imperfectly paired termini allowed the development of oligonucleotide ligation and amplification systems to detect mutations in genes of medical interest.

BOX 1. CONDENSING AND CROWDING REAGENTS

Condensing reagents, like hexaminecobalt chloride, and crowding reagents, such as polyethylene glycol, have two effects on ligation reactions.

- They accelerate the rate of ligation of blunt-ended DNA by one to three orders of magnitude. This increase allows ligation reactions to be performed at lower concentrations of enzyme and DNA.
- They alter the distribution of ligation products. Intramolecular ligation is suppressed, and the ligation products are created exclusively by intermolecular joining events. Thus, even at concentrations of DNA that favor circularization, all of the DNA products are linear multimers.

Different batches of PEG 8000 stimulate the ligation of blunt-ended DNAs to different extents. It is a good idea to test several batches of PEG 8000 to determine which one yields a maximum stimulation of ligation, and then to dedicate the chosen bottle for use in ligation reactions only. The maximum stimulation of ligation usually occurs between 3% and 5% PEG 8000 in the ligation reaction; however, this value can fluctuate between different batches and should be determined empirically. The PEG stock (13%) solution should be warmed to room temperature before being added to the ligation reaction, as DNA can precipitate at cold temperatures in the presence of PEG 8000. In addition, it is important that the PEG stock solution be added as the final ingredient to the ligation reaction. Stimulation of blunt-ended DNA ligation by PEG 8000 is highly dependent on the concentration of magnesium ions, which should be maintained in the 5–10-mM range in the ligation reaction.

In our hands, PEG gives more reproducible stimulation of blunt-end ligation than hexaminecobalt chloride.

- High concentrations (>100 mM) of monovalent cations such as Na⁺ and K⁺ inhibit the activity of T4 DNA ligase, the extent of the inhibition varying with the terminal sequences of the substrate DNAs (Hayashi et al. 1985a). However, in the presence of crowding agents such as 10% PEG, monovalent cations have a paradoxical effect and stimulate the activity of the enzyme.

E. coli DNA LIGASE

- *E. coli* DNA ligase is encoded by the *lig* gene that lies at 52 min on the *E. coli* genetic map (Gottesman et al. 1973; Bachmann 1990).
- The *lig* gene (Gottesman 1976) and *lop11 lig*⁺ (Cameron et al. 1975), a regulatory mutant over-producing the enzyme, have been cloned into bacteriophage λ vectors, thus facilitating large-scale purification of the enzyme (Panasencko et al. 1977, 1978). The nucleotide sequence of the *lig* gene (Ishino et al. 1986) shows that *E. coli* DNA ligase consists of 671 amino acids with a molecular weight of 73,690.
- For several years, it was believed that *E. coli* DNA ligase would not ligate blunt-ended dsDNA. However, following cloning and expression of the ligase gene, highly active preparations of the enzyme became available that were able to catalyze blunt-end ligation with moderate efficiency (Barringer et al. 1990). Blunt-end ligation is stimulated ~10-fold when the reaction mixture contains 10%–15% PEG and high concentrations of K⁺ (Hayashi et al. 1985b). Nevertheless, *E. coli* DNA ligase is not widely used in molecular cloning procedures, because T4 DNA ligase is capable of efficiently joining blunt-ended DNAs in the absence of crowding agents.
- Unlike T4 DNA ligase, *E. coli* DNA ligase will not efficiently join RNA to DNA and is therefore unable to join adjacent RNA and DNA segments that arise during replacement synthesis of second-strand cDNA (Okayama and Berg 1982). The bacterial enzyme can therefore be used to generate long strands of cDNA that are uninterrupted by segments of RNA.

THERMOSTABLE DNA LIGASES

- The genes encoding thermostable ligases from several thermophilic bacteria have been cloned, sequenced, and expressed to high levels in *E. coli* (e.g., see Takahashi et al. 1984; Barany and Gelfand 1991; Lauer et al. 1991; Jónsson et al. 1994). Several of these enzymes are available from commercial sources.
- Like the *E. coli* enzyme, almost all thermostable ligases use NAD⁺ as a cofactor and work preferentially at nicks in double-stranded DNA. In addition, thermostable ligases, like their mesophilic homolog, can catalyze blunt-end ligation in the presence of crowding agents, even at elevated temperatures (Takahashi and Uchida 1986).
- Because thermostable ligases retain activity after multiple rounds of thermal cycling, they are used extensively in the ligase amplification reaction to detect mutations in mammalian DNAs.

UNITS OF LIGASE ACTIVITY

The standardization of ligase activity in units that are meaningful to both biochemists and molecular cloners has proven to be an elusive goal. For the last 20 yr, at least three different units have been used to measure ligase activity.

- A Weiss unit (Weiss et al. 1968b) is defined as the amount of ligase that catalyzes the exchange of 1 nmol of ³²P from inorganic pyrophosphate to ATP in 20 min at 37°C.

- The Modrich–Lehman unit (Modrich and Lehman 1971), now rarely used, is based on the conversion of radiolabeled d(A-T)_n copolymer with 3′-hydroxyl and 5′-phosphoryl termini to a form resistant to digestion with exonuclease III. One Modrich–Lehman unit is defined as the amount of enzyme required to convert 100 nmol of d(A-T)_n to an exonuclease-III-resistant form in 30 min under standard assay conditions.
- Arbitrary units, defined by commercial suppliers, are based on the ability of ligase to ligate cohesive ends of DNA. These units are often more subjective than quantitative and provide little guidance to an investigator who values precision. In the absence of meaningful information, most investigators setting up ligation reactions must resort to guesswork, which inevitably means that more ligase will be used than is necessary. This, of course, is exactly what the commercial companies seek. As a rough guide, 1 Weiss U is approximately equivalent to 60 cohesive end units (as defined by New England Biolabs). Thus, in 30 min at 16°C, 0.015 Weiss U of T4 DNA ligase should ligate 50% of fragments derived by digestion of 5 µg of bacteriophage λ DNA with HindIII.

T4 RNA LIGASE

T4 RNA ligase, first described by Silber et al. (1972), catalyzes the joining of single-stranded RNA or DNA to either oligoribo- or oligodeoxyribonucleotides (England et al. 1977; Uhlenbeck and Gumpert 1982). The reaction requires a donor molecule with a 5′-terminal phosphate, an acceptor with a 3′-terminal hydroxyl group and ATP, unless the 5′ terminus of the donor is already in an activated intermediate form. An oligonucleotide carrying a 5′-phosphate group and a 3′-hydroxyl group can serve both as an acceptor and as a donor, generating circular and/or multimeric products.

T4 RNA ligase is available from several commercial suppliers. NEB sells several forms of the enzymes that differ in their efficiency to catalyze the ligation of different forms of RNA and oligodeoxynucleotide templates.

T4 RNA ligase has been used for labeling the 3′-termini of RNA (England and Uhlenbeck 1978a), attaching oligoribonucleotides to the 5′ termini of RNAs in preparation for amplification by PCR (Schaefer 1995), inter- and intramolecular ligation of RNAs and DNAs (Romaniuk and Uhlenbeck 1983; Moore and Sharp 1992), and enzymatic synthesis of oligoribonucleotides of defined sequence (England and Uhlenbeck 1978b).

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