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Edited by PAUL D. BOYER

Department of Chemistry and
Molecular Biology Institute
University of California
Los Angeles, California

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List of Contributors

Numbers in parentheses indicate the pages on which the authors' contributions begin.

SIDNEY ALTMAN (469), Department of Biology, Yale University, New Haven, Connecticut 06510

L. ANDREW BALL (281), Biophysics Laboratory and Biochemistry Department, University of Wisconsin, Madison, Wisconsin 53706

EDMUND W. BENZ, JR. (155), Department of Developmental Biology and Cancer, Albert Einstein College of Medicine of Yeshiva University, Bronx, New York 10461

PETER BLACKBURN (317), The Rockefeller University, New York, New York 10021

THOMAS BLUMENTHAL (267), Department of Biology, Indiana University, Bloomington, Indiana 47401

RICHARD R. BURGESS (109), McArdle Laboratory for Cancer Research, Madison, Wisconsin 53706

MICHAEL J. CHAMBERLIN (61, 87), Department of Biochemistry, University of California, Berkeley, California 94720

MURRAY P. DEUTSCHER (183), Department of Biochemistry, University of Connecticut Health Center, Farmington, Connecticut 06032

JOHN J. DUNN (485), Department of Biology, Brookhaven National Laboratory, Upton, New York 11973

MARY EDMONDS (217), Department of Biological Sciences, University of Pittsburgh, Pittsburgh, Pennsylvania 15260

MICHAEL J. ENGLER (3), Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts 02115

RICHARD I. GUMPORT (31), Department of Biochemistry and School of Basic Medical Sciences, University of Illinois, Urbana, Illinois 61801

JERARD HURWITZ (155, 245), Department of Developmental Biology and Cancer, Albert Einstein College of Medicine of Yeshiva University, Bronx, New York 10461

LARRY K. KLINE (557, 567), Department of Biological Sciences, State University College, Brockport, New York 14420

RYSZARD KOLE* (469), Department of Biology, Yale University, New Haven, Connecticut 06510

MARTIN K. LEWIS (109), McArdle Laboratory for Cancer Research, Madison, Wisconsin 53706

U. Z. LITTAUER (517), Department of Neurobiology, The Weizmann Institute of Science, Rehovot, Israel 76100

STANFORD MOORE (317, 435), The Rockefeller University, New York, New York 10021

DANNY REINBERG (155), Department of Developmental Biology and Cancer, Albert Einstein College of Medicine of Yeshiva University, Bronx, New York 10461

CHARLES C. RICHARDSON (3), Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts 02115

T. RYAN (87), Department of Biochemistry, University of California, Berkeley, California 94720

D. SCHLESSINGER (501), Department of Microbiology and Immunology, Washington University School of Medicine, St. Louis, Missouri 63110

V. SHEN (501), Department of Microbiology and Immunology, Washington University School of Medicine, St. Louis, Missouri 63110

STEWART SHUMAN (245), Department of Developmental Biology and Cancer, Albert Einstein College of Medicine of Yeshiva University, Bronx, New York 10461

DIETER SÖLL (557, 567), Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut 06511

H. SOREQ (517), Department of Neurobiology, The Weizmann Institute of Science, Rehovot, Israel 76100

KENJI TAKAHASHI (435), Primate Research Institute, Kyoto University, Inuyama Aichi 484, Japan

OLKE C. UHLENBECK (31), Department of Biochemistry, University of Illinois, Urbana, Illinois 61801

* Present address: Department of Human Genetics, Yale University School of Medicine, New Haven, Connecticut 06510.

Preface

This is the second of two volumes that cover nucleic acid enzymology. The striking advances and crucial importance of this rapidly developing area made review at this time imperative, even though there is still much to be learned about the molecular enzymology involved.

With the exception of the chapter on DNA ligases, this volume centers on enzymes involved in the formation, degradation, and modification of RNA. Present information is extensive, and readers will likely recognize an indebtedness to the excellent authors for their authoritative coverage.

It is a distinct pleasure to record appreciation for the guidance provided by the Advisory Board members of this and the preceding volume. Their exceptional professional competence and breadth of knowledge made essential contributions to the excellence of the volumes.

This volume records a milestone along the path of one of the most vital and revealing areas of biological research of all times.

Paul D. Boyer

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Section I

Ligases

1

DNA Ligases

MICHAEL J. ENGLER • CHARLES C. RICHARDSON

I. Introduction

DNA ligases are enzymes that catalyze the formation of a phosphodiester linkage between DNA chains. Condensation of the 5′-phosphoryl group with the adjacent 3′-hydroxyl group is coupled with the hydrolysis of a pyrophosphate moiety of NAD (bacterial enzyme) or ATP (phage or eukaryotic enzymes).

Prior to the discovery of DNA ligase several experimental observations suggested the existence of an enzyme that could catalyze the covalent

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joining of polynucleotides. Studies on bacteriophage had shown that genetic recombination involved the breaking and rejoining of polynucleotide strands (*1, 2*), and physical and genetic studies on the repair of ultraviolet-irradiated DNA suggested a terminal step requiring strand joining (*3*). A more specific demonstration of a joining activity came from the observation that the linear DNA molecule of phage lambda was converted to a covalently closed duplex circle shortly after injection into its host, *Escherichia coli* (*4, 5*). An added impetus to search for such an enzyme activity was the growing realization that a novel mechanism might exist for the overall 3′–5′ directional growth of a DNA strand (lagging strand) during replication [see Ref. (*6*)]. One postulated mechanism, the synthesis of small chromosomal units on both strands in an antiparallel manner (*7*), required a subsequent joining event to yield DNA of high molecular weight. It was not until later, concurrent with the discovery of DNA ligase, that direct evidence was obtained to support a discontinuous mechanism for DNA replication (*8*).

DNA ligase was first identified in extracts of uninfected and T-phage-infected *E. coli* in 1967 (*9–14*). Initially, the major feature that distinguished the bacterial from phage-induced enzymes was their cofactor specificity; *E. coli* ligase requires NAD, whereas the phage enzyme requires ATP. Another bacterial ligase, isolated from *Bacillus subtilis,* also requires NAD (*15*). DNA ligases have been found in a large variety of eukaryotic cells [see review, Ref. (*16*)]; all have a requirement for ATP.

Of the DNA ligases that have been described, the *E. coli* and phage T4-induced enzymes have been most thoroughly characterized. There-

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fore, this chapter focuses on these two enzymes and refers to studies on ligases from other sources only when they supplement, or differ from, those obtained with the *E. coli* and T4 enzymes. This series (*17*) and other reviews (*18, 19*) have already covered earlier studies on DNA ligases. This chapter places major emphasis on (1) the purification and physical properties of the ligases; (2) the properties and substrate specificities of the reactions catalyzed by the enzyme, including the intermediates in the reactions; (3) the *in vivo* roles of DNA ligases; and (4) the research applications of the enzyme.

II. Isolation and Physical Properties

A. Assays

1. *Alteration of the Properties of Polynucleotide Chains*

DNA ligase activities have been measured by a number of procedures. Ligase activity was initially detected in extracts of *E. coli* by measuring the conversion of hydrogen-bonded circles of phage λ DNA to covalently bonded ones using a sedimentation assay (*9*). A more rapid assay, which also utilizes the cohesive ends of λ DNA, involves the joining of radioactively labeled λ DNA to cross-linked unlabeled λ DNA (*20*). Although the cross-linked DNA will renature after treatment with alkali, the labeled DNA will not, unless it has become covalently attached to the cross-linked DNA. The single-stranded and native DNA reaction products are then quantitated by hydroxylapatite chromatography.

Another assay (*14*) that measures the covalent joining of one duplex polymer to another makes use of a polynucleotide chain covalently linked to cellulose, thus permitting it to be isolated by sedimentation or filtration. By adding the appropriate complementary polymers to the cellulose-linked polymer, a duplex substrate can be prepared with which to measure ligase activity.

A rapid and convenient assay measures the conversion of ^{3}H-labeled d(AT) copolymer to a form resistant to exonuclease III (*21*). In this reac-

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tion DNA ligase catalyzes an intramolecular joining reaction with linear self-complementary d(AT) oligomers, leading to the formation of circular molecules (*22*).

2. *Direct Measurement of Phosphodiester Bond Formation*

A more direct type of assay for ligase activity measures the conversion of internally located ^{32}P-labeled 5′-phosphomonoesters to diesters, which are resistant to *E. coli* alkaline phosphatase. After limited digestion with pancreatic DNase, duplex DNA contains single-strand breaks bearing 5′-phosphoryl groups. All such phosphomonoesters are removed by treatment with phosphatase at elevated temperatures, and the external and internal 5′-hydroxyl groups are then radioactively labeled by phosphorylation using [γ-^{32}P]ATP and polynucleotide kinase (*10, 23*). If these ^{32}P-labeled phosphomonoesters are incorporated into phosphodiester linkages in a ligase reaction, they are converted to a phosphatase-resistant form.

A similar assay (*11, 24*) uses as substrate a double-stranded homopolymer pair consisting of multiple oligo(dT) units labeled with [5′-^{32}P]phosphomonoester hydrogen-bonded to a long poly(dA) chain. A novel variation of this type of assay is the covalent joining of a [5′-^{32}P](dA-dT) oligomer to yield phosphatase-resistant radioactivity (*22*), a reaction dependent on the ability of poly(dA-dT) to form intramolecular circles.

3. *Detection of Biological Activity*

Several biological assays for measuring ligase activity have also been described. Ligase will restore marker activity of transforming DNA that has been inactivated by the introduction of single-strand breaks with pancreatic DNase (*15, 25*). Similarly, ligase activity has been measured by following the restoration of biological activity in a transfection assay (*26*). In this case, phage DNA, previously inactivated by a single restriction enzyme cleavage, is repaired by covalent joining via the short cohesive ends generated by the restriction cut.

4. *Measurement of a Partial Reaction*

More rapid assays, which do not require the preparation of a special DNA substrate, have been used to monitor the purification of DNA ligase.

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These assays measure the first step in the ligase-catalyzed reaction. Both *E. coli* ligase (*27*) and T4-induced ligase (*28*) can be assayed by measurement of the formation of the acid-precipitable ligase-AMP intermediate using NAD or ATP, respectively, radioactively labeled in the AMP moiety.

T4 DNA ligase has been assayed during purification by measuring the exchange reaction between ATP and $^{32}PP_i$ (*29*). This assay measures the conversion of $^{32}PP_i$ into a form that adsorbs to charcoal. In principle such an assay could also be used to measure the exchange reaction between NAD and NMN in the *E. coli* DNA ligase reaction.

5. *Other Assays*

Although all of the assays discussed above have been used to monitor DNA ligase activity during purification, many other possibilities exist. Such assays are limited only by the expertise and ingenuity of the investigators. For example, the joining of restriction fragments and the reformation of covalently closed circular molecules, reactions catalyzed by DNA ligase, can be followed by such diverse techniques as pycnographic analysis, electron microscopy, and gel electrophoretic analysis.

6. *Choice of an Assay*

Which assay should be used to measure DNA ligase activity during purification? For detecting normal amounts of ligase activity in extracts of cells, the most suitable assay procedures are probably those that most directly measure phosphodiester formation by the conversion of a phosphatase-sensitive ^{32}P-labeled 5′-phosphomonoester to a phosphatase-resistant form. Equally satisfactory and sensitive is the conversion of linear poly(dA-dT) copolymer to an exonuclease III resistant form. Since extracts of cells may contain other enzymes that catalyze an ATP–PP_i exchange and NAD–NMN exchange, these assays cannot always be used in the early stages of purification. However, the purification procedure developed for the T4 DNA ligase is sufficiently reproducible in the early steps to permit postponing an assay of the enzyme until Step V (chromatography on DEAE-cellulose) when the exchange assay is reliable (*29*). When overproducing strains of cells are used as a source of enzyme any of the assays should provide a sufficiently reliable method to identify the peaks that contain ligase activity during column chromatography.

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