ENZYME BIOTECHNOLOGY

Protein Engineering, Structure Prediction and Fermentation

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Protein Engineering, Structure Prediction and Fermentation

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ELLIS HORWOOD
NEW YORK LONDON TORONTO SYDNEY TOKYO SINGAPORE

First published in 1990 by
ELLIS HORWOOD LIMITED
Market Cross House, Cooper Street,
Chichester, West Sussex, PO19 1EB, England

A division of
Simon & Schuster International Group

Typeset in Times by Ellis Horwood
Printed and bound in Great Britain
by The Camelot Press, Southampton

British Library Cataloguing in Publication Data

Enzyme biotechnology: protein engineering, structure prediction and fermentation
1. Industrial chemicals: Enzymes
I. Crabbe, M. James C. *1951–* II. Series
661'8
ISBN 0–13–262393–4

Library of Congress Cataloging-in-Publication Data

Enzyme biotechnology: protein engineering, structure prediction and fermentation/editor, M. James C. Crabbe.
p. cm. — (Ellis Horwood book in the biological sciences. Series in biochemistry and biotechnology)
ISBN 0–13–262393–4
1. Enzymes–Biotechnology. 2. Protein engineering.
I. Crabbe, M. James C. II. Series.
TP248.65.E59E59 1988
660'.63–dc19 89–1653
CIP

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* *In preparation*

Table of contents

Coloured illustrations appear together in the colour section between pages 64 and 65.

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Preface

The technical achievements of gene synthesis, together with controlled or random mutagenesis, now allow us to 'engineer' proteins to our specific requirements. Slowly we are working towards *predictive* models for enzyme structure and catalysis. This in turn should permit *theoretical* and *practical* advances, both of which are important for the development of enzymes for biotechnology. This book is an attempt to bring together some of the recent advances in our understanding of enzyme structure and function, through protein engineering, that will help in that development.

Oxford, 1989

M. James C. Crabbe

1

Strategies for enzyme engineering

M. James C. Crabbe

INTRODUCTION

Perhaps the main achievement of the techniques developed for isolating, characterizing, sequencing and manipulating genes has been the development of 'protein engineering'. This has allowed the cloning and expression in microorganisms of genes coding for rare or valuable mammalian proteins, which have then been produced on an industrial scale (Scragg 1988). Insulin, interferon and growth hormones became available for study, testing and therapeutic use. The next step, of engineering proteins so that they could be distributed, stored, administered and used under conditions far from those encountered *in vivo*, demands an intimate knowledge of the relationships between a protein's structure and its function. Such mutations may be of use in a whole variety of applications, including the design of new therapeutic compounds (Hol 1987), the production of enzyme biocatalysts suitable for use in industrial non-aqueous media (Deetz & Rozzell 1988), the modification of substrate specificity of deoxyribonucleic acid (DNA) restriction endonucleases (Cowan *et al.* 1989), studies on the enzyme catalysis in solid crystals (Hajdu *et al.* 1988), and the design of molecular switches and 'biochips' (Robinson & Seeman 1987). In the future, we may see the production of bizarre multifunctional enzymes with a number of catalytic domains grafted onto sections of stable proteins (Ringe 1989). When such applications are used in a large production scale, then the term 'protein engineering' does appear to be appropriate. Engineered enzymes are of potential use in the fine chemical, pharmaceutical, toxic waste and food industries. Large-scale commercial enterprises use proteases for conversion of proteins (casein and soya) into peptides and amino acids for food, animal

food, cheese production and pharmaceuticals, invertase for conversion of sucrose into invert sugar for confectionery, glucose oxidase for conversion of glucose into gluconic acid for food and drinks, and lactase to convert lactose into glucose and galactose for milk treatment. Fungal and bacterial enzymes (peroxidase, polyphenol oxidase, cyanide hydratase, lipase and hydrolase) are used for toxic waste conversions.

The technology for introducing defined mutations at specific sites is available, and a number of enzymes have been used as models to indicate how specific mutations effect enzyme stability, specificity, substrate binding and catalysis. The methodology is essentially as follows.

(1) To define the required change in function.
(2) To clone the gene or cloned DNA (cDNA) for the protein so that it can subsequently be mutated and expressed.
(3) To obtain a knowledge of the protein's structure.
(4) To propose specific residues which should be altered to achieve the desired functional change, and to model the consequences of such mutations.
(5) To effect promising mutations based on the modelling studies.
(6) To express and process the mutant proteins.
(7) To determine the functionality of the new protein.

Any novel information gained from steps (5)–(7) can then be fed in at step (3) and iterate the process, which is shown diagrammatically in Fig. 1.1.

This chapter outlines some of the strategies used to prepare enzymes for engineering and gives a number of examples where this has been successful. Other chapters discuss some of the techniques in more detail. Modern methods for protein structure prediction and the assessment of structure in solution are covered in Chapter 2. In Chapter 3 we move into the industrial sector, with discussions on methods for fermentation biotechnology.

GENETIC MANIPULATION

Techniques

Protein engineering involves the use of genetic manipulations to alter the coding sequence of a cloned gene. The mutations may be *insertions*, *deletions* or *base substitutions*. Such changes may be **random** or **site specific**. Insertion and deletion mutations are useful, often to define regions of interest in the protein. Random mutagenesis has the advantage that large numbers of mutations may be produced. This can be very useful, particularly in the early stages of analysis, provided that a rapid and specific screening method is available. A number of methods are available for the production of a series of random mutations localized to a specific region of DNA. Predomi-

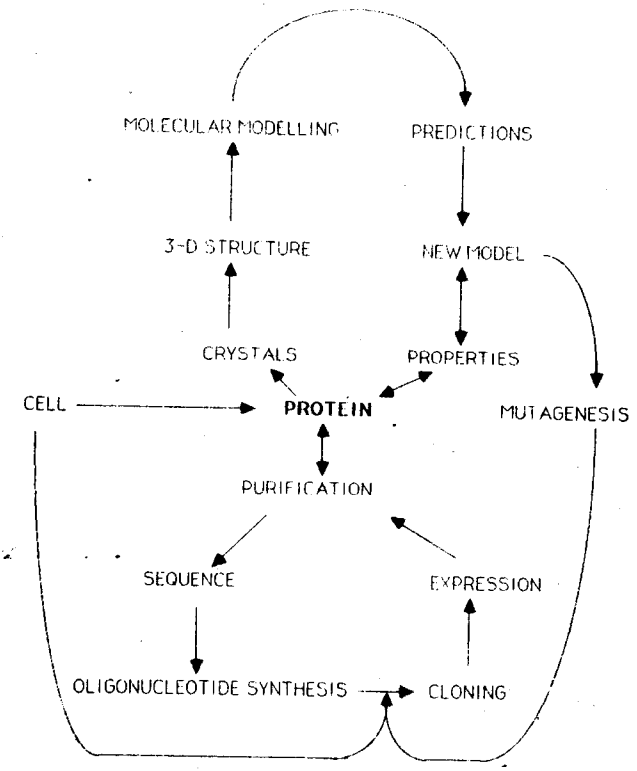

Fig. 1.1 — Diagrammatic iterative scheme for obtaining protein structure and function information.

nantly single base substitutions are produced, so that amino acid substitutions are not likely, and deletions and insertions are not possible. Specific or site-directed mutagenesis has the advantage that specific known mutations are produced as a single site in the DNA, so that single amino acid changes may be made in the protein sequence. Direct automated chemical synthesis of DNA fragments — oligodeoxyribonucleotides — is now possible. Here we shall now discuss some of the methods that have been of use in enzyme mutagenesis. For comprehensive reviews on methods and methodology see Morrow (1979), Shortle *et al.* (1981), Maniatis (1980), Smith & Gillam (1981), Shortle & Botstein (1985), Lathe *et al.* (1983), Itakura *et al.* (1984), Craik (1985), Smith (1982, 1985), Williams & Patient (1988) and Murrell & Roberts (1989).

(1) Insertion or deletion mutations

A number of methods have been developed for the excision or insertion of defined DNA sequences in a gene. All involve enzymatic methods to cleave the DNA, to remove or insert a DNA sequence, and to ligate the ends (Fig. 1.2). Cleavage may be achieved using restriction endonucleases that produce blunt ends (e.g. *Pvu*II) or cohesive ends (e.g. *Eco*RI for a 5′ overhang, *Kpn*I for a 3′ overhang). These ends (5′-phosphate and 3′hydroxyl) may then be ligated with T4 DNA ligase. Unwanted ligation may be prevented by using calf intestinal phosphatase, which removes the 5′-phosphate to generate a hydroxyl group. This enzyme, together with T4 polynucleotide kinase, may be used to end-label DNA fragments with ^{32}P. A series of overlapping deletion or insertion mutants may be constructed. This is useful in analysing transcriptional control and identifying discrete functional elements in proteins (Sakonju *et al.* 1980, Bogenhagen *et al.* 1980) (Fig. 1.3). Appendix A lists a number of restriction nucleases together with their base recognition sequences.

(2) Base substitution methods

There are two main types of base substitution as shown in Fig. 1.4: those leading to substitutions over a targeted segment of DNA, and those causing specific determined base pair changes.

(a) Classical fragment mutagenesis

Here the entire genome or a fragment is exposed to a mutagen. Agents such as hydroxylamine (Hong & Ames 1971, Chu *et al.* 1979), methoxyamine (Borrias *et al.* 1976), nitrosoguanidine (Volker & Showe 1980), nitrogen mustards (Salganik *et al.* 1980) and nitrous acid (Solnik 1981) may be used.

(b) Site mutagenesis

(i) *Incorporation of nucleotide analogues.* This technique was first applied using *N*4-hydroxycytidine 5′-triphosphate to replace either uridine triphosphate or cytidine 5′-triphosphate (Flavell *et al.* 1974, Weissman *et al.* 1979). Modified bases may be incorporated to produce a mixture of products. For example, A:T→G:C transitions may be produced by incorporation of *N*4-hydroxy-dCTP into a primer-extension reaction (see Shortle *et al.* 1978).

(ii) *Chemical methods.* Sodium bisulphite catalyses the deamination of cytosine to uracil in single-stranded DNA (Hayatsu 1976). As double-stranded DNA is relatively unreactive, treatment of gapped DNA results in a number of C:G→T:A transition mutants in the single-stranded region (Shortle & Nathans 1978, Shortle & Botstein 1983). Formaldehyde resembles bisulphite ion in that conditions can be chosen that allow preferential attack on single-stranded DNA. The predomi-

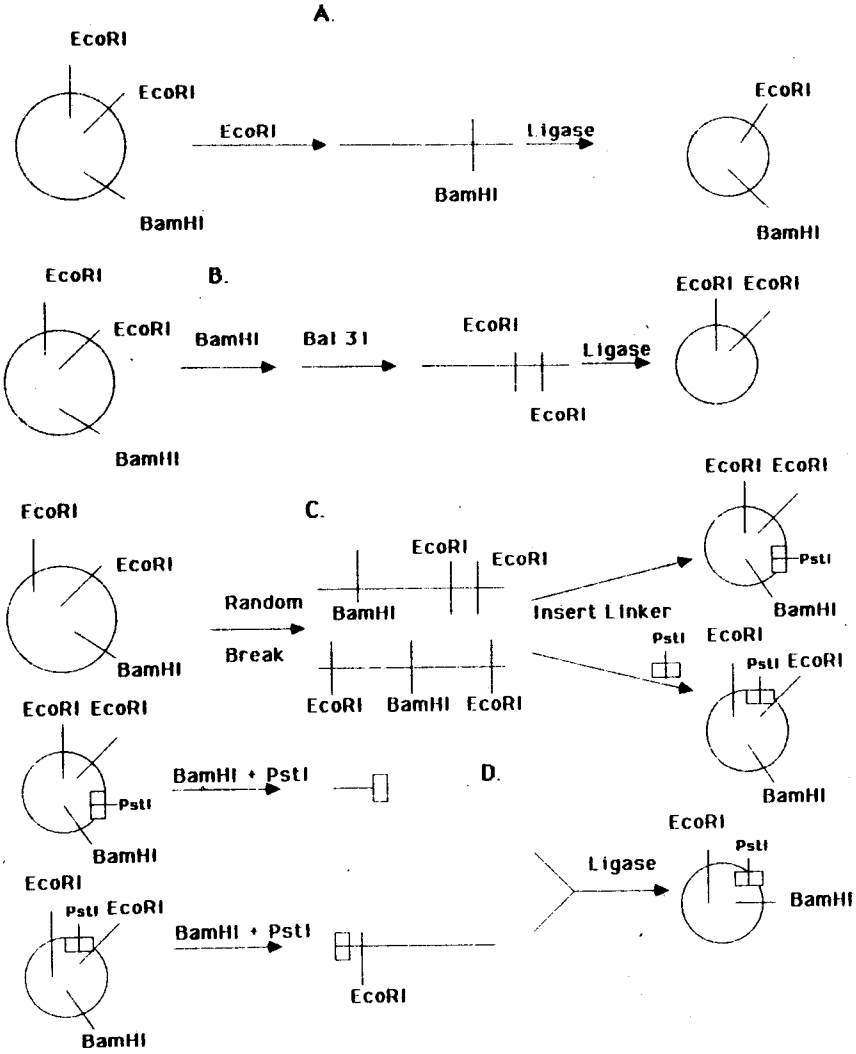

Fig. 1.2 — Schemes for construction of deletion and insertion mutations. (Adapted from Shortle *et al.* (1981)).

nant mutagenic reaction appears to be the formation of a Schiff's base at N6 of adenine, resulting in a derivative exhibiting ambiguous base-pairing properties.

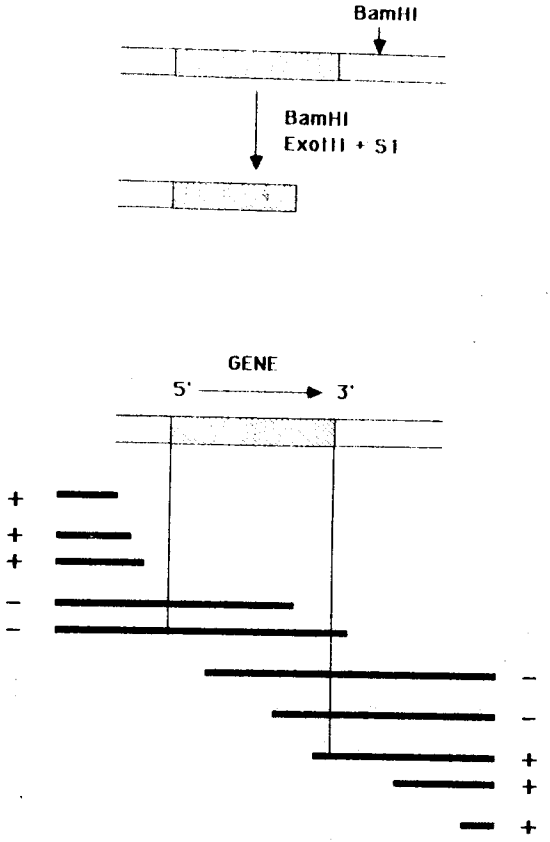

Fig. 1.3 — Construction of overlapping deletion mutations. (Adapted from Shortle *et al.* (1981).

(3) Oligonucleotide-directed mutagenesis

An oligonucleotide that is partly complementary to the nucleotide sequence in the target DNA is synthesized. At an appropriate temperature, after the newly synthesized strand has been closed using T4 ligase, it can hybridize with the target sequence to form a heteroduplex, even though one or more bases are mismatched. The synthetic oligonucleotide can then be used as a primer for the Klenow fragment of DNA polymerase I to produce, for example, a plasmid with a specific mispaired region. On introduction of such a plasmid into a foreign host (e.g. a bacterium), replication will produce two