

Understanding Enzyme Mechanism through Protein Chimeragenesis

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Abstract The preparation of chimeras, proteins that contain segments from two or more different parent proteins, is a valuable tool in protein engineering yielding structures with novel properties. In addition to the obvious practical value of hybrid proteins as catalysts and biopharmaceuticals, their careful analysis can be used to understand the role of specific domains in enzymatic catalysis and protein evolution in a unique way that complements other structure-function studies. The study of hybrid enzymes can reveal, for example, the role specific subunits and/or domains play in dictating substrate specificity, catalytic activity, processivity, and stability. Popular chimeragenesis methods, including noncombinatorial and combinatorial methods, that can be used to generate hybrid proteins, are discussed here and four case studies are presented that beautifully demonstrate how hybrids can be studied to gain detailed understanding about substrate selectivity, enzymatic activity,

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enzyme chemistry, and protein evolution. The examples highlight the power of chimeragenesis as a tool for gaining insights into enzyme mechanism as well as the need to combine this technology with other methods such as random mutagenesis and DNA shuffling, especially because the replacement of a domain can yield unpredicted perturbations to structural and functional parameters.

1 Introduction

Understanding how enzymes form stable structures, catalyze chemical reactions at greatly enhanced rates, show remarkable substrate specificity, and evolve is a long-standing quest in biochemical research. Proteins influence practically all biological processes and only by investigating protein structure and function can we begin to fully appreciate how they are integrated into living organisms. Elucidation of structure–function relationships in enzymatic catalysis was traditionally accomplished by observing the effect of reaction conditions, substrate structure, and/or natural or unnatural amino acid replacements on the rate, specificity, or three-dimensional structure of an enzyme. Recently, such investigations of enzymes have been enhanced by advances in structural, computational, biophysical, and protein engineering methods (Eisenmesser et al. 2002; Garcia-Viloca et al. 2004; Mittermaier and Kay 2006). This review focuses on the use of hybrid approaches, which are providing increasingly important avenues for obtaining novel insights into structure–function relationships in enzymatic catalysis (Armstrong 1990). Discussed herein is the preparation and use of hybrid enzymes with altered function or mechanism to gain detailed insights into enzyme structure, specificity, catalytic efficiency, and molecular evolution. The importance of combining novel hybrid generation technologies with other methods, such as rational and random mutagenesis, novel screening and selection approaches, X-ray crystallography, gene alignments, and pre-steady-state and steady-state kinetics, is highlighted.

1.1 Terminology

Throughout this review, the terms “hybrid” and “chimera” are used interchangeably. Hybrid proteins contain segments (domains or subdomains) from at least two different natural or man-made parent proteins (Armstrong 1990). Domains and subdomains are loosely defined terms that refer to structural motifs of various sizes and complexity, including small units of approximately ten to 30 amino acids, folded functional units, and large domains of several hundred amino acids that may have enzymatic activity (Ostermeier and Benkovic 2001). There are several types of hybrids. Single crossover hybrids consist of the N-terminal section of one protein and the C-terminal section of another (Fig. 1). In multiple crossover hybrids, one or more internal stretches of amino acid sequence have been replaced by the corre-

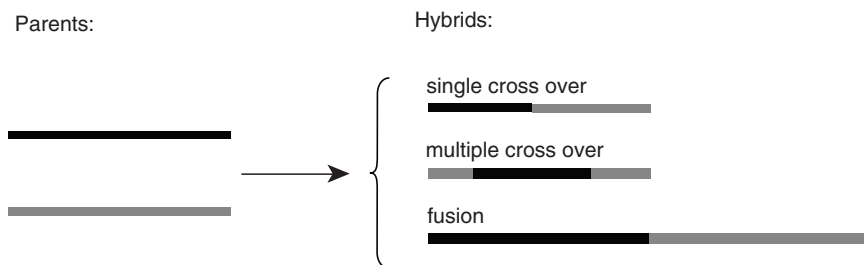


Fig. 1 Common types of hybrids created from two parent enzymes

sponding segment(s) from another enzyme(s). In fusion hybrids, a functional domain of one protein is linked with a domain(s) from another protein(s), creating a fused product that is larger than any of the parents alone. Figure 1 illustrates some of the types of hybrids; for the sake of simplicity, only hybrids derived from two parents are shown. Hybrids can of course contain amino acid mutations, deletions, and/or insertions.

1.2 What Can Hybrid Proteins Contribute to Understanding Enzyme Catalysis?

Section 2 of this review briefly outlines the common experimental methods for hybrid generation. In Sect. 3, recent examples from the literature are provided to demonstrate how chimeragenesis, combined with thorough biochemical studies, yields novel insights into enzyme mechanism. The case studies in Sect. 3 are not meant to be comprehensive, but rather are presented to illustrate the types of hybrid approaches used and the kind of information that can be obtained about protein function. We discuss how careful study of hybrid enzymes can lead to the identification of structural/functional domains and help determine which domains between two structurally similar proteins are interchangeable (Mas et al. 1986; Gurvitz et al. 2001). Hybrid studies can answer the question of whether a structural module can contribute a defined functional characteristic to the hybrid enzyme. The study of hybrid enzymes can reveal the role subunits/domains play in dictating substrate specificity, catalytic activity, processivity, and stability in a protein (Brock and Waterman 2000; Du et al. 2001; Stevenson et al. 2001; Lee et al. 2003).

Rational design of hybrids requires the preliminary identification of functionally important modules (Hopfner et al. 1998; Schneider et al. 1998) by inspection of the three-dimensional structure of either parent enzyme. In the absence of structural information, amino acid sequence alignments can be used to identify potentially important segments. Moreover, DNA sequences can be inspected to locate exon-intron

interfaces, which may define the boundaries of structural or functional units. Recent advances in hybrid methods have made it possible to generate chimeric libraries in a random fashion, removing the need for structure or sequence alignments (Ostermeier et al. 1999b; Stevenson and Benkovic 2002). Thus, random methods make it possible to examine the contribution of protein segments to function without preconceived bias. These methods also have the advantage that the fusion points between structural or functional domains or subunits can be located precisely. Finally, inspection of hybrids can provide valuable understanding about the elusive but important residue–residue contacts and long-distance residue networks which are crucial for protein activity (Agarwal et al. 2002; Rajagopalan et al. 2002; Benkovic and Hammes-Schiffer 2003). In Sect. 2, we outline some of the popular chimera-genesis methods used to prepare hybrid proteins for the purpose of further understanding structure–function relationships.

2 Chimera-genesis Methods

Several excellent reviews in the literature comprehensively cover current chimera-genesis methods (Nixon et al. 1998; Lutz and Benkovic 2000, 2002; Stevenson and Benkovic 2002; Horswill et al. 2004). This section presents a short overview, focusing on techniques that have been used to generate chimeric proteins for structure–function relationship studies. These techniques can be divided into noncombinatorial and combinatorial approaches. Noncombinatorial methods are considered “rational” because it is necessary to choose both the domain targeted for swapping and the crossover points that define the domain. Combinatorial or random methods, on the other hand, produce large libraries of chimeric genes with fragments of random size inserted into or deleted from random positions of the target scaffold. A disadvantage of rational methods is that choosing the module for swapping and the precise end points of the fragment is difficult, and success depends on the level of understanding of the structure, folding, and catalytic mechanism of the enzyme under study. Much work is required to create a single or a few hybrid proteins, while combinatorial methods can yield more than 10^8 different hybrids in one experiment. However, rational approaches often lead to more definite information regarding the role of a specific domain in protein function, while combinatorial methods often do not yield instantly recognizable answers and rely heavily on development and execution of genetic selection protocols.

2.1 Noncombinatorial Domain Swapping Methods

The actual construction of rationally designed domain-swapped genes can be accomplished by several techniques. In the first method, *cassette mutagenesis*, restriction sites flanking the DNA particular sequence to be replaced are digested with the cognate restriction enzymes and a replacement sequence is inserted between these sites (Wells et al. 1985).

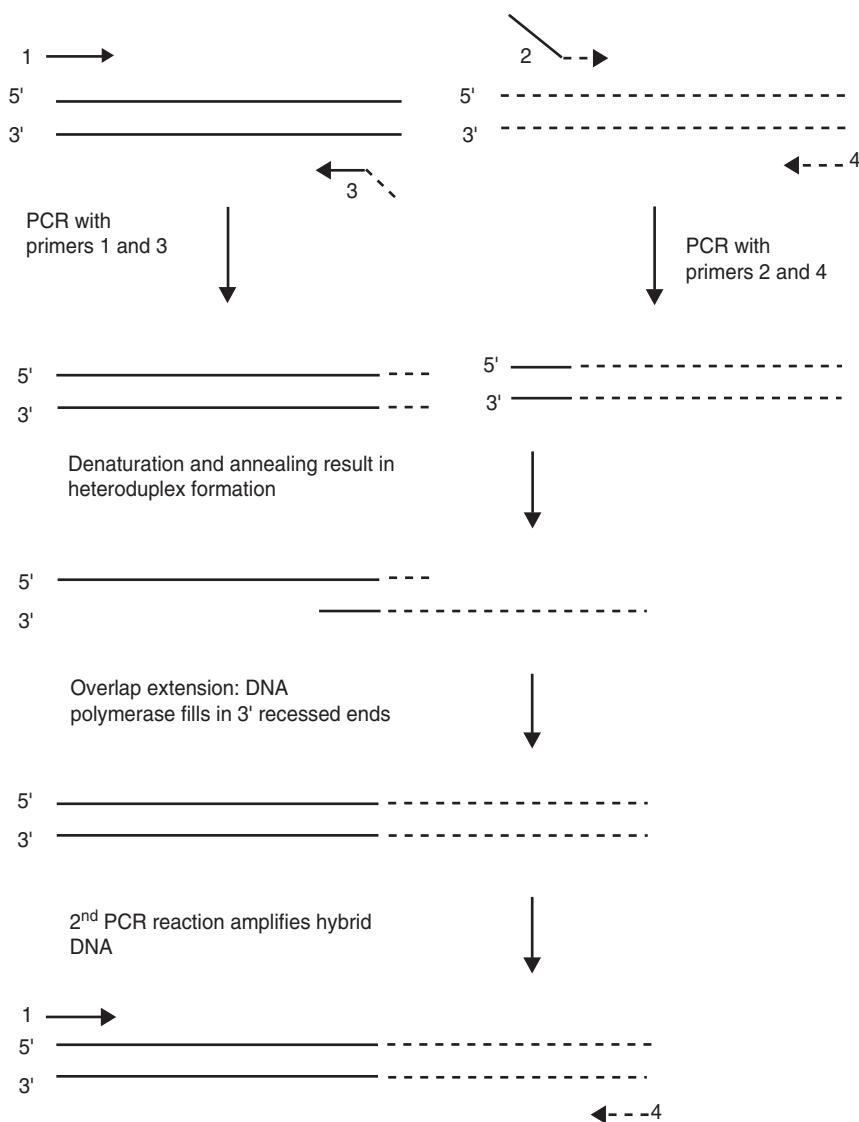


Fig. 2 Gene splicing by overlap extension polymerase chain reaction (PCR). *Arrows* represent primers (1–4), the *solid line* and *dotted lines* represent DNA sequences from two different parent genes

The restriction sites can be either naturally occurring or artificially introduced to the desired location in the target gene by oligonucleotide-directed mutagenesis. The choice of restriction sites is based on their uniqueness to the plasmid and conservation of the final amino acid coding sequence. In the second method, *gene splicing by overlap extension*, DNA molecules are recombined without the use of restriction endonucleases (Fig. 2) (Horton et al. 1989). Fragments from the genes



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