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A Discussion of Site-directed Mutagenesis Versus Chemical Modification
蛋白质分子的定点突变和化学修饰

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Abstract: Recent researches together with development and application advances of the molecular protein engineering are reviewed. The site-directed mutagenesis and chemical modification are discussed. The advantages of site-specific mutagenesis over chemical modification are its high specificity of site mutation and almost free approach of engineering proteins by fusing new and foreign functional groups or deleting some original groups.

Key words: protein engineering, site-directed mutagenesis, chemical modification

摘要: 回顾蛋白质工程研究及应用技术、手段和方法,分析定点突变及化学修饰在改造蛋白质分子结构与功能方面的异同。定点突变技术可以随心所欲地在已知 DNA 序列中取代、插入或缺失一定长度的核苷酸片段。该方法与使用化学因素导致突变的方法相比,具有突变率高、简单易行、重复性好的特点。

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Proteins are the main functional bio-molecules which are so useful that they can be employed as therapeutic agents, catalysts, and materials. A large number of diseases stem from mutations in proteins, which make these proteins malfunction. In some cases, catalysis may be damaged and so metabolic pathways may be changed, e.g., phenylketonuria. In other cases, structural properties may be impaired, leading to a loss of physiological function, e.g., muscular dystrophy. Some transmissible encephalopathies result from proteins which have been changed in shape and forming of polymers. Many cancers result from mutation proteins, and about 50% of human cancers are caused by mutations in the tumor suppressor p53 that has lower stability. Enzymes and signal receptors are the major targets of drugs used for either to restore function or to destroy infectious factors of cancers. Meanwhile, in the aspects of fine-chemical synthesis for the resolution of racemic mixtures into one enantiomer, biosensors, pharmaceuticals for enzyme replacement therapy, and commercial catalysts in food processing and detergent applications, the great potential application of biocatalysts have necessitated the development of biocatalysts in natural catalytic characteristics. The ultimate goal of protein science is to elucidate the relationship of structure and activity of proteins and to design and synthesize novel catalysts, materials and drugs that will be able to eliminate diseases, minimize ill health, and improve our life in diverse aspects. To achieve the goal, chemical modification and site-directed mutagenesis are the key approaches. Combined with mass spectrometry, X-ray crystallography and NMR spectroscopy, these approaches would speed up structural characterization at a rapid pace.

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1 Chemical modification

Amino acid side chains of protein react with a variety of chemical reagents to form covalent bonds. The principal

chemically reactive groups in proteins are nucleophiles. These are generally founded in the same groups at the active sites of enzymes, and are responsible for catalysis. The nucleophiles are the potent toward "hard" electrophilic centres, such as the carbonyl, phosphoryl, and sulfuryl groups, including the —OH of serine, threonine, and tyrosine; the ε-NH_2 group of lysine and α-amino groups of the N-termini; the imidazole ring of histidine; the —S^- of cysteine; and the —CO_2^- of aspartate, glutamate, and the C-termini. The nucleophiles are also reactive with varying degrees toward "soft" electrophiles such as saturated carbon. Additionally, the S atom in the side chain of methionine is nucleophilic toward "soft" electrophiles, as is the aromatic ring of tyrosine. Two important classes of reagents that are used to modify the nucleophilic side chains are the acylating and similarly reacting agents, and the alkylating agents. However, the reagents tend to be nonspecific and to react with any accessible amino acid residue that has the appropriate chemical nature, with variable consequences. Covalent modification of an enzyme may lead to an irreversible loss of activity in such situations as a catalytically essential residue is blocked, substrate binding is sterically impeded, the protein is distorted or its mobility is impaired. Alternatively, modification may not affect activity if an unimportant residue is modified, or the inhibition may be only transitory if the chemical reaction is reversible.

1.1 Protein chemical modification reactions

Chemical modification of proteins was once mainly used in the amino acid sequence analysis and the identification of residues at the catalytic and binding sites of protein. Glazer et al.[1] presented the reactions and quantitative methods for each amino acid as well as the end-group reactions for the determination of NH_2-terminal and COOH-terminal residues with specific chemical reagents. Spectroscopic probes may be covalently linked to certain regions of proteins, e.g., fluorescent derivatives such as the dansyl group may be linked to amino groups, spin labels such as nitroxide derivatives may be attached to cysteine residues and tyrosine residues may be modified by nitration. These probes are used to examine local structure or overall conformation.

However, the profound importance of protein chemical modification in affecting protein biological functions has attracted lots of interest of chemists and re-merged as a powerful complementary approach to site-directed mutagenesis and directed evolution for tailoring proteins and enzymes.

Prior to the site-directed mutagenesis, chemical modification was used to convert the active site serine residue in subtilisin to a cysteine residue, producing thiolsubtilisin[2,3]. The effect of this change was to eliminate the enzyme's amide hydrolysis activity and preserve esterase capability. Interestingly, this mutated catalyst also showed a useful peptide ligase activity since it lacks the ability to degrade the resulting amide product[4]. An exciting extension of this work produces a selenosubtilisin variant that contains a selenocysteine at the same position[5]. The hydrolytic and acetyltransferase properties of this modified enzyme paralleled the reactivity profile observed for thiolsubtilisin. The inclusion of the redox active selenium atom, however, produces an enzyme with a peroxide-reducing activity similar to that of glutathione peroxidase[6]. An important development in the "chemical mutagenesis" of enzymes is the preparation of flavopapains by Kaiser and his colleagues[7,8]. These novel mutants were prepared by the alkylation of a specific cysteine residue presented in papain (Cys25) by reactive flavin analogs. As flavins can catalyze a variety of transformation, a number of different reactions can be studied using flavopapains. Capitalizing on the concept developed with flavins, several other types of catalytic groups have been attached to proteins in order to either alter the chemical reactions performed by specific enzymes or introduce reactive functionality into proteins devoid of catalytic activity.

1.2 Strategies for the covalent modification of proteins with cofactors and metal-chelating groups

(a) Flavin cofactor, (b) Thiazolinium cofactor, (c) Bipyridine metal-binding ligand, (d) Phenanthroline metal-binding ligand, (e, f) EDTA-based metal-binding ligands and (g) Pyridoxamine cofactor. Protein attachment to form proceeds (a ~ d and f) by cysteine alklation, whereas conjugation to produce forms (e, g) via disulfide-bond formation.

It is also desirable to control specificity by chemical modification. Schultz and co-workers modified *Staphylococcal* nuclease, an enzyme that cleaves DNA with little sequence specificity, a deoxyoligonucleotide of define sequence 15 bases in length near the enzyme active site.

The resulting conjugate cleaved a complementary single-stranded DNA segment 64 nucleotides in length almost exclusively at a single site[9].

Chemical modifications are also actively employed in following ways: the cross-linking of proteins by bi- and poly-functional reagents such as dimethylsuberimidate, in order to increase stability, particularly for nonaqueous application; the insertion of radioactive tags into molecules; the searching of unusually reactive groups by high reaction rates; the measuring of the exposure degree of groups to solvent from their activity with reagents; the assessment of groups by the effect of modification on reaction rates in catalysis; the irreversibly inhibiting activity. Protein engineering via DNA manipulation has largely replaced chemical modification in assessing the role of side chains in catalysis and binding.

A unique advantage of chemical modification, which can not be achieved by site-directed mutagenesis alone, is to incorporate a protein with a non-natural amino acid. The resulting novel protein may possess some new or enhanced properties[10, 11].

2 Site-directed mutagenesis

Until 1980s, the chemical modification is the only way to test mechanisms by altering the groups in catalysis. This technique is restricted to chemically reactive side chains, and is often nonspecific, and also frequently makes significant changes such as producing steric bulk and grossly changing the properties of the site chain. Site-directed mutagenesis can be used to make rational design and construction of novel proteins. For instance, it can be used to do the dissection of structure and the examination of the changes of properties of novel proteins, and produce novel proteins for use in medicine and industry. A good example is the 'humanization' of monoclonal antibodies. Monoclonal antibodies are produced from rodents, which can not be used therapeutically because the human antibody system may recognize a rodent antibody as foreign and neutralize it. The antigen-binding loops from a mouse antibody may be grafted onto a human framework with hardly loss of binding activity.

There are a variety of methods specifically to mutate the codon for a specific amino acid in a protein. Most are based on the procedure of oligdeoxynucleotide directed mutagenesis. Probing into the structure-function relationship of proteins and nucleic acids by site-directed mutagenesis has become an important strategy in functional studies and genetic engineering[12]. The gene is cloned into a double-stranded vector, and one of the constituent circles of single-stranded DNA is isolated. A short oligodeoxynucleotide has already been synthesized to be complementary to the region of the gene to be mutated, except for a single-base (or double-base) mismatch. The mismatch is designed to change the codon of the target amino acid residue into the codon of the desired mutant residue. The oligodeoxynucleotide is annealed to the gene in the single-stranded vector and becomes a primer for Pol Ⅰ (the Klenow fragment) in replicating the rest of the genome. The replicated strand is then ligated, and the result is a heteroduplex containing one strand of mutant and one strand of unmutant DNA. The hetroduplex is used to transform a host and produce colonies of cells that contain either the vector with the mutant or the one with unmutant gene. The probe, radioactively labeled original oligodeoxynucleotide primer, is used to screen the colonies in a DNA hybridization assays. The probe anneals preferentially to the mutant DNA, to which it is absolutely complementary, rather than to the unmutant DNA, in which it has a mismatch. One of the best methods for mutagenesis is to use PCR (Polymerase chain reaction), a circular plasmid, and two mutant primers that are complementary in sequence at the site of mutation. In the first cycle of mutation, one primer introduces the mutation into one strand; the other primer causes the complementary mutation in the other strand. In the following rounds of amplification, the mutants are subsequently amplified because all the primers are complimentary to the mutants. After certain amount of cycles of PCR, there should be all mutant progeny.

Recently Andreas Seyfang and Huaqian Jin[13] have employed a novel method using two terminal tailed primers with a unique 25-nucleotide tail each that are simultaneously annealed to template DNA together with the set of mutagenic primers in between. Following synthesis of the mutant strand by primer extension and ligation with T_4 DNA polymerase and ligase, the unique mutant strand-specific tails of the terminal primers are used as anchors to specifically amplify the mutant strand by high-fidelity polymerase

chain reaction.

It is possible to change amino acid residue at any given sites in protein sequence to another by above-described techniques. It is not difficult to synthesize the genes for proteins *de novo* using cassettes of double-strand oligodeoxynucleotides with sticky ends. Alternatively, longer, single-stranded oligodeoxynucleotides can be linked together by shorter strands that are complementary to the end of one long strand and the beginning of the next. The gaps can then be filled in by a polymerase, followed by ligation. Mutants of subtilisin are made in such a way, whereby a double-stranded oligodeoxynucleotide is synthesized and inserted into the subtilisin gene using restriction enzymes and ligation.

The protein mutants created by site-specific mutagenesis are the basis of the rational and systematic analysis of proteins and their redesign by protein engineering. The prerequisite for these studies is that the enzyme has been cloned and expressed. Further, unless relatively crude information is required, it is essential that the structure has been solved at high resolution. Accurate structure-activity studies require even more stringent criteria: absolute values of rate constants.

In 1982, the catalytic mechanism of tyrosyl-tRNA synthetase was completely unknown. Many years of investigation of the 20 different aminoacy-tRNA synthetases by classical protein chemistry and kinetics had failed to reveal acid or basic groups in catalysis. The crystal structure of the tyrosyl-tRNA synthetase had given no clues to its reaction mechanism or the groups in catalysis. But just as the solution of the first X-ray structure of an enzyme, lysozyme, elucidated a previously unknown mechanism, so the first application of protein engineering, using site-directed mutagenesis, to an enzyme revealed its mechanism.

There are simple rules to follow in order to produce mutants that have a chance of being analyzed simply. The aim is to minimize reorganization of the structure of the enzyme, either locally or globally. Structure reorganization or distortion is accompanied by unknown energy changes , and that complicating changes are arising from the direct interactions of the target side chain. In principle, a mutation should be chosen to try to achieve the following goals.

(1) Deleting part of a side chain or leading to an isosteric change.

(2) Avoiding creating buried unpaired changes.

(3) Deleting the minimal number of interaction.

(4) Avoiding addition of new functional group to side chains.

The ideal mutation is a deletion in which just an interaction is removed without causing a disruption or reorganization of structure. The following mutations are highly suitable for a simple analysis:

Ile→Val, Ala→Gly, Thr→Ser. The loss of a $-CH_2$-group is a good probe of hydrophobic interactions. There is no change of stereochemistry or increase in branching, and only a cavity is formed.

Ile→Ala, Val→Ala, Leu→Ala. A large loss of energy and a greater likelihood of surrounding side chains moving into the cavity or the ingress of solvent. There is on smaller deletion.

Ser→Ala, Tyr→Phe, Cys→Ala, they are good for probing hydrogen bonds.

His→Asn, His→Gln, an NH of the $-CONH_2$ of Asn can sometimes substitute for the NδH of the imidazole ring, or an NH of the $-CONH_2$ of Gln can substitute for the NεH as hydrogen bond donors. Complicated results probably will come out when mutation of Asp→Asn, Glu→Gln and vice visa are made because the $-CO_2$-group is just a hydrogen bond acceptor, while the $-CONH_2$ is both a donor and an acceptor.

Based on the understanding of catalysis mechanism of an enzyme, site-directed mutagenesis is further employed for redesigning a new one, which could be different from the original in terms of specificity, stability, and other properties. The specificities of subtilisin, hydrolyzing peptides and esters by the same acylenzyme mechanism as for chymotrypsin, are dependent on the Gly-166 in some extent. Mutations increasing the size of the side chain of residue 166 will decrease the activity toward tyrosine of the substrate. Substitution of Met-222, adjacent to the activity site Ser-221 by other amino acids leads to less active but more oxidation-resistant subtilisin[14].

Unlike chemical modification which has to be carried out, in many cases, in vitro, the proteins achieved by site-directed mutagenesis always exist in the living cells and can be easily located at a specific site in the cells by fusing the mutated gene with a certain signal peptide. This is extraordinarily important thing for the biological studies. The big advantages of site-specific mutagenesis over chemical modi-

fication are its highly specificity of site mutation and almost free approach of engineering proteins by fusing new and foreign functional groups or deleting some original groups.

3 Combination of site-directed mutagengsis and chemical modification

Even though chemical modification is a more rapid and unexpensive method to stabilize enzymes by crossing linking or by introduction of monomeric or polymeric moieties, chemical modification can introduce functional groups and specificity-determining groups that are inaccessible by mutagenesis techniques, and by mutagenesis techniques, the design of new catalytic activities or binding sites by systematic changes of single amino acids is still impossible[14]. It has been widely accepted that site-directed mutagenesis has transcended all traditional approaches for protein mutation. Combined with chemical modification technique, site-directed mutagenesis has emerged as a rapid, controlled, and versatile strategy that can yield well-characterized homogeneous protein products. Distefano et al.[15] and Desantis et al.[16] reviewed the new development of combination of site-directed mutagenesis and chemical modification in protein engineering.

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