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Short Communication

Quest for a turnover mechanism in peptide-based enzyme mimics

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ABSTRACT

Creation of synthetic structures with an enzyme-like mechanism and turnover remains a significant challenge. In this study, peptides containing a cysteine thiol and histidine imidazole group were designed to mimic the active site of the cysteine protease papain. Ellman's reagent trapping experiments showed that rapid acetyl group exchange exists between the thiol and imidazole groups. This exchange rate increased significantly in peptides with bulky R-groups (phenylalanine) between the cysteine and histidine. A reduction of the cysteine thiol pKa and NMR results further supports closer proximity of the thiol and imidazole groups in peptides with faster acetyl group exchange.

1. Introduction

Of all the macromolecules in living organisms, enzymes represent those that are the most complicated in terms of mechanistic properties. Enzymes are able to catalyze the transformation of all other biomolecules, providing the dynamics and very essence of life. In this era of nanotechnology, enzymes can aptly be considered bio-nanomachines that do chemistry. These bio-nanomachines catalyze reactions with high specificity and enormous rate accelerations; some producing millions of turnovers per second [1].

Over the last several decades, considerable efforts have been made to create synthetic versions of enzymes, which are sometimes called synzymes, chemzymes, or nanozymes [2–4]. Although, some progress has been made with enzyme mimics, most attempts have failed, and the few successes exhibit only marginally catalytic properties. A few examples include supramolecular structures (e.g. cyclodextrins), polypeptides, metal complexes, and nanoparticles and nanostructures [5–12], which have become of more interest in the past decade.

1.1. Catalytic mechanism of protease

In the cysteine protease papain and serine protease chymotrypsin, the area within the enzyme that is responsible for catalysis is known as the active site. Within the active site of these proteases is an arrangement of three key amino acids known as the catalytic triad. In papain, the triad consists of Cys-25, His-159, and Asp-175, while in chymotrypsin the triad consists of Ser-195, His-57, and Asp-102 [13–15]. The enzyme's three-dimensional structure produces the precise location and arrangement of these three groups within the active site. Binding of the substrate within the active site triggers subtle mechanical movements that produce catalysis. The ubiquitous occurrences of these triad arrangements in many related proteases and esterases emphasize the importance of this structural motif and the key amino acid R-group interactions. The basic reaction mechanism for papain involves a nucleophilic attack by the cysteine thiol anion ($-S^-$) on the amide or ester bond of the substrate forming a covalent acyl-thiol intermediate. The histidine imidazole group provides general acid–base assisted catalysis for proton abstraction and donation during the reaction. The aspartate carboxyl group plays a more secondary role in the overall catalytic reaction. A similar type of reaction mechanism exists for chymotrypsin, where a serine hydroxyl anion ($-O^-$) now carries out the nucleophilic attack on the amide or ester bond of the substrate. While there are still some aspects of papain and chymotrypsin catalysis that are not completely understood, acceptance of the basic triad mechanism has remained relatively constant [16–20].

1.2. Studies on enzyme mimics using amino acid structures

In early work on synzymes, Heller et al. designed and synthesized cysteine and histidine peptides that were intended to mimic the catalytic site of papain [21]. While no significant turnover was observed, there

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was evidence that a reversible intramolecular transfer of the acetyl group was occurring between the cysteine thiol and histidine imidazole groups, where the equilibria favor the acetyl-thiol intermediate (Fig. 1). Such results suggest that if the back-attack by the cysteine thiol could be prevented, subsequent hydrolysis of the acyl-imidazole intermediate would produce a leaving group mechanism that leads to turnover. This is because the acetyl-imidazole intermediate is more than two orders of magnitude more labile to hydrolysis than the acetyl-thiol intermediate [22,23]. While the formation of a covalent acyl-imidazole intermediate is not believed to be part of the actual papain or chymotrypsin reaction mechanisms, enzymes do overcome similar back-attack reactions and do exhibit distinct mechanical movements. Indeed, evidence exists for major dynamic movement of the histidine imidazole group in chymotrypsin catalysis [24]. More recently, Kisailus et al. designed and synthesized nanostructures as mimics for the catalytic site of the hydrolase enzyme silicatein, which has a serine and histidine at its active site. In this work, aliphatic hydroxyl and imidazole groups were placed in parallel arrangements on a gold surface. The interface between chemically distinct monolayers provided juxtaposition of nucleophilic (hydroxyl) and hydrogen-bonding (imidazole) groups. Such arrangements should have the potential to catalyze the hydrolysis of a gallium oxide precursor, and template the condensed product formation of gallium oxohydroxide (GaOOH) and gamma-gallium oxide (–Ga_2O3) [8]. While this study demonstrates a form of template-based heterogeneous catalysis, which is different from homogeneous ester and amide hydrolysis, it again shows the importance of the imidazole group in performing hydrolysis reactions. The study also demonstrates a new role that nanotechnology may ultimately play in the development of viable synthetic catalysts. Several other enzyme-like nanostructures derived from short-sequence peptides have now also been studied that show further promise for peptide-based catalysts [11,12,25,26].

The goal of our study was to further investigate the acetylation and deacylation mechanism in new peptide constructs where the cysteine thiol and histidine imidazole groups are in close proximity. The peptides also contained aspartate, which provided a carboxyl group in the vicinity of the histidine imidazole. A better understanding of the dynamics of these catalytic groups in the acyl exchange mechanism is of key importance to the further development of viable synzymes which can produce turnover.

2. Experimental

2.1. Peptides studied

Listed in Table 1 are the nine peptides used in this study (95% purity verified by HPLC from Genscript). The peptides were combinations of cysteine (sulfhydryl/thiol), serine (hydroxyl), histidine (imidazole) and aspartate (carboxyl), along with seven other amino acids which included alanine, phenylalanine, glycine, arginine, lysine, asparagine, and proline. Peptide 1 consisted of a cysteine and serine but excludes the histidine, which leads to the loss of the acetyl-transfer effect. Peptide 2 contains a cysteine, histidine and aspartate, and produces some acetyl group transfer. Peptides 3 and 4 are variations of Peptides 1 and 2, respectively, with an arginine at the C-terminus introducing a positively charged group. Peptides 5 and 6 are further variations of Peptide 4, with phenylalanines adjacent to cysteine to provide a bending of the peptide backbone structure to produce a closer proximity of cysteine thiol and histidine imidazole groups (Fig. 2). Peptide 7 has the cysteine and histidine in reverse order and examines the effects of arginine on aspartate. Peptide 8 acts as a control for Peptide 7 by replacing the histidine with asparagine, which lacks reactivity for acetyl group exchange. Peptide 9 is composed of fourteen amino acids, and incorporates two extra histidines and a proline that produces further bending of the backbone (Fig. 2). All nine peptides have their N-terminus acetylated in order to avoid nonspecific nucleophilic reaction by the α-amino group. Each of the peptides was reacted with the substrate p-nitrophenyl acetate (pNPA) or acetylated by acetic anhydride and the corresponding acylation/deacylation rate constants were calculated.

2.2. Acetylation

Peptides 1 through 9 were initially diluted to 1 mM in DI water. The substrate p-nitrophenyl acetate (pNPA) was diluted in ethanol, also to 1 mM. Each peptide was further diluted in 0.1 M Tris–Borate (TB) buffer pH 8.5, and then the pNPA was added to start the reaction, which was carried out at 20 °C for 20 min. The reaction concentration for both peptide and substrate was 100 μM in a final volume of 600 μL. The peptide reaction rates were determined along with: Controls 1 and 2—each substrate for acetylation and deacylation alone; Control 3–100 μM n-acetyl l-cysteine (AC); Control 4–100 μM n-acetyl l-histidine (AH); and

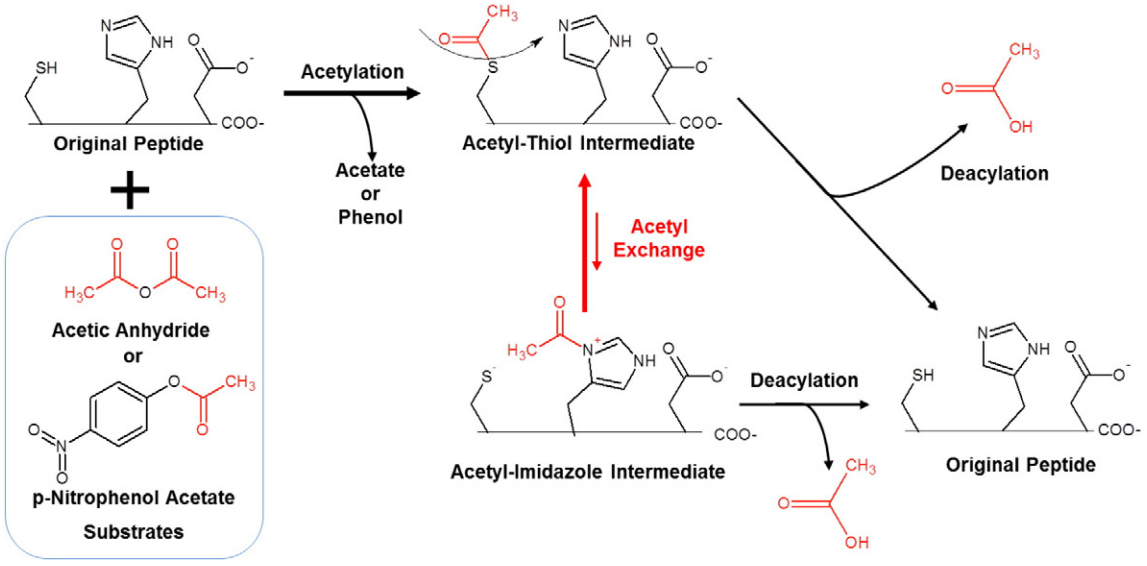

Fig. 1. Acetyl group exchange and back-attack in peptide enzyme mimetics. After acetylation of the peptide by the substrate, rapid acetyl group exchange can occur between the thiol and imidazole groups. Back-attack by the thiol group significantly reduces the deacylation rate and prevents turnover.