An Investigation into the Minimum Requirements for Peptide Hydrolysis by Mutation of the Catalytic Triad of Trypsin

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Abstract: The catalytic triad of rat anionic trypsin has been systematically altered by site-directed mutagenesis to determine the activity of alternate combinations of peptide bonds. Genetically modified rat trypsins H57A, H57D, H57E, H57K, H57R, H57A/D102N, H57D/D102N, H57L/D102N, H57K/D102N, H57A/S195A, H57K/S195A, and H57A/D102N/S195A have been generated. Rigorous steps were taken to ensure that the resultant catalysis was due to the mutant enzymes and not contaminants. Each of the variants exhibited measurable activity toward the hydrolysis of peptide bonds. The rate constants for the mutant trypsins were subsequently assayed for their ability to hydrolyze the unactivated amide linkages of protein substrates. Trypsins D102N, H57K, and H57K/D102N exhibited the highest level of activity. The rate constant for the D102N enzyme was $4 \text{ h}^{-1}$ (0.003% of wild-type). At pH 10.5, the rate constant for the mutant trypsins was $0.011$ to $1.3 \text{ min}^{-1}$ (0.0004-0.04% of wild-type). The H57A/D102N double mutant was not as active as the wild-type enzyme.

The development of peptidases with designed specificities would facilitate the manipulation of peptides and proteins. The challenge in designing such catalysts is the inclusion of interdependent binding and catalytic motifs within a common structural framework to achieve the energetically demanding hydrolysis of peptide bonds. Initial studies have involved the derivatization of small molecules with reactive moieties to partially or fully mimic the chemistry of the serine protease catalytic triad. These catalysts have helped elucidate some aspects of the interactions between members of the triad but have not yet been shown to catalyze the cleavage of amide linkages. Recently, this approach has been extended with a de novo designed four helix bundle polypeptide bearing catalytic serine, histidine, and aspartic acid residues.


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These have been shown to hydrolyze both esters and activated amide bonds with efficiencies which approach natural serine protease function for some substrates. In the future, this catalysis may be further enhanced by genetically introducing strategically placed catalytic residues. The selective and efficient hydrolysis of unactivated peptide bonds by designed catalysts has not been reported.

This report seeks to determine that the development of agents which hydrolyze peptides by a serine protease-like mechanism is a realistic goal and to discover how this catalysis might be maximized. To accomplish this, the catalytic triad of the serine protease trypsin (His 57, Asp 102, Ser 195) has been mutated to discover a minimal basis for peptidase function. This strategy is similar in its goals to the minimalistic approach to protein engineering in that it seeks to simplify the initial basis for catalyst design.

Trypsin was chosen as a target for this analysis because it has already been optimized for highly specific peptidase activity. It contains an oxyanion hole and a highly selective substrate binding site (Figure 1), so that the stage is set for catalysis, even when the catalytic triad is absent. Alternate combinations of triad residues can then be auditioned within a framework that maximizes their potential for catalysis. Thus, the trypsin scaffold is a good starting point for defining elements which may be utilized to endow catalytic antibodies or de novo engineered proteins with peptidase activity.

Analysis of point mutations within the catalytic triad of trypsin and subtilisin has provided information on the interplay of the three amino acids in catalysis by serine proteases. The replacement of aspartic acid 102 with asparagine in trypsin yields a mutant which retains 0.1% of the wild-type esterase activity at pH 8.0 and up to 10% of the activity when assayed at pH 10.5. Similarly, studies of the serine protease subtilisin have shown that mutant enzymes in which one or more of the catalytic residues have been replaced with alanine retain activity toward activated amide substrates. Moreover, a detailed understanding of the active site histidine in serine protease amidolysis has lead to the development of a mutant of subtilisin capable of substrate assisted catalysis and to a trypsin variant whose activity could be controlled by the presence of transition metals.

We report here the mutation of trypsin to produce the H57A, H57D, H57E, H57K, H57R, H57A/D102N, H57D/D102N, H57K/D102N, H57L/D102N, H57D/D102N, H57A/D102N, H57S, H57T, and H57V mutant enzymes in which one or more of the catalytic residues have been replaced with alanine retain activity toward activated amide substrates. Moreover, a detailed understanding of the active site histidine in serine protease amidolysis has lead to the development of a mutant of subtilisin capable of substrate assisted catalysis and to a trypsin variant whose activity could be controlled by the presence of transition metals.

Table I. Kinetic Parameters of Wild-Type and Mutant Trypsins at pH 8.0 and 10.1

<table>
<thead>
<tr>
<th>Variant</th>
<th>pH 8.0 kcat (min⁻¹)</th>
<th>KM (µM)</th>
<th>kcat/KM</th>
<th>pH 10.1 kcat (min⁻¹)</th>
<th>KM (µM)</th>
<th>kcat/KM</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type</td>
<td>3200</td>
<td>15</td>
<td>210</td>
<td>2700</td>
<td>19</td>
<td>140</td>
</tr>
<tr>
<td>H57A</td>
<td>0.054</td>
<td>17</td>
<td>0.0032</td>
<td>0.11</td>
<td>20</td>
<td>0.0055</td>
</tr>
<tr>
<td>H57L</td>
<td>0.075</td>
<td>20</td>
<td>0.0037</td>
<td>0.16</td>
<td>21</td>
<td>0.0076</td>
</tr>
<tr>
<td>H57D</td>
<td>0.78</td>
<td>13</td>
<td>0.06</td>
<td>0.71</td>
<td>17</td>
<td>0.0042</td>
</tr>
<tr>
<td>H57E</td>
<td>0.69</td>
<td>21</td>
<td>0.033</td>
<td>0.63</td>
<td>25</td>
<td>0.0025</td>
</tr>
<tr>
<td>M57K</td>
<td>0.83</td>
<td>41</td>
<td>0.020</td>
<td>5.2</td>
<td>48</td>
<td>0.108</td>
</tr>
<tr>
<td>H57R</td>
<td>0.017</td>
<td>67</td>
<td>0.00025</td>
<td>0.65</td>
<td>160</td>
<td>0.0041</td>
</tr>
<tr>
<td>D102N</td>
<td>1.3</td>
<td>4.2</td>
<td>0.30</td>
<td>140</td>
<td>13</td>
<td>11</td>
</tr>
<tr>
<td>H57A, D102N</td>
<td>0.17</td>
<td>87</td>
<td>0.0019</td>
<td>7.5</td>
<td>130</td>
<td>0.058</td>
</tr>
<tr>
<td>H57D, D102N</td>
<td>0.18</td>
<td>62</td>
<td>0.0029</td>
<td>0.48</td>
<td>130</td>
<td>0.0037</td>
</tr>
<tr>
<td>H57D, H57E</td>
<td>0.41</td>
<td>18</td>
<td>0.023</td>
<td>6.2</td>
<td>130</td>
<td>0.048</td>
</tr>
<tr>
<td>H57L, D102N</td>
<td>0.13</td>
<td>41</td>
<td>0.0031</td>
<td>4.9</td>
<td>230</td>
<td>0.021</td>
</tr>
<tr>
<td>S195A</td>
<td>0.079</td>
<td>41</td>
<td>0.0019</td>
<td>0.057</td>
<td>45</td>
<td>0.0013</td>
</tr>
<tr>
<td>S195T</td>
<td>0.011</td>
<td>21</td>
<td>0.00052</td>
<td>0.012</td>
<td>15</td>
<td>0.0008</td>
</tr>
<tr>
<td>H57A, D102N, S195A</td>
<td>0.038</td>
<td>89</td>
<td>0.00043</td>
<td>0.041</td>
<td>170</td>
<td>0.0024</td>
</tr>
</tbody>
</table>

The error in these determinations was 10-20%. The buffers were 100 mM NaCl/20 mM CaCl₂ and either 50 mM Tris-Cl, pH 8.0, or 50 mM glycine, pH 10.1. Similar results were observed at pH 8.0 with 50 mM MOPS. The experiments were allowed to proceed until more than one turnover had occurred.


(11) However, it is important to note that neither trypsin nor any other model system can be ideal, since they differ structurally from the antibody or other protein frameworks which will be the focus of future design efforts. Each framework will have its own intrinsic potential for catalysis.


and H57A/D102N/S195A variants. Initial kinetic analysis of these proteins has been performed using the sensitive fluorogenic substrate Z-GPR-AMC. The variants were then screened against insulin β-chain and galanin peptide to define their activity toward unactivated peptide bonds.

Results and Discussion

Rat anionic trypsin has been modified via site-directed mutagenesis of its catalytic triad, His 57, Asp 102, and Ser 195. The goals of this strategy are (1) to determine the capacity of amino acid substitutions to restore catalytic activity to active site variants and (2) to determine if any of the mutant trypsins thus generated retain the ability to hydrolyze unactivated peptide bonds.

Substitutions for Histidine 57. His 57 has been replaced with either acidic (Asp, Glu), basic (Lys, Arg), or neutral (Ala, Leu) amino acids to assess the effect of these substitutions on catalysis. Kinetic analysis was performed by following the hydrolysis of the fluorogenic substrate Z-Gly-Pro-Arg-7-amino-4-methylcoumarin (Z-GPR-AMC). At pH 8.0, the H57D, H57E, H57K, and H57R mutants retained a limited amount of activity, ranging from 4000- to 190000-fold below wild-type (Table I; Figure 2a). At best, this catalysis was only ~10-fold greater than that exhibited by the nonpolar H57L or H57A variants, indicating that the polarity of the substitution did not have a substantial effect. At pH 10.1, $k_{cat}$ for the H57E and H57D variants was unchanged, reflecting the constant ionization of the acidic side chains over the pH range, while $k_{cat}$ for the H57K and H57R trypsins increased 6.3- and 38-fold, respectively (Figure 2b). These increases may reflect the assumption of some general-base function by these residues, or they may be due to a solvent-mediated hydroxide-dependent mechanism.

Substitutions for Histidine 57 and Asparagine 102. Asp 102 effects catalysis by influencing the polarity of His 57, so that the removal of the histidine negates the catalytic rationale for the presence of the aspartic acid. Therefore, we believed that the mutation D102N should not drastically lower $k_{cat}$ further for mutants already lacking His 57. Moreover, the negative electrostatic potential of Asp 102 may act to destabilize the developing negative charge in the transition state for hydrolysis by the trypsin variants lacking His 57. Alternatively, the removal of Asp 102 may increase the rate of hydroxide-catalyzed deacylation by...

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Figure 3. C18 reversed-phase HPLC analysis of the cleavage of insulin β-chain by D102N trypsin. The cleavage reaction was incubated in 20 mM CaCl₂/100 mM NaCl/50 mM Tris-Cl, pH 8.0, buffer containing 4 μM enzyme and 240 μM peptide substrate for 16 h at 37 °C. Peak 1 was identified by mass spectral analysis as FVDQHLCGSHLVEALYLCOS0GGERFFYPKA. Peak 2 was GFFYPKA. Peak 3 was the parent peptide. The gradient was 20-80% buffer B over 10 min (buffer A = 0.1% trifluoroacetic acid/99.9% doubly distilled H₂O; buffer B = 95% CH₃CN/5% H₂O/0.08% TFA).

removing negative potential, thereby making the active site more accessible to hydroxide. In either case, substitution for Asp 102 may actually increase substrate hydrolysis.

A series of variants was constructed containing D102N and either alanine, aspartic acid, lysine, leucine, or histidine at position 57. The values for the H57A/D102N and H57L/D102N variants at pH 8.0 were slightly higher and at pH 10.1 were much higher than those for the analogous H57A and H57L enzymes (Figure 2b; Table I). This increase in kcat may support an inhibitory effect of Asp 102 on catalysis by the latter variants. However, the kcat/Km values do not reflect this increase, leaving the effect of Asp 102 on catalysis by these trypsin variants uncertain.

At pH 8.0 the D102N mutant was approximately 10-fold more active than the other variants (Table I) (3000-fold lower than wild-type) (Figure 2a), perhaps reflecting a residual role for the retained histidine. At pH 10.1 the variants, with the exception of H57D/D102N, exhibited substantial increases in activity (Figure 2a). The observation that the H57A/D102N and H57L/D102N variants exhibited rate enhancements at pH 10.1 that are similar to that of the H57K/D102N enzyme suggested that the Lys 57 did not play a pivotal role in catalysis and that the increase in kcat was primarily due to a hydroxide-mediated reaction. The D102N variant possessed a much greater kcat than the other mutants (140 m⁻¹, 20-fold higher), suggesting that His 57 continued to contribute to catalysis in the absence of Asp 102.

Substitutions for Serine 195. Serine 195 is the residue most directly involved in catalysis. We assessed the effects on catalysis of its replacement with either threonine or alanine. Both mutants resulted in large reductions in kcat (Table I). Previously the replacement S195C had been shown to reduce kcat to 0.0036 m⁻¹, possibly due to obstruction of the oxyanion hole. Similarly, the mutation of serine to alanine in subtilisin reduced catalysis by a factor of 10⁻³. The H57A, D102N, and S195A mutant, the simplest variant included in these studies, was also constructed. It also exhibited a greatly decreased kcat (85,000-fold) relative to wild-type.

Hydrolysis of Peptide Substrates. The hydrolysis of unactivated amide bonds was assayed using oxidized insulin β-chain. This peptide was chosen as a substrate because it contains an internal arginine which is flanked by residues which can fill the S2, S3, S1', S2', and S3' subsites. This should maximize the binding energy available for catalysis. The progress of the digestions was monitored by C18 reversed-phase HPLC, and the resultant products were identified by mass spectral analysis. No hydrolysis was observed in the absence of added protease. Kinetic analysis

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Table II. Effect of Leaving Group on Catalysis by Wild-Type and D102N Trypsins

<table>
<thead>
<tr>
<th>Substrate</th>
<th>wild-type</th>
<th>D102N</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>kcat (μM)</td>
<td>kcat (min⁻¹)</td>
</tr>
<tr>
<td>Z-GPR-S-benzyl</td>
<td>9.2</td>
<td>2900</td>
</tr>
<tr>
<td>Z-GPR-p-nitroanilide</td>
<td>7.1</td>
<td>3800</td>
</tr>
<tr>
<td>Z-GPR-AMC</td>
<td>15</td>
<td>3300</td>
</tr>
<tr>
<td>insulin peptide, β-chain</td>
<td>140</td>
<td>2500</td>
</tr>
</tbody>
</table>

The error in these determinations was 10% for the ester and the activated amide substrate and 20% for insulin β-chain. The buffer was 100 mM NaCl/20 mM CaCl₂/50 mM Tris-Cl, pH 8.0.

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protein preparations. This is a minute quantity, but given the low activity of H57R trypsin at pH 8.0, it was sufficient to yield misleading kinetic data. The presence of contamination was further indicated by the observation that the CGC-encoded protein readily cleaved oxidized insulin β-chain within 12 h, while the AGA-encoded enzyme required a much longer incubation (70 h) to achieve a perceptible amount of hydrolysis. These observations emphasize that, for a mutant enzyme preparation, a pH-rate profile that is different from that of the wild-type enzyme does not necessarily indicate that there is no contribution at all pHs from wild-type contamination.

Precautions against Contamination. As noted above, the low activities of the mutant trypsins required that contaminating enzymes be eliminated as a possible explanation of the observed catalysis. Escherichia coli cultures containing a truncated trypsin gene exhibited no proteolytic activity, indicating that endogenous bacterial enzyme activity was not significant.21 Nondenaturing SDS gel electrophoresis of the mutants followed by application of a substrate-impregnated overlay membrane22 showed that enzyme activity migrated at the same location as with wild-type trypsin, further suggesting that the activity was not due to any endogenous contamination. Finally, the cleavage of either insulin β-chain or porcine galanin peptide occurred at arginine, confirming that the mutant preparations possessed a trypsin-like specificity.

Another source of contamination could be small amounts of wild-type trypsin, introduced either through codon level misincorporation or by plasmid contamination. Several lines of evidence indicate that the observed activity is due to the mutant protein: (1) All mutants titrate with 4-methylumbelliferyl p-guanidino-benzoate (MUGB) except for the H57E, H57A, and H57L proteins and those mutants lacking Ser 195. (2) Several mutants have Km's which significantly differ from wild type (H57K, H57R, all mutants with the D102N mutation). (3) All mutants have pH-rate profiles different from those of the wild-type except for H57E, H57L, and those mutants lacking serine 195.

Taken together, these results indicate that the activity of each mutant enzyme preparation was predominantly due to protein with the mutation. However, they do not rule out the presence of an amount of wild-type contamination too small to be observed in the kinetic assays. Such a minute amount of activity might account for the activity against insulin β-chain. One line of evidence against this is that those mutants containing Ser 195 uniformly retained at least a minimal level of activity against the peptide, while those lacking Ser 195 showed no activity. In addition, variants containing mutations at both His 57 and Asp 102 retained activity, eliminating any source of contamination involving amino acid misincorporation since two misincorporation events would be needed to generate wild-type protein. The possibility of contamination was further tested by the treatment of the H57A/D102N variant with TLCK, a compound which inactivates wild-type trypsin by labeling the active site histidine. Both H57A/D102N and wild-type bovine trypsin were treated with 1 mM TLCK and then subsequently dialyzed against 1 mM HCl to remove unreacted label. Titration with MUGB demonstrated that the wild-type protein had lost 94% of its original reactivity, while the H57A/D102N protein was fully active. The treated mutant also showed no alteration in its activity toward Z-GPR-AMC or insulin β-chain (see above).

Conclusion. Proteolytic function depends on the complex interaction of amino acid chemistry and transition-state stabilization to hydrolyze amide bonds, which otherwise would have a half-life of 7 years. For trypsin and other similar serine proteases this involves three finely balanced catalytic residues, His 57, Asp 102, and Ser 195, an oxyanion hole formed by backbone amides, and transition-state stabilization due to substrate binding. The incorporation of all of these characteristics into catalytic antibodies, designed peptides.

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(22) Smith, R. E. J. Histochem. Cytochem. 1984, 33, 1265–1274.
or preexisting unrelated proteins would be a very demanding project. Each addition would need to be fit in a precise spatial orientation while simultaneously avoiding the disruption of substrate binding or preexisting catalytic features.

Our results indicate that this problem can be simplified because, of the three members of the triad, only serine is essential for the cleavage of unactivated amide bonds by trypsin. The presence of an adjacent basic residue, lysine or histidine, can increase this catalysis further. Moreover, designed systems need not be limited to serine since it is the intrinsic structural framework of trypsin which prevents other nucleophilic side chains from functioning. For other protein scaffolds the presence of cysteine, threonine, or tyrosine may also be sufficient. Thus, a small, but measurable level of activity can be obtained by a simplified protease, indicating that initial attempts at introducing peptidease function into proteins should focus on the introduction of an oxygen anion hole,2324 transition-state stabilization via substrate binding, and a strategically positioned nucleophile instead of an entire triad. Subsequent studies could then be undertaken to maximize this catalysis.

Experimental Section

Total Synthesis of (±)-Chondrillin, (±)-Plakorin, and Related Peroxy Ketals. Development of a General Route to 3,6-Dihydro-1,2-dioxin-3-ols

Barry B. Snider* and Zhongping Shi

Contribution from the Department of Chemistry, Brandeis University, Waltham, Massachusetts 02254-9110. Received July 22, 1991

Abstract: Seven-step syntheses of the antitumor cyclic peroxy ketals 1a, 2a, chondrillin (1b), and plakorin (2b) from (methoxymethoxy)benzene (8) have been achieved in 26-82% overall yields. The key step is the photooxygenation of enone 4 with a sun lamp using rose bengal lactone or CuSO₄ as a sensitizer which gives a mixture of peroxy hemiketals 15 and 16 in 75-85% yields. Acetal formation in acidic methanol completes the syntheses of 1 and 2. The mechanism of photooxygenation was ascertained using 3-nonen-2-one which can undergo photoenolization to give a dienol. Peroxy hemiketals of this last step is not known, although singlet oxygen is probably not involved. This reaction is general for any enone or enal which can undergo photoenolization to give a dienol. Peroxy hemiketals 33a, 41, and 43-46 were prepared in 30-80% yields. Peroxy ketals can be used for the synthesis of furans, diones, and pyridazines.

Introduction

A wide variety of biologically active cyclic peroxides have been isolated from marine organisms. Chondrillin (1b) was first isolated in 1976 from a sponge of the genus Chondrilla by Wells. More recently, xestins A (2c) and B (1c) have been isolated from a sponge of the genus Xestospongia by Crews, chondrillin (1b) and a series of related ketals (2a,f-h) have been isolated from Plakortis lita by Higa and Christophersen, chondrillin (1b), epi-chondrillin (2b), and a series of unsaturated analogues (1d,e, 2d,e) have been isolated from P. lita by De Guzman and Schmitz, and epi-chondrillin (called plakortin) (2b) has been isolated from a Plakortis species by Kobayashi.

Peroxy ketals 2a,c,f-h have been shown to be active against P388 mouse leukemia cells in vitro with IC₅₀ values of 0.05-0.3 μg/mL. The isomers 1b,c are approximately 1 order of magnitude less active. Plakorin (2b) (10⁻⁵ M) activated SR Ca²⁺-ATPase activity by 30% and exhibited antineoplastic activity against L1210 cells and KB cells in vitro with IC₅₀ values of 0.85 and 1.8 μg/mL, respectively. A variety of related cyclic peroxides with branched skeletons, including the norsterterpenes trunclins A and B, plakortic acid, plakoric acid, and plakinic acid B, have been shown to possess antitumor and antimicrobial activity.

The structural novelty and potent biological activity of peroxy ketals 1 and 2 prompted us to undertake their synthesis. Despite the biological activity and ostensible simplicity of these cyclic

Acknowledgment. We are grateful to Dr. John Vasquez for helpful advice. This work was supported by NSF Grants DMB-8904956 and EET-8807179 to C.S.C. and a Damon Runyon-Walter Winchell Fellowship (DRG 1076) to D.R.C. The mass spectral data were prepared by the Bio-organic Biomedical Mass Spectrometry Resource supported by NIH Division of Research Resources, Grant 001614.