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Importance of hydrogen-bond formation in stabilizing the transition state of subtilisin

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Structural studies on serine proteinases have shown that hydrogen bonds are involved in stabilizing the charged tetrahedral intermediate in the transition-state complex. However, little is known about the quantitative contribution of these interactions to transition-state stabilization. X-ray crystallographic studies of subtilisin (Robertus, J. D., Kraut, J., Alden, R. A. & Birktoft, J. J. *Biochemistry, Wash.* **11**, 4293–4303 (1972)) have suggested that the amide side chain from asparagine-155 forms a hydrogen bond with the oxyanion produced on the substrate carbonyl oxygen in the tetrahedral intermediate. To study the importance of the Asn-155 hydrogen bond in stabilizing the tetrahedral intermediate, Asn-155 was substituted with Thr, His, Gln and Asp by using site-specific mutagenesis of the cloned subtilisin gene from *B. amyloliquefaciens*. These substitutions were intended to alter the position and charge of the potential hydrogen-bonding group at 155.

Mutations of Asn-155 caused large decreases in substrate turnover, k_{cat} (200- to 4000-fold), with marginal decreases in substrate binding, K_M (up to 7-fold). The most dramatic effects were seen with Thr-155, where k_{cat} was reduced 4000-fold with a slight increase in K_M. Mutations of Asn-155 caused a loss in transition-state stabilization energy of 9.2–20 kJ mol^{-1}

Simple enrichment methods are described which greatly facilitate the isolation of mutant sequences. These methods depend upon the introduction or elimination of a unique and silent restriction site near the site of mutagenesis.

1. Introduction

Serine proteases are a diverse class of enzymes having a wide range of specificities and biological functions (for a review see Stroud (1974)). Despite their functional diversity, the catalytic machinery of serine proteases has been approached by at least two genetically distinct families of enzymes: the *Bacillus* subtilisins and the mammalian and homologous bacterial serine proteases (for example, trypsin and *S. gresius* trypsin). These two families of serine proteases show remarkably similar mechanisms of catalysis (for a review see Kraut (1977)). Furthermore, although the primary structure is unrelated, the tertiary structure of these two enzyme families brings together a conserved catalytic triad of amino acids, consisting of a serine, a histidine and an aspartic acid (see figure 1). These residues are positioned to facilitate nucleophilic attack by the serine hydroxylate on the carbonyl of the scissile peptide bond.

At least three hydrogen bonds may help to stabilize the transition-state complex for the tetrahedral intermediate (figure 1; for a review see Kossiakoff 1985). One hydrogen bond is between the aspartic acid and the positively charged histidine, ND1 (Kossiakoff & Spencer

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FIGURE 1. Mechanistic diagram of the catalytic site of *B. amyloliquefaciens* subtilisin. The scissile peptide bond

$$\overset{\text{O}}{\underset{|}{\overset{\|}{-\text{C}}}}\!-\!\overset{\text{H}}{\overset{|}{\text{N}}}-$$

is oriented in the Michaelis complex (E·S) to facilitate nucleophilic attack by the serine-221 hydroxylate on the carbonyl carbon. The serine-221 hydroxyl proton is transferred to the imidazole of histidine-64. Two of the hydrogen bonds which are proposed to develop in the transition-state complex are indicated by the broken lines. Although not shown, an additional hydrogen bond has been proposed to form between the oxyanion in the transition-state complex and the main-chain amide proton from serine-221 (Robertus *et al.* 1972).

1981). A second hydrogen bond forms between the scissile amide nitrogen of the substrate and the (NE2) proton on the histidine. A third set of hydrogen bonds forms between the enzyme and the oxyanion that is produced from the carbonyl oxygen of the substrate. This latter set of hydrogen bonds is formed differently by mammalian serine proteases and by subtilisin. Specifically, X-ray crystallographic studies of chymotrypsin (Henderson 1970) indicate that two hydrogen bonds form between the substrate oxyanion and two main-chain amide protons from the enzyme (Gly-193 and the catalytic Ser-195). Crystallographic studies of subtilisin (Robertus *et al.* 1972; Matthews *et al.* 1975; Poulos *et al.* 1976) show that two hydrogen bonds are also formed with the oxyanion; one hydrogen-bond donor is from the catalytic serine-221 main-chain amide while the other is from one of the NE2 protons of the asparagine-155 side chain.

Despite structural evidence for the formation of hydrogen bonds to the tetrahedral intermediate, very little is known about their free-energy contribution to transition-state stabilization. Among the serine protease class of enzymes, subtilisin is best suited to test the importance of hydrogen bond to stabilization of the oxyanion. Because one of the hydrogen bonds in subtilisin (i.e. Asn-155) is contributed by an amino-acid side-chain, not main-chain, amide, site-directed mutagenesis can be used to replace this hydrogen-bonding group. The subtilisin gene has been cloned and expressed (Wells *et al.* 1983). Here we describe studies where Asn-155 was altered by a modified site-directed mutagenesis method to estimate the contribution to the transition-state stabilization energy of the presumed hydrogen bond between Asn-155 and the oxyanion.

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2. Materials and methods

(a) Materials

T4 DNA ligase, *Eco*RI, and *Bam*HI were obtained from New England Biolabs. *Kpn*I and T4 polynucleotide kinase were from Bethesda Research Laboratories and *Escherichia coli* DNA polymerase I large fragment (Klenow) was from Boehringer–Mannheim. Succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide was from Vega Biochemicals. LB media and plates (Miller 1972) contained 12.5 μg ml^{-1} chloramphenicol. Transformation of *E. coli* MM 294 rec^+ cells (for plasmids) or JM 101 cells (for M-13) was performed as previously described (Mandel & Higa 1970). The protease deficient *B. subtilis* strain, BG2036 (Yang *et al.* 1984) was transformed as previously described (Anagnostopoulos & Spizizen 1961). Oligodeoxyribonucleotides were synthesized by using phosphoramidite chemistry and purified by polyacrylamide gel electrophoresis.

(b) Site-specific mutagenesis

The overall strategy for mutagenesis with the use of mutant enrichment by restriction–purification is outlined in figure 2. The 1.5 kb (kilobase) *Eco*R1-*Bam*HI fragment containing

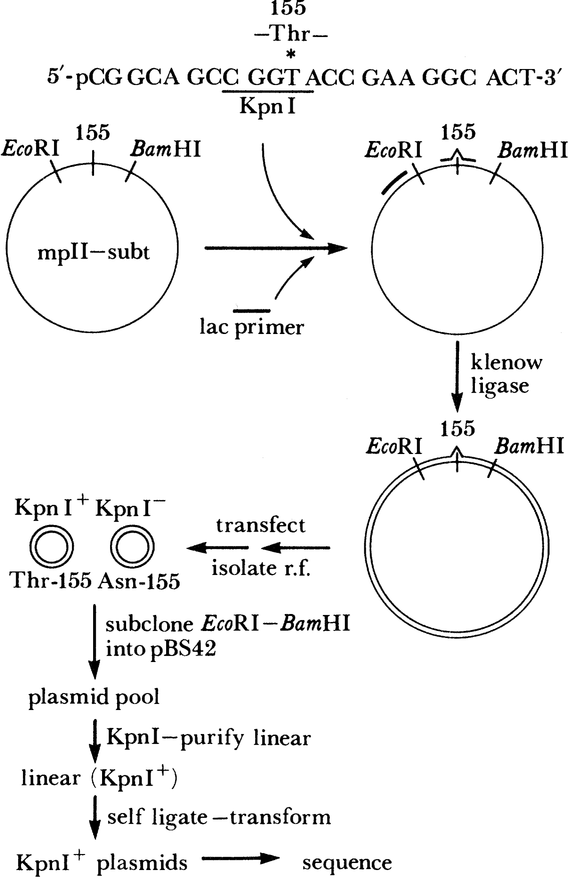

Figure 2. Strategy for mutagenesis of Asn-155→Thr in subtilisin. A primer directing the Asn-155→Thr mutation (figure 3) and the universal sequencing primer were annealed to an M13mp11-SUBT template containing the complementary subtilisin gene sequence inserted between the *Eco*RI and *Bam*HI sites. Following heteroduplex synthesis, transfection, and subcloning, the Asn-155→Thr mutant DNA was isolated from wild-type by digestion with *Kpn*I and purification on polyacrylamide gels. See §§2*b*, 3*a* for further details.

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the *B. amyloliquefaciens* subtilisin gene from the plasmid pS4.5 (Wells *et al.* 1983) was ligated into M13mp11 (Messing & Vierra 1982; Messing 1983). The phosphorylated primer directing the Asn-155 → Thr mutation (figure 3) was annealed to the single-stranded template m13mp11-SUBT, along with the universal sequencing primer (lac primer) and extended with Klenow and ligase (Adelman *et al.* 1983). The heteroduplex was transformed into *E. coli* JM101 and

```
                                      155
codon sequence: Ala  - Ala  - Ala  - Gly  - Asn - Glu  - Gly - Thr

(1)  wild type   5′CG-GCA-GCC-GGT-AAC-GAA-GGC-ACT-3′

                                       *
(2)  Thr-155      CG-GCA-GCC-GGT-ACC-GAA-GGC-ACT
                                 KpnI

                                   *   *
(3)  Gln-155      CG-GCA-GCC-GGT-CAA-GAA-GGC-ACT

                                   *
(4)  His-155      CG-GCA-GCC-GGT-CAC-GAA-GGC-ACT

                                   *    *
(5)  Asp-155      CG-GCA-GCC-GGT-GAT-GAA-GGC-ACT
```

FIGURE 3. Sequence of wild-type (Asn-155) mutagenic primers used to produce codon-155 mutants. Asterisks indicate the nucleotide differences from the wild-type coding sequence. The Asn-155 → Thr primer (line 2) introduces a unique *Kpn*I site which was used to isolate first the Thr-155 mutant (*Kpn*I^{+}) (figure 2). The Thr-155 mutant template was then used for mutagenesis with the remaining primers (lines 3–5). The His, Asp, and Gln mutant plasmids (*Kpn*I^{-}) were efficiently isolated from the Thr-155 background (*Kpn*I^{+}) by *Kpn*I restriction digestion and retransformation (see §§2*b*, 3*b* for details).

the resulting pool of replicative form (r.f.) DNA was isolated from a 5 ml overnight phage culture (Birnboim & Doly 1979). The *Eco*RI-*Bam*HI fragment containing the subtilisin gene insert was cut out of the M13 r.f. and ligated between the *Eco*RI-*Bam*HI sites of the *B. subtilis*–*E. coli* shuttle plasmid, pBS42 (Band & Henner 1984). This ligation mixture was used to transform *E. coli* 294 cells and the mixture of recombinant plasmids was purified as above. The Asn-155 → Thr mutation introduces a unique *Kpn*I site (figure 3). Thus, digestion of the plasmid pool with *Kpn*I produces linear molecules for the Asn-155 → Thr mutant plasmid. Linear molecules were purified by electrophoresis on 7.5% polyacrylamide gels and DNA was electro-eluted (Maniatis *et al.* 1982). Although linear molecules this size (*ca.* 5 kb) are poorly resolved by acrylamide, circular molecules (i.e. Asn-155 wild type) do not enter the gel. The linearized molecules were recircularized by ligation and used to transform *E. coli* 294 cells. Colonies were picked and plasmid DNA was screened for the presence and location of the *Kpn*I site expected for the Asn-155 → Thr mutant. The sequence of the mutant was confirmed in the plasmid (Chen & Seeburg 1985) or in M13mp11 (Sanger *et al.* 1980) by using a primer having the sequence 5′-GGCAGTTAGATAAGCCG-3′, which terminates 45 nucleotides 5′ to codon-155.

An Asn-155 → Thr mutant M13mp11-SUBT template was prepared and used for generation

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of mutants having Asp, His or Gln codons at position 155. These primers eliminate the *Kpn*I site introduced by the Asn-155 → Thr mutation (figure 3). From separate mutagenesis reactions for each mutant, r.f. DNA was isolated and the mutagenized subtilisin-gene insert was cloned into pBS42 as before. The mixture of purified plasmids was digested with *Kpn*I to reduce greatly the Thr-155 background and the DNA was used to transform *E. coli* 294 cells. Plasmid DNA lacking the *Kpn*I site was prepared from individual colonies and mutant sequences were confirmed as above.

(*c*) *Expression, purification, and kinetics of mutant subtilisins*

Recombinant pBS42-SUBT plasmids from *E. coli* were used to transform the protease deficient *B. subtilis* BG2036 (Yang *et al.* 1984). The transformants were grown in shake flasks under conditions which would express the mutant enzymes. Mutant and wild-type subtilisins were purified from culture supernatants by chromatography on carboxymethyl cellulose as described by Estell *et al.* (1985) and Philipp & Bender (1983). Enzymes were greater than 95% pure as judged by SDS–p.a.g.e. (Laemmli 1970). Kinetic constants (V_{max} and K_M) were determined from a modified progress-curve analysis (Estell *et al.* 1985). Enzyme concentrations were determined spectrophotometrically (ϵ_{280} (0.1%) = 1.17; Matsubara *et al.* (1965)), k_{cat} was calculated from the relation $k_{cat} = V_{max}/[\text{enzyme}]_{total}$.

3. Results

(*a*) *Mutant enrichment by restriction–purification*

The enrichment strategy outlined in figure 2 for obtaining the Asn-155 → Thr mutation took advantage of the unique *Kpn*I site introduced by the Asn-155 → Thr primer. When plasmids from ten colonies were randomly screened for the presence of the *Kpn*I site before enrichment by restriction–purification, none of the plasmids contained the *Kpn*I site, whereas after enrichment six plasmids of ten examined contained the *Kpn*I site. This simple enrichment procedure has given mutagenesis efficiencies that range from 25 to 75% mutant for mutagenesis primers at several other sites in the subtilisin gene. This compares with frequencies of mutagenesis that range from 0.1 to 10% without this enrichment. The efficiency of mutagenesis after enrichment did not reach 100%. One reason for this might be that the wild-type plasmids which were linearized by physical shearing copurified with mutant plasmids linearized by restriction digestion.

The restriction–purification method is limited to finding a unique restriction-site sequence that is silent to the amino-acid coding sequence and in close proximity to the site of mutation. Assuming a random nucleotide sequence, the frequency of introducing a particular six base-pair recognition sequence by a single base-pair change that is silent to the coding sequence is roughly one in 500 base pairs. However, because of the large number of commercially available restriction enzymes (more than 40 enzymes with six base-pair recognition sequences), it is often possible to introduce a unique and silent restriction site in close proximity to the target site of mutagenesis. For example, in the *B. amyloliquefaciens* subtilisin coding sequence, such restriction sites can be introduced roughly every 25 base pairs. Although this enrichment method cannot be conveniently used for mutagenesis at any site, it can always be applied when one is mutagenizing a plasmid to produce unique restriction sites (for example, for introduction of synthetic DNA cassettes (Wells *et al.* 1985)).

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(b) *Mutant enrichment by restriction–selection*

The remaining mutations (His, Gln and Asp) were produced from the Thr-155 template by restriction–selection. After heteroduplex synthesis and transfection, the *Eco*RI-*Bam*HI subtilisin gene fragment from the pool of M13 r.f. DNA was subcloned into pBS42 (figure 2). Because the His, Gln and Asp-155 primers lacked the *Kpn*I site (figure 3), the resulting pools of plasmids were restricted with *Kpn*I to reduce the *Thr*155 plasmid contamination. Eight colonies were screened after restriction–selection from each of the three mutagenesis reactions. In each case, six or seven of the cloned plasmids lacked the *Kpn*I site and all *Kpn*I$^-$ plasmids sequenced contained the desired mutation. The restriction–selection can be applied directly on the pool of M13 r.f. DNA (see figure 2), followed by retransfection. Because the efficiency of this selection is high (more than 50%), templates prepared from a small number of plaques can be sequenced directly to identify the mutant. This method has been previously applied to enrich for deletion mutants by eliminating a *Pst*I site in human growth hormone (Adelman *et al.* 1983).

(c) *Kinetics of mutant subtilisins*

Kinetic constants for the purified wild-type and mutant subtilisins are shown in table 1. For the two-step reaction mechanism of serine proteases, $K_M = K_s\,[k_3/(k_2+k_3)]$ and $k_{cat} = k_2 k_3/(k_2+k_3)$, where K_s is the enzyme–substrate dissociation constant, k_2 is the rate of acylation, and k_3 is the rate of de-acylation (Gutfreund & Sturtevant 1956). For hydrolysis

TABLE 1. KINETIC CONSTANTS FOR WILD-TYPE AND CODON-155 SUBTILISIN MUTANTS AGAINST A SUC-L-ALA-L-ALA-L-PRO-L-PHE-*p*-NITROANILIDE SUBSTRATE[a]

codon-155	Asn (wild type)	Thr	Gln	Asp	His
structure	H_2N, =O	HO, CH_3	NH_2, O=	O^-, =O	HN, N
k_{cat} s^{-1}	50	0.02	0.06	0.02	0.2
$10^{-4}\,K_M$/M	1.4	2	0.3	0.3	0.2
$10^{-3}\,k_{cat}/K_M/(\mathrm{Ms}^{-1})$	360	0.1	2	0.8	9
$\Delta\Delta G\ddagger$ (mutant wild-type)[b]/(kJ mol^{-1})	0	20	13	15	9.2

[a] Enzymes were purified and kinetics were evaluated as described in §2*c*.
[b] Calculated from the relation

$$\Delta\Delta G\ddagger = -RT\ln\frac{(k_{cat}/K_M)_{\text{mutant}}}{(k_{cat}/K_M)_{\text{Asn-155}}}$$

(see Fersht *et al.* 1985).

of amide bonds by subtilisin, acylation is rate limiting (Markland & Smith 1971); for hydrolysis of the *p*-nitroanilide substrate used here, the ratio of the rate constants for de-acylation (k_3) to acylation (k_2) is 33 (Estell & Graycar, unpublished results). Under conditions where $k_2 \ll k_3$, these equations simplify to $K_M \approx K_s$ and $k_{cat} \approx k_2$. If one assumes this is valid for the mutant enzymes, the large decrease observed in k_{cat} (200- to 4000-fold) for mutant-155 versus Asn-155 can be interpreted as the loss of stabilizing interaction(s) as E·S changes to E–S‡ (figure 1). The small decrease in K_M suggests those mutant enzymes have a greater affinity for substrate than wild-type enzymes.

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4. Discussion

X-ray crystallographic studies of subtilisin reacted with chloromethyl ketone peptide inhibitors (Robertus *et al.* 1972; Poulos *et al.* 1976) and boronic acid inhibitors (Matthews *et al.* 1975) clearly show the presence of a hydrogen bond of *ca.* 2.5 Å† between the ND2 of the amide side chain of Asn-155 and an oxygen of the tetrahedral oxyanion analogue produced by these transition-state inhibitors. In addition, the Asn-155 is oriented by a hydrogen bond between the OD1 and the proton from the main-chain amide nitrogen of Thr-220.

Substitutions were made for Asn-155 that were intended to alter the potential hydrogen-bond donor (i.e. Thr, Gln and His) or place a charge at position 155, depending on the pH of the assay (i.e. His and Asp). Assuming the structure of the mutant enzymes is not significantly altered except for the substitution of the side chain at position 155, molecular graphics can aid in the structural interpretation of these data (table 1). By using this analysis, the Thr-155 hydroxyl group would be at least 3.5 Å from the oxyanion. This distance is outside that required to form a good hydrogen bond. Although the glutamine side chain can be built to form a good hydrogen bond with the oxyanion, the OD1 of Gln-155 cannot hydrogen bond to the main-chain amide of Thr-220. Furthermore, in forming a hydrogen bond to the oxyanion, the glutamine side chain would sterically interfere with substrate binding. This structural interpretation does not fit with the interpretation of the kinetic results. Thus, from both structural and functional grounds it is more likely that the glutamine side chain is positioned out in the solvent and does not significantly participate in hydrogen bonding to the carbonyl oxyanion. This interpretation is even more applicable to the aspartic acid substitution. Although Asp is isosteric with Asn and can form a hydrogen bond to the Thr-220 main-chain amide, the charge repulsion with the oxyanion at a distance of only 2.5 Å should have led to a lower k_{cat} than observed for the non-hydrogen-bonding Thr mutant (table 1). Instead, the kinetic results for the Asp and Gln mutations are comparable. Modelling shows that the aspartyl side chain can be positioned to extend into the solvent at a distance greater than 4.5 Å from the oxyanion (Rick Bott, personal communication). If these analyses are correct, it can be estimated that the loss of the hydrogen bond to the oxyanion results in a reduction of 1000–4000 in the rate of acylation (i.e. k_{cat} values obtained from the Thr, Gln, and Asp-155 enzymes). This is a significant proportion of the ratio of the rate constants for subtilisin-catalysed (Polgar & Bender 1967) to hydroxide-catalysed (Edwards & Pearson 1962) acylation of *p*-nitrophenylacetate, which is 10^{10}–10^{11}.

The k_{cat} for the His-155 enzyme suggests that it is less deleterious to transition-state stabilization than the other mutations (table 1). It should be noted that at pH 8.6, where the kinetic analysis was performed, the imidazole should be predominantly of neutral charge. Modelling shows that a protonated ND1 of the His side chain can form a hydrogen bond to the oxyanion. However, the His side chain can also be built to occupy a position out in solution away from the oxyanion.

Several explanations can account for the lowering of K_M by five- to sevenfold for the Asp-155, Gln-155, and His-155 mutations. If these side chains were to extend out into the solvent, computer modelling shows that the active site is more exposed to bind substrate than are the Asn or Thr-155 enzymes. The lowering of K_M could also result from indirect structural perturbations or solvent effects caused by these mutations. Deletion of a poor hydrogen bond between tyrosyl-tRNA synthetase and ATP actually improves substrate binding by as much as 100-fold (Wilkinson *et al.* 1984). An explanation provided for this result was that the wild-type

† 1 Å = 10^{-1} nm = 10^{-10} m.

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enzyme was exchanging a good hydrogen bond with water for a poor one with the bound substrate. Thus, an alternative explanation for the lowering of K_M for the His-155, Asp-155 and Gln-155 mutations in subtilisin is that a poor hydrogen bond has been eliminated in the Michaelis complex (i.e. between the scissile carbonyl oxygen and Asn-155).

The kinetic data indicate that the effect of mutations of Asn-155 on the energy difference between the transition state (E–S‡) and the ground state (E+S) was to cause a loss of 9–20 kJ mol^{-1} stabilization energy compared with wild-type subtilisin ($\Delta\Delta G$‡, table 1). From mutations in tyrosyl-tRNA synthetase, empirical estimates have been made for the contribution of various hydrogen bonds to transition-state stabilization energy (Fersht *et al.* 1985). Specifically, hydrogen bonds between neutral donors and charged versus uncharged acceptors contribute 15–21 versus 2.1–6.2 kJ mol^{-1}, respectively, to transition-state stabilization. The $\Delta\Delta G$‡ values reported here are slightly below the range predicted for the loss of a hydrogen bond between a neutral donor and a charged acceptor. Kinetic studies of chymotrypsin suggest that only a partial charge develops in the transition state during catalysis (Jencks 1971), in which case the hydrogen bond to Asn-155 may be expected to contribute stabilization energy that is intermediate between the neutral and fully charged case. Furthermore, it is possible that disruption of the hydrogen bond between the oxyanion and Asn-155 may affect the hydrogen bond between the oxyanion and the main-chain amide of Ser-221.

In conclusion, the data presented here are consistent with the crystallographic interpretation (Robertus *et al.* 1972; Matthews *et al.* 1975; Paulos *et al.* 1976) that a hydrogen bond forms between the transition-state oxyanion and Asn-155; a hydrogen bond forms only weakly, if at all, between the scissile amide carbonyl oxygen and Asn-155 in the Michaelis complex. It should be emphasized that inferring the function of a particular amino-acid side chain (i.e. Asn-155) by amino-acid substitution is a multivariable experiment. X-ray crystallographic studies and further kinetic analyses will be necessary to determine the structures and better define the functional properties of these mutant proteins.

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