The Importance of a Distal Hydrogen Bonding Group in Stabilizing the Transition State in Subtilisin BPN'*

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Stabilization of an oxyanion transition state is important to catalysis of peptide bond hydrolysis in all proteases. For subtilisin BPN', a bacterial serine protease, structural data suggest that two hydrogen bonds stabilize the tetrahedral-like oxyanion intermediate: one from the main chain NH of Ser221 and another from the amide side chain of Asn155. These structural observations were corroborated by site-directed mutagenesis (Bryan et al., 1986; Wells et al., 1986) which demonstrated that replacement of Asn155 with a variety of other side chains resulted in 3–5 kcal/mol reductions in transition state stabilization energy with only minor effects upon substrate binding.

In the x-ray structure of subtilisin BPN', the side chain of Thr220 (Fig. 1) is oriented so that the γ-hydroxyl is about 4 Å away from the developing oxyanion in the transition state complex (E-S2). Molecular dynamic simulations and free energy calculations (Rao et al., 1987) suggested that the γ-hydroxyl of Thr220 may move to form a direct hydrogen bond with the oxyanion in the transition state. It has been suggested that enzymes can also provide oriented dipoles that stabilize charged transition states without being in direct H-bonding distance (Warshel, 1987; Warshel et al., 1988; Hwang and Warshel, 1988). Thr220 is such an oriented dipole and one of the most highly conserved residues in the subtilisin family (Fig. 2). Its side chain makes van der Waals contact with Asn155 and thus may affect the functional role of Asn155.

Therefore, to evaluate the functional importance of Thr220 to catalysis versus substrate binding and its functional independence from Asn155, we have systematically replaced Thr220 separately and together with Asn155.

EXPERIMENTAL PROCEDURES

Construction and Purification of Subtilisin Variants—The Thr220 mutations were introduced into the S24C variant (Carter and Wells, 1987) of the Bacillus amyloliquefaciens subtilisin gene (Wells et al., 1983) cloned in to the phagemid vector pSS5 (Carter and Wells, 1988) by site-directed mutagenesis (Carter et al., 1985). Mutagenesis was performed on a single-stranded pSS5 template containing a KpnI site near codon 220 and S221C mutation with the following oligonucleotides: T220S, 5' GGCTTACACGGGAGCTCTATGGCATCTC, NheI site; T220A, 5' GGCGTACAACGGCGCCTCTATGGCATCTC, SacI site; T220V, 5' GCGTACAACGGCGCCTCTATGGCATCTC, NarI site, which regenerated the active site Ser221 (restriction sites introduced are underlined). Restriction-selection (Wells et al., 1986) against the KpnI site over the parent (KpnI/S221C) template was used to enrich for mutant plasmids. The variant phagemids were verified by dideox sequencing (Sanger et al., 1977) and double-stranded DNA was transformed into a peptide-deficient strain (BG2036) of Bacillus subtilis (Yang et al., 1984). Subtilisin mutants were cultured (in the absence of wild type) and purified on an SP-Sephadex ion exchange column followed by activated thiol affinity chromatography as described (Carter and Wells, 1988).

Kinetic Characterization—Enzymes were assayed with the sub-

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† Mutants are named according to the wild type residue (using single-letter amino acid code) followed by its sequence position and then the mutant residue. For example, T220S designates that Thr220 is replaced by serine (Wetzel, 1988). Multiple mutants are indicated by the series of single mutants separated by slashes.
Oxyanion stabilization by Thr^{200}

![Stereo view of the active site of subtilisin showing the Asp^{32}, His^{64}, Ser^{221} catalytic triad and the Asn^{155}, backbone NH of Ser^{221} and Or of Thr^{200} in the oxyanion hole (designated by stippled lines). Distances labeled are in angstroms. The model transition state is based upon the crystal structure of the Eglin C inhibitor bound at the active site of subtilisin (McPhalen and James, 1988).](image)

FIG. 1. Stereo view of the active site of subtilisin showing the Asp^{32}, His^{64}, Ser^{221} catalytic triad and the Asn^{155}, backbone NH of Ser^{221} and Or of Thr^{200} in the oxyanion hole (designated by stippled lines). Distances labeled are in angstroms. The model transition state is based upon the crystal structure of the Eglin C inhibitor bound at the active site of subtilisin (McPhalen and James, 1988).

FIG. 2. Sequence comparison among 15 subtilins showing that Thr^{200} is absolutely conserved.

| 220       | Reference |
|-----------|-----------|
| B. amyloliquefaciens (BPN') | P G N K Y G A Y N G T S M A S P H V A G 1 |
| B. amylosacchariticus | P G T Y G A Y N G T S M A T P H V A G 2 |
| B. mesentericus | P G T Y G A Y N G T S M A T P H V A G 3 |
| B. DY | P S N T Y T S L N G T S M A S P H V A G 4 |
| B. licheniformis (Carlsberg) | P T N Y A T L N G T S M A S P H V A G 5 |
| B. subtilis 168 | P G T Y G A Y N G T S M A T P H V A G 6 |
| Protease K | I G S T R S I S G T S M A T P H V A G 7 |
| Aquasyn l | Y T S D T A T G L N G T S M A T P H V A G 8 |
| B. subtilis bacillolipidase F | P G Q T Y D G W D G T S M A G P H Y S A 9 |
| Streptococcal C5A | A N K Y A K L S G T S M S A P L Y A G 10 |
| B. subtilis ISP | P N K K Y G K L T G T S M A A P E V S G 11 |
| B. extracellular protease | L N Q Y Y A T G S G T S Q A T P H A A A 12 |
| Streptococcus cremoris Wg2 | N N N G Y T N M S G T S M A S P F I A G 13 |
| Lactococcus lactis SKII | N N N G Y T N M S G T S M A S P F I A G 14 |
| Thermin | P T S T Y A S L S G T S M A T P H V A G 15 |

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Kinetic constants for wild-type and codon 220 subtilisin mutants 
against N-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-p-nitroanilide substrate

All mutants contain S24C to aid in purification.

| Mutant          | \(k_{\text{cat}}(\pm \text{S.D.})\) | \(K_{\text{M}}(\pm \text{S.D.})\) | \(k_{\text{cat}}/K_{\text{M}}(\pm \text{S.D.})\) | \(\Delta G^\ddagger\) kcal/mol |
|-----------------|-----------------------------------|---------------------------------|---------------------------------|-------------------------------|
| Wild type       | 56 (±0.9)                         | 162 (±14)                       | 3.5 (±0.21) \(\times 10^6\)     | (0)                           |
| T220S           | 32 (±0.5)                         | 215 (±6)                        | 1.5 (±0.02) \(\times 10^6\)     | 0.5                           |
| T220C           | 2.9 (±0.05)                       | 239 (±12)                       | 1.2 (±0.04) \(\times 10^6\)     | 2.0                           |
| T220V           | 2.7 (±0.16)                       | 280 (±38)                       | 9.6 (±1.1) \(\times 10^4\)      | 2.1                           |
| T220A           | 6.8 (±0.19)                       | 386 (±24)                       | 1.8 (±0.7) \(\times 10^4\)      | 1.8                           |

Effect of separate and combined substitutions at Asn\(^{155}\) and Thr\(^{220}\) on the kinetics 
for hydrolysis of N-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-p-nitroanilide

| Mutant          | \(k_{\text{cat}}(\pm \text{S.D.})\) | \(K_{\text{M}}(\pm \text{S.D.})\) | \(k_{\text{cat}}/K_{\text{M}}(\pm \text{S.D.})\) | \(\Delta G^\ddagger\) kcal/mol | \(\sum \Delta G^\ddagger\) of component single mutants |
|-----------------|-----------------------------------|---------------------------------|---------------------------------|-------------------------------|-----------------------------------------------------|
| Wild type       | 56 (±0.9)                         | 162 (±14)                       | 3.5 (±0.21) \(\times 10^6\)     | (0)                           | 0.37                                               |
| T220A           | 32 (±0.5)                         | 215 (±6)                        | 1.5 (±0.02) \(\times 10^6\)     | 0.5                           | 0.37                                               |
| N155A           | 0.14 (±0.01)                      | 480 (±15)                       | 2.9 (±0.05) \(\times 10^6\)     | 4.2                           | 4.2                                                |
| N155A/T220A     | 0.011 (±0.001)                    | 790 (±21)                       | 1.4 (±0.2) \(\times 10^4\)      | 6.0                           | 6.0                                                |
| N155S           | 0.13 (±0.000)                     | 500 (±50)                       | 2.6 (±0.02) \(\times 10^4\)     | 4.3                           | 4.3                                                |
| N155S/T220A     | 0.035 (±0.002)                    | 680 (±50)                       | 5.1 (±0.3) \(\times 10^4\)      | 5.2                           | 5.2                                                |
| N155Q           | 0.06                              | 30                              | 2.0 \(\times 10^5\)             | 3.1                           | 3.1                                                |
| N155Q/T220A     | 0.009 (±0.00005)                  | 1,400 (±170)                    | 6 (±0.6)                        | 6.5                           | 6.5                                                |

Results and Discussion

A series of side chain replacements was produced by site-directed mutagenesis to probe the role of Thr\(^{220}\) in catalysis (Table I). Removal of the γ-methyl group by the T220S mutation causes only a small reduction (<2-fold) in \(k_{\text{cat}}\) and essentially no change in \(K_{\text{M}}\). In contrast, replacing the hydrogen bond donating γ-hydroxyl with a γ-thiol, which is nonpolar and a poor hydrogen bond donor (Crampton, 1974; Paul, 1974), results in a 20-fold drop in \(k_{\text{cat}}\) for the T220C mutant relative to wild-type, with essentially no change in \(K_{\text{M}}\). The reduction in activity for the T220C mutation is not likely due to deprotonation of Cys at pH 8.6, because even at pH 7.0 the reduction in \(K_{\text{M}}\) relative to wild-type is the same (data not shown). Similarly, replacing the γ-hydroxyl with a γ-methyl group as in the T220V mutation causes a 20-fold reduction in \(k_{\text{cat}}\) relative to the wild-type enzyme. The T220A mutation is slightly less deleterious than substituting the γ-hydroxyl with nonpolar side chains as in the T220C and T220V mutants. Perhaps a water molecule can fit in the space left when T220 is replaced by alanine, and this polar water can stabilize the oxyanion better than a nonpolar substituent. In any case, these data suggest that the γ-hydroxyl group of Thr\(^{220}\) functions predominantly in the catalytic step to stabilize the oxyanion transition state complex.

Assuming that the enzyme mechanism and structure are not substantially altered by mutations at Thr\(^{220}\), the reduction in transition state stabilization energy (\(\Delta G^\ddagger\); Table I) can be a useful estimate of the functional importance of the Thr\(^{220}\) γ-hydroxyl group (Wilkinson et al., 1983). The data suggest the hydroxyl imparts 1.8–2.0 kcal/mol to stabilize the oxyanion transition. This is substantially less than the stabilization energy imparted by Asn\(^{155}\), estimated to be 3.5–5 kcal/mol (Table II; Wells et al., 1986; Bryan et al., 1986). The structural basis for these differences could reside in the fact that the N62 of Asn\(^{155}\) is within hydrogen bonding distance of the developing oxyanion (≤2.7 Å), whereas the Oγ of Thr\(^{220}\) is too far away to hydrogen bond (≤4.0 Å). Alternatively, because of the Oγ of Thr\(^{220}\) makes van der Waals contact with Asn\(^{155}\), the role of Thr\(^{220}\) could only be to support the side chain orientation of Asn\(^{155}\) or to polarize its amide side chain.

Double mutants were constructed to distinguish these possibilities. If the role of Thr\(^{220}\) is to support the function of Asn\(^{155}\), then once the N62 of Asn\(^{155}\) is altered, mutations at Thr\(^{220}\) should no longer have an effect upon \(\Delta G^\ddagger\) (Carter et al., 1984; for review see Wells, 1990). On the other hand, if both residues function independently then the \(\Delta G^\ddagger\) value for the double mutant at positions 155 and 220 should be equal to the sum of the \(\Delta G^\ddagger\) values for the two single mutants. When two alanine substitutions are combined at positions 155 and 220 the effect upon the \(\Delta G^\ddagger\) for the double mutant (N155A, T220A) is the sum of the two single mutants (Table II). Assuming the absence of compensating effects, these mutations are functionally independent, which suggests that the γ-hydroxyl of Thr\(^{220}\) stabilizes the oxyanion transition state separately from the amide of Asn\(^{155}\).

It is important to point out that the above additivity analysis was performed using small alanine substitutions to minimize structurally disruptive or alternate H-bonding effects that could complicate the interpretation of the results. For example, when N155S is substituted for N155A (Table III), the reduction in \(\Delta G^\ddagger\) for the double mutant (N155S/T220A) is about 1 kcal/mol less than expected (5.2 kcal/mol actual; 6.1 kcal/mol expected). By contrast, when N155Q is substi-
Oxyanion stabilization by Thr$^{220}$

The developing oxyanion. Moreover, mutations of Thr$^{220}$ may be rationalized in one of two ways. Dynamic simulations over a short time period (<20 ps; Rao et al., 1987) indicate that Thr$^{220}$ can move to donate an H-bond to the oxyanion intermediate. In this case, the smaller contribution of Thr$^{220}$ as compared with Asn$^{155}$ (1.8 kcal/mol versus 4.2 kcal/mol) may represent strain or reorganization energy involved in placing the γ-hydroxyl of Thr$^{220}$ near the oxyanion.

A second possibility is that Thr$^{220}$ may stabilize the oxyanion by longer range dipolar effects (Warshel, 1987; Warshel et al., 1988; Hwang and Warshel, 1988). Charge-dipole potentials fall off as 1/r$^2$ (for review see Adamson, 1979) and may provide significant electrostatic stabilization (Hwang and Warshel, 1988; Warshel, 1987). For this effect, the contribution of Thr$^{220}$ to transition state stabilization can be estimated in kcal/mol using equation 1

$$\Delta G = 332 \frac{Q}{\epsilon r^4}$$

(1)

where Q is the charge on the oxyanion (−1.0; Warshel and Russell, 1986), $\mu$ is the effective charge of a hydroxyl dipole (0.427; Warshel and Russell, 1986), r is the separation distance in Å (4.0 Å; Matthews et al., 1975), and $\epsilon$ is the effective dielectric constant (−4.5). For Thr$^{220}$, $\Delta G$ is about −2.0 kcal/mol. The same calculation for Asn$^{155}$, with a separation of 2.7 Å between the Nδ2 and the oxyanion, gives $\Delta G$ as about −4.5 kcal/mol. Thus, the smaller effect upon mutating Thr$^{220}$ compared with Asn$^{155}$ may only reflect its being further away from the developing oxyanion. Moreover, mutations of Thr$^{220}$ or Asn$^{155}$ affect $K_w$ and not $K_{eq}$. Electrostatic effects can account for this preferential stabilization of the oxyanion (in the E$^-$/S$^-$) as compared with the carbonyl (in E$^+$) because a charge-dipole interaction is stronger and more long range compared with orientated dipole-dipole interaction (which decays as 1/r$^6$).

A concern in analyzing mutant enzymes with such low catalytic activity is that artifacts could occur from a consistent low level contamination by other proteases or mistranslation leading to a wild-type enzyme contamination. However, the $K_w$ values for most of the mutants analyzed usually differ significantly (albeit slightly) from wild-type enzyme and the other mutants. In addition, the multiple mutants differ substantially from each other, the single mutants, and the wild type. Each of the mutants contains an additional S24C mutation that allows affinity purification of the mutant enzyme by thiol-Sepharose chromatography (Carter and Wells, 1987). This purification procedure has been shown to reduce contamination by cysteine-free wild-type enzyme below detectable limits (<10$^{-7}$) (Carter and Wells, 1988). All subtilisin mutants were purified to homogeneity as judged by Coomassie-stained