Biochemistry

© Copyright 2001 by the American Chemical Society Volume 40, Number 35 September 4, 2001

Accelerated Publications

A Novel Engineered Subtilisin BPN′ Lacking a Low-Barrier Hydrogen Bond in the Catalytic Triad†

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Received May 7, 2001; Revised Manuscript Received July 9, 2001

ABSTRACT: The low-barrier hydrogen bond (LBHB) between the Asp and His residues of the catalytic triad in a serine protease was perturbed via the D32C mutation in subtilisin BPN′ (*Bacillus* protease N′). This mutant enzyme catalyzes the hydrolysis of *N*-Suc-Ala-Ala-Pro-Phe-SBzl with a k_{cat}/K_m value that is only 8-fold reduced from that of the wild-type (WT) enzyme. The value of k_{cat}/K_m for the corresponding *p*-nitroanilide (*p*NA) substrate is only 50-fold lower than that of the WT enzyme ($\Delta\Delta G^{\ddagger} = 2.2$ kcal/mol). The pK_a controlling the ascending limb of the pH versus k_{cat}/K_m profile is lowered from 7.01 (WT) to 6.53 (D32C), implying that any hydrogen bond replacing that between Asp32 and His64 of the WT enzyme most likely involves the neutral thiol rather than the thiolate form of Cys32. It is shown by viscosity variation that the reaction of WT subtilisin with *N*-Suc-Ala-Ala-Pro-Phe-SBzl is 50% (sucrose) to 100% (glycerol) diffusion-controlled, while that of the D32C construct is 29% (sucrose) to 76% (glycerol) diffusion-controlled. The low-field NMR resonance of 18 ppm that has been assigned to a proton shared by Asp32 and His64, and is considered diagnostic of a LBHB in the WT enzyme, is not present in D32C subtilisin. Thus, the LBHB is not an inherent requirement for substantial rate enhancement for subtilisin.

The extraordinary rate accelerations associated with enzymes (up to 10^{17}-fold; *1*) have generated a very large body of theoretical and experimental work attempting to combine the known factored catalytic commodities of physical organic chemistry to account for the totality of enzymatic catalysis. In addition to contributions such as nucleophilic and general acid−base catalysis, which are well understood from model reactions, several factors that are more specific to enzymes have been discussed in recent years. These include entropy traps (e.g., the Circe effect) (*2*) and preorganization of active sites (*3*, *4*). Hydrogen tunneling, while not unique to enzymes, appears to be disproportionately applicable to enzyme catalysis (*5*, *6*).

The possible importance of strong low-barrier hydrogen bonds, which may contribute as much as 5−10 kcal/mol in rate acceleration in the preferential stabilization of enzymatic transition versus ground states, has drawn considerable attention over the past several years (*7*−*16*). This special type of hydrogen bond has large gas-phase free energies of formation. Hibbert and Emsley (*17*) quote a range of experimental and theoretical values for the hydrogen bond in HF_2^- between −58 and −30 kcal/mol. LBHBs[1] are characterized by short internuclear distances ($r_{D-A} \leq 2.6$ Å), large downfield NMR chemical shifts exhibited by the shared proton (17−22 ppm), and unusually low fractionation factors

† This work was supported by NIH Grant GM35393 to J.F.K.

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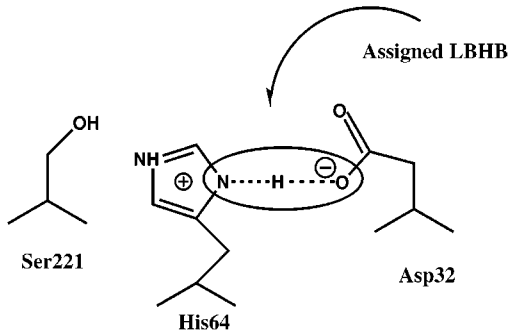
10.1021/bi015542n CCC: $20.00 © 2001 American Chemical Society
Published on Web 08/08/2001

Scheme 1



discriminating against deuterium in the LBHB (as low as 0.3 for *p*-nitrophenol and *p*-nitrophenolate ion) (*18*). One or more of these diagnostic indicators have been observed in a number of enzymes, e.g., the serine proteases (*8*), triosephosphate isomerase (*19*, *34*), ketosteroid isomerase (*15*), citrate synthase (*20*), and various pyridoxal phosphate-dependent enzymes (*21*). While the experimental observations are not disputed, the quantitative importance of the LBHB in catalysis is controversial. Alternative interpretations have been advanced for most of these enzymes; however, a very strong case remains that the LBHB contributes significantly in the catalytic mechanism of the serine proteases, where the LBHB is between the Asp and His components of the catalytic triad (Scheme 1).

We report here the construction and kinetic characterization of D32C subtilisin. The typical pK_a of Cys-SH in proteins is ~9.0−9.5, while that of the β-carboxylate of Asp is 3.9−4.0 (*22*). Thus, the pK_a values if matched in WT subtilisin would not be in D32C (see the Discussion). This mutant subtilisin is shown by NMR not to possess a LBHB yet retains substantial catalytic power.

EXPERIMENTAL PROCEDURES

Substrates. *N*-Succinyl-Ala-Ala-Pro-Phe (*N*-Suc-A-A-P-F) with *p*NA and SBzl leaving groups was purchased from Sigma (St. Louis, MO) and Bachem (Torrance, CA), respectively.

Mutagenesis, Expression, Purification, and Refolding of Subtilisins. WT subtilisin BPN′ was expressed and purified according to the method of Estell et al. (*23*). D32C subtilisin was prepared by standard recombinant PCR using the mutagenic oligonucleotides: 5′-CGGTTATCTGCAGCGG-TATC-3′ and 5′-GATACCGCTGCAGATAACCG-3′. This mutant construct contains the 23-amino acid histidine tag and enterokinase cleavage site that is present in the Novagen vector pET19b. This sequence was fused in frame to the N-terminus of the WT pro region of subtilisin (the *Bacillus subtilis* secretion signal was deleted, and the pro region started at Ser34). The presence of the mutation was verified by DNA sequencing of the entire gene. D32C subtilisin was expressed in *Escherichia coli* strain BL21(DE3)pLysS (Novagen, Madison, WI) with a 4 h induction after addition of 0.5 mM isopropyl α-thiogalactopyranoside.

Cell pellets were collected and lysed. The inclusion bodies containing D32C subtilisin were solubilized (2 h at 25 °C, with stirring) with 6 M guanidine hydrochloride, 0.1 M sodium phosphate (pH 8.0), and 0.01 M Tris base. Cell debris was removed by centrifugation at 10000*g*, and the resulting supernatant was batch processed with pre-equilibrated Ni−NTA resin overnight (Qiagen, Valencia, CA), and then washed with the same buffer. Two subsequent washes were carried out at pH 8.0 and 7.0 with solutions containing 8 M urea, 0.1 M sodium phosphate, and 0.01 M Tris base. The bulk of the mutant subtilisin was eluted in the same buffer adjusted to pH 4.5.

The denatured mutant subtilisins were refolded as follows. The purified fractions from the Ni−NTA column were combined, and diluted to 0.5 mg/mL with 4 M urea, 0.5 M ammonium sulfate, 1 mM $CaCl_2$, 10 mM Tris base (pH 7.0), and 5 mM β-mercaptoethanol. The solution was placed in a prepared dialysis bag (6000−8000 molecular weight cutoff), and dialyzed against the above buffer (1:40, v/v), and four subsequent buffers that varied only in the amount of urea (from 4 to 0 M urea) each for 2 h. The final dialysate was kept at 4 °C for 6 days to allow autocleavage of the pro region.

Viscosity Variation. The values of k_{cat} and k_{cat}/K_m were determined as a function of viscosity by the method of Brouwer and Kirsch (*24*). The reactions were carried out in 0.1 M Tris buffer (pH 8.6) and 4% DMSO at 25 °C, with glycerol and sucrose as viscosogenic agents each at 14, 24, and 32% (w/w). Substrate concentrations were varied between 0.05 and 6 mM. The rates of hydrolysis were measured over a range of substrate concentrations that varied: 0.2−15K_m (*N*-Suc-A-A-P-F-SBzl with WT), 0.3−3K_m (*N*-Suc-A-A-P-F-SBzl with D32C), 0.2−23K_m (*N*-Suc-A-A-P-F-*p*NA with WT), and 0.02−2K_m (*N*-Suc-A-A-P-F-*p*NA with D32C) with viscosity (η_{rel}) varied from 1 to 3.4. The weighted data were fit to eq 1 for the SBzl derivatives, while the *p*NA data were fit to a straight line (see Table 2 for definitions).

$$\left(\frac{k_{cat}}{K_m}\right)_{obs} = \frac{k_1^0\eta^0/\eta}{1 + \frac{k_{-1}^0\eta^0}{k_2\eta}} \quad (1)$$

NMR Spectroscopy. WT and D32C subtilisins were prepared for NMR measurements by extensive dialysis against 10 mM ammonium bicarbonate buffer (pH 5.0) followed by lyophilization. The NMR samples contained 200 μL of 2.9 mM protein solutions dissolved in 10 mM ammonium bicarbonate (pH 5.0).

One-dimensional ^{1}H NMR spectra of WT and D32C subtilisins were recorded with an 11-echo sequence (*25*) on a Bruker AMX spectrometer operating at 600 MHz and 11 °C. A total of 16 384 scans were signal averaged using 8192 points, a spectral width of 26 315 Hz, and a recycle delay of 0.3 s for each spectrum. The excitation maximum was set to 18 ppm. All other experimental parameters were identical in both experiments. Prior to Fourier transformation, 10 Hz of exponential line broadening was applied to the data, followed by zero filling to 16 384 points using the program FELIX97 (Biosym Technologies, San Diego, CA).

[1] Abbreviations: LBHB, low-barrier hydrogen bond; *N*-Suc-A-A-P-F-SBzl, *N*-succinyl-Ala-Ala-Pro-Phe-thiobenzyl ester; *p*NA, *p*-nitroanilide; NMR, nuclear magnetic resonance; WT, wild type; TFK, trifluoromethyl ketone.

Table 1: Kinetic Parameters for the Reactions of WT and D32C Subtilisins with the SBzl and *p*NA Derivatives of *N*-Suc-A-A-P-F (Standard Errors in Parentheses)

	WT			D32C			ratio (D32C/WT)		
	k_{cat}[a] (s^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} s^{-1})	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} s^{-1})	k_{cat}	K_m	k_{cat}/K_m
N-Suc-A-A-P-F-$SCH_2\Phi$	1400 (21)	215 (10)	6.5 (0.3) $\times$ 10^6	1200 (30)	1600 (80)	0.75 (0.03) $\times$ 10^6	0.85 (0.02)	7.4 (0.52)	0.12 (0.01)
N-Suc-A-A-P-F-*p*NA	33 (1)	230 (30)	1.4 (0.1) $\times$ 10^5	5.9 (0.2)	1960 (130)	3.0 (0.1) $\times$ 10^3	0.18 (0.01)	8.4 (1.1)	0.021 (0.002)

[a] The kinetics were followed in 0.1 M Tris buffer (pH 8.6) (in 4% DMSO) at 25 °C. The substrate concentrations were varied from 0.01 to 4 mM and from 0.01 to 5 mM for the WT and D32C subtilisins, respectively.

Table 2: Values of the Association Rate Constant at a Relative Viscosity (η/η_0) of 1 and Partition Ratio for the Reactions of WT and D32C Subtilisin with *N*-Suc-A-A-P-F-SBzl (Standard Errors in Parentheses)

	glycerol		sucrose	
	k_1^0 (M^{-1} s^{-1} $\times 10^{-6}$)	k_{-1}^0/k_2	k_1^0 (M^{-1} s^{-1} $\times 10^{-6}$)	k_{-1}^0/k_2
D32C subtilisin	1.2 (0.2)	0.3 (0.4)	2.8 (0.9)	2.4 (1.4)
WT subtilisin	4.0 (1.4)	−0.1 (0.4)	8.2 (1.7)	0.9 (0.5)

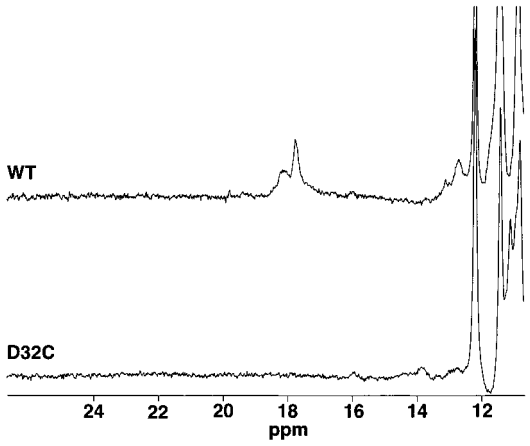


FIGURE 1: Downfield region of ^{1}H NMR spectra of WT (top) and D32C subtilisins (bottom) recorded under identical conditions and with the same experimental parameters (pH 5.0). The two peaks near 18 ppm arise from the Hδ1 proton of His64 (*26*). The multiplet pattern was observed previously and was attributed to conformational heterogeneity associated with the His64 ring.

RESULTS

Both proteins have the expected molar quantities of cysteine from quantitative amino acid analysis (WT, 0.0 observed, 0 theoretical; D32C, 0.8 observed, 1 theoretical). This result was confirmed by 5,5′-dithiobis(2-nitrobenzoic acid) analysis, which gave 0.8 equiv of SH in D32C and 0 equiv SH in the WT control. The enzyme was stored in the presence of 1 mM β-mercaptoethanol to prevent oxidation of Cys32. The fraction of SH did not change over several months of storage at 4 °C.

The characteristic downfield chemical shift of the LBHB proton (His64 Hδ1; *26*) near 18 ppm in the NMR spectrum of WT subtilisin is shown in Figure 1. This peak is missing in the D32C mutant; therefore, by this criterion, the LBHB is absent in D32C subtilisin.

The kinetic parameters characterizing the reactions of WT and D32C subtilisins with both the SBzl and *p*NA derivatives of *N*-Suc-A-A-P-F are collected in Table 1. The D32C mutation results in only modest changes in the thiol ester hydrolysis kinetics (15% reduction in k_{cat}, 8-fold decrease in k_{cat}/K_m). Larger effects are observed for the *p*NA substrate (6-fold in k_{cat} and 50-fold in k_{cat}/K_m).

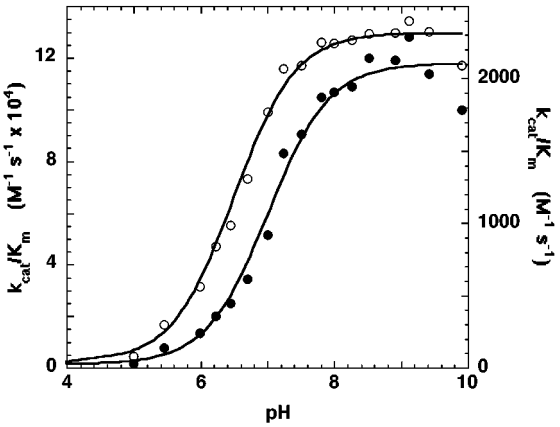


FIGURE 2: pH dependence of k_{cat}/K_m for WT (●, left ordinate) and D32C (○, right ordinate) subtilisin BPN′. The pH dependencies of the reaction of the subtilisins were measured in 100 mM acetate-MES-Tris buffer and 3% DMSO at 25 °C with the *p*NA substrate. Data were fit by nonlinear regression to the equation $k_{cat}/K_m = [(k_{cat}/K_m)_{lim}/(1 + 10^{pK_a - pH})]$ with the Kaleidagraph application (Synergy Software, Reading, PA).

The k_{cat}/K_m versus pH profiles for the reactions of WT and D32C subtilisins with *N*-Suc-A-A-P-F-*p*NA are shown in Figure 2. The apparent pK_a values obtained from the equation in the Figure 2 legend are 7.01 ± 0.07 (WT) and 6.53 ± 0.04 (D32C). The WT value can be compared with the literature value (7.17 ± 0.02) (*27*). The D32C mutation lowers the pK_a by 0.5 unit. The values of K_m are identical within error at pH 6.5 and 8.6 for both enzymes (data not shown). The K_m values for the reactions of subtilisin with the ester substrate *p*-nitrophenylbutyrate are invariant from pH 5 to 10 (*28*).

The normalized values for k_{cat}/K_m are plotted versus relative viscosity (η_{rel}) in Figure 3. The value of k_{cat}/K_m for the reaction of WT subtilisin with the SBzl ester of 6.5 $\times$ 10^6 M^{-1} s^{-1} is consistent with that expected for a diffusion-controlled reaction (*24*), while the k_{cat}/K_m values for the *p*-nitroanilide are much lower. The values of k_{cat} for both mutant and WT subtilisins are relatively insensitive to viscosity (data not shown). The variation was only 15%, while the k_{cat}/K_m values exhibit the dependencies on viscosity shown in Figure 3. The values of k_{cat}/K_m for the *p*NA substrate are insensitive to η_{rel} as indicated by the nearly horizontal lines.

DISCUSSION

The LBHB as a Factor Contributing to Enzyme Catalysis. The formation of a LBHB in the active site of an enzyme presumably requires special circumstances. The strong hydrogen bonding properties of water compete with the

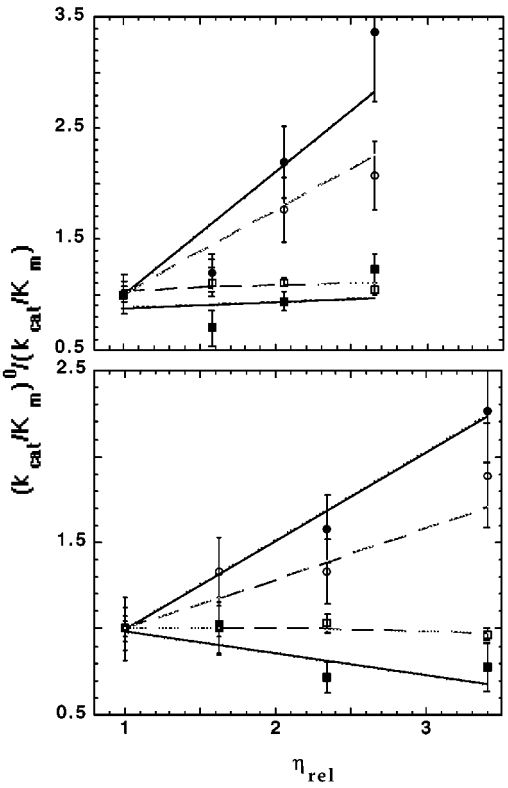


FIGURE 3: $(k_{cat}/K_m)^0/(k_{cat}/K_m)$ vs η_{rel} in glycerol (top) or sucrose (bottom) for WT (● and ■ with solid lines) and D32C (○ and □ with dashed lines) subtilisins. Both peptide substrates are represented: SBzl (● and ○) and *p*NA (■ and □). Note that data points overlap when $\eta_{rel} = 1.6$ in both plots.

formation of LBHBs where such a bond might be exposed to solvent. The intramolecular hydrogen bonds in the maleic and mesaconic monoanions have ΔG's of formation equal to −4.4 kcal/mol in DMSO, but only −0.5 kcal/mol in water (*29*). Therefore, an enzyme active site containing a LBHB should be relatively solvent inaccessible or contain only ordered water molecules. However, Zhao et al. (*15*) and Lin and Frey (*30*) report evidence for intramolecular LBHBs in aqueous solution. Hydrogen bond strength increases inversely with the dielectric constant when $\epsilon < 20$ (*32*); therefore, the contribution to catalysis might be substantially enhanced were the active site to have a low-dielectric microenvironment. It has further been argued on theoretical grounds that a LBHB is less stable than a normal hydrogen bond in water (*9*). Another important tenet of LBHB theory is that the pK_a's of the hydrogen bond donor and conjugate acid of the acceptor should be nearly matched for the proton to be shared equally between the two heteroatoms (*7*). The free energy of hydrogen bond formation is found to be linearly correlated with ΔpK_a with the largest value occurring at Δp$K_a = 0$. However, there is no positive discontinuity near this point either experimentally observed (*31*) or theoretically calculated (*32*).

For the LBHB to enhance enzymatic rate constants significantly, it should not exist in the ground state, but be formed only in the transition state to reduce the free energy differential between the two states. An important enzymatic example was thought to be provided in triosephosphate isomerase where the hydrogen bond to the developing enediolate anion is donated by the free imidazole of His95 (pK_a estimated to be 13−14) rather than the more strongly acidic imidazolium form (p$K_a \cong 6$) (*7*, *19*, *33*). Subsequent NMR investigations found, however, that the His95 hydrogen bond to phosphoglycohydroxamate, an analogue of the enediol, has a normal NMR shift (13.5 ppm). Harris et al. (*34*) suggest an alternate LBHB between Glu165 and the hydroxyl moiety of the hydroxamate. A reviewer points out that the pK_a of the enediol might match that of the neutral histidine while that of the hydroxamate is much lower.

A highly deshielded proton (18.15 ppm) is observed in the NMR spectrum of the Δ^5-3-ketoisomerase (*Pseudomonas testosteroni*) with the transition state analogue dihydroequilenin (*35*, *36*). The assignment and role of this hydrogen bond in catalysis are controversial. One NMR study places the shared proton between Tyr14 and Asp99 in a catalytic dyad where the hydroxyl group of Tyr14 also hydrogen bonds to the 3-OH of dihydroequilenin (*36*). In contrast, the crystallographic data are interpreted in terms of both Asp99 and Tyr14 participating directly in a double hydrogen bond to the 3-OH (*35*), but a LBHB is assigned to the contact between the 3-O of the inhibitor and the Tyr14 hydroxyl in the D38N complex (*53*).

Short internuclear distances are characteristic of LBHBs; however, short hydrogen bonds are not necessarily indicative of large free energies of formation. A reported 2.432 Å hydrogen bond is found in a 0.98−1.05 Å resolution structure of ligand-bound phosphate binding protein between a phosphate oxygen atom and Oδ2 of Asp56 (*37*). However, the K_d value is increased <2-fold in the D56N mutant. A reviewer suggested that the mutation might increase the pK_a of the bound phosphate ion with an enhancement of affinity. Citrate synthase provides an additional example; although the distance between Asp375 Oδ1 and a transition state analogue oxygen atom is not so precisely defined in this structure, the crystallographic (*20*) and NMR data (*38*) place r_{O-O} at <2.5 Å and the chemical shift at 22 ± 5 ppm, respectively. Replacement of the carboxylate moiety of the analogue with a carboxamide reduces the affinity by a factor of only 20 ($\Delta\Delta G = 1.9$ kcal/mol) (*20*). In contrast, k_{cat}/K_m is reduced by $\sim 10^4$ in the D375N mutant (*39*). The effect of the mutation on k_{cat}/K_m is a true measure of the free energy differences between the ground and transition states, while the effect on the dissociation energy of a transition state analogue is only an approximation of indeterminate quality. Thus, it remains possible that a LBHB may contribute significantly to the kinetics of citrate synthase.

The highly deshielded protons, which have been observed in the NMR spectra of a variety of pyridoxal phosphate-dependent enzymes, bridge the pyridinium nitrogen atom of the cofactor with an enzyme carboxylate ion (*21*, *40*). The latter was converted to cysteine sulfinate by a combination of site-directed mutagenesis and chemical modification in aspartate aminotransferase (*41*). This procedure reduces the pK_a of the enzyme site by ~2.5 pK_a units, and moves the low-field peak (16.52 ppm) in the NMR spectrum to 14.69 ppm (*41*), but has no effect on k_{cat}/K_m. Thus, the LBHB may exist for this class of enzymes, but if so, it contributes negligibly to catalysis.

LBHB in Subtilisin. The evidence for the existence of the LBHB in subtilisin includes the low-field shift of the proton shared by an imidazole nitrogen and a carboxylate oxygen atom of the catalytic triad in both the free enzyme and trifluoromethyl ketone (TFK) complexes (18 ppm ≤ δ ≤

19 ppm) (*13*, *42*), the deuterium fractionation factors (ϕ) of 0.32−0.68 (*13*, *43*), and the short N−O distance [r_{N-O} = 2.6 Å (*44*); r_{N-O} = 2.5 Å (*45*)]. Although the above observations satisfy the characteristic diagnostics of the LBHB, they do not address its importance in catalysis. The attribution of a significant LBHB contribution to differential transition state stabilization rests largely on two linear free energy relationships: (1) the correlation between log K_i and $\Delta H^{\ddagger}$ for exchange of the proton in question in TFK complexes (*43*) and (2) the correlation for the pK_a of His57 with log k_{cat}/K_m or log K_i for *N*-acetylpeptide methyl esters or the corresponding TFKs, respectively (*46*).

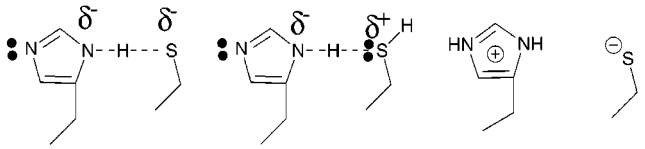
There is strong structural evidence that the putative LBHB proton is not shared equally between the Nδ1 and Oδ2. A 0.78 Å high-resolution structure of *Bacillus lentus* subtilisin shows a short, but not unusually so, r_{N-O} of 2.6 Å; however, at pH 5.9, r_{N-H} = 1.2 Å and r_{O-H} = 1.5 Å (*44*). The state of protonation of the His Nϵ2 is not known. It is expected to be protonated at this pH, which is ~1 unit lower than the pK_a controlling enzyme activity, but that proton is not visible in the structure. These observations are consistent with the $^1J_{NH}$ spin-coupling constants of α-lytic protease, which show the proton as 100 and 85% associated with the His nitrogen at neutral and acidic pH, respectively (*12*). Similar results were obtained for subtilisin BPN′ (*12*) and *B. lentus* subtilisin TFK complexes (*13*).

Mutations of the catalytic triad residues, S221A and H64A, reduce the activity by ~10^6-fold [with the substrate *N*-Suc-F-A-A-F-*p*NA (*47*)]. Given that previous mutations of D32 [D32N (*48*) and D32A (*47*)] resulted in enzymes that exhibited activities reported as inactivated and $<2 \times 10^{-4}$ of that of WT (k_{cat}/K_m), respectively, it was surprising that the D32C construct loses at most a factor of 50 in catalytic activity (Table 1). To account for this large difference, two alternative explanations can be proposed. (1) The alanine and/or asparagine substitutions seriously perturb the geometry and/or function of the catalytic triad, while cysteine does not. More likely, (2) the thiol group of cysteine substitutes functionally for aspartate in a way that is precluded for alanine and asparagine. One possibility is that a weak hydrogen bond to the thiol serves to orient the Nϵ2 atom of His64 to accept a proton from the β-hydroxyl group of Ser221. The maximum additional contribution of the LBHB in the WT enzyme over the possible hydrogen bond present between Cys32 and His64 in the D32C mutant is $-RT$ log 50 (=2.2 kcal/mol) in k_{cat}/K_m for the *p*NA substrate. There are negligible differences in the catalytic parameters for the SBzl ester substrate. The 2% activity recorded for the D32C mutant with the *p*NA substrate is not a result of WT contamination, because (1) the k_{cat} values for the SBzl ester are very close and the K_m values for both substrates differ (Table 1) and (2) the k_{cat}/K_m versus pH profiles are 0.5 pH unit apart (Figure 2).

Interestingly, Corey et al. (*50*) showed that a substantial fraction of the inactive D102S mutant of trypsin could be rescued by a S214D mutation. The newly introduced carboxylate does hydrogen bond with His57, but the geometry of the hydrogen bonding pattern is substantially altered from that of the WT enzyme. No NMR investigation of the D102S/S214D construct has been reported.

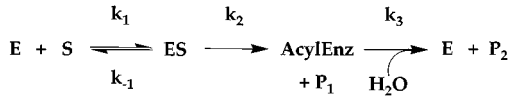
Kinetic Mechanism of D32C Subtilisin. The value of k_{cat}/K_m for WT subtilisin exhibits a pK_a near 7.0 that is assigned to His64 (*51*). The results shown in Figure 2 for the reaction of WT subtilisin with *N*-Suc-A-A-P-F-*p*NA yield a pK_a of 7.01 ± 0.07, but that for the D32C enzyme is 6.53 ± 0.04. This pK_a change is the opposite of that expected were the more basic thiolate ion substituted for a carboxylate oxygen (Scheme 2A). It is therefore likely that either no hydrogen bond exists between the cysteine and histidine or a weak one is formed with a nonbonded electron pair of the neutral thiol (Scheme 2B).[2] A configuration like that of the catalytic dyad of papain (Scheme 2C) would require that the thiolate of Cys32 function as the nucleophile, which would entail a complete change of both mechanism and active site topology. Such a large perturbation is inconsistent with the relatively small changes in K_m observed between the WT and D32C enzymes (Table 1).

Scheme 2[a]



[a] Possible interactions of the introduced thiol group of Cys32 with His64. (A) The thiolate hydrogen bonds to the neutral imidazole. The thiolate ion is a stronger base than the β-carboxylate of aspartate; therefore, the pK_a value controlling k_{cat}/K_m for the conjugate acid of this species should be higher than that of the WT enzyme. (B) The nonbonded electron pair of the neutral thiol accepts a hydrogen bond from the neutral imidazole. This hydrogen bond is expected to be very weak because of the low basicity of the thiol, but could serve to orient the imidazole correctly to abstract the proton from the nucleophilic serine. The pK_a of the conjugate acid would be lower than that of the WT enzyme, which is observed. (C) A thiolate imidazolium ion pair is the catalytically active form of the papain type of cysteine protease (*49*).

Scheme 3



Diffusion-Control Considerations. The rate-determining step for the hydrolysis of ester and thiol ester substrates is the reaction of water with the acyl enzyme, while acylation is normally rate-determining for anilides (Scheme 3) (*51*).

The viscosity variation results in Table 2 and Figure 3 support the mass of extant data showing that formation of the acyl enzyme is rate-determining for this class of substrates (*51*). The value of k_{cat} for the SBzl ester is insensitive to η_{rel} as expected, because this kinetic parameter reflects the rate of hydrolysis of the acyl enzyme, a chemical rather than a diffusive step. The values of k_{cat}/K_m, however, are viscosity-dependent (Table 2 and Figure 3). The magnitudes of k_{-1}°/k_2 show that the reaction rates are approximately 100 and 50% diffusion-controlled (eq 2), measured in glycerol and sucrose-containing buffers, respectively (*24*).

$$\% \text{ diffusion control} = 100 \times \frac{k_2}{k_{-1}^0 + k_2} \qquad (2)$$

[2] One of the reviewers of the manuscript raised the possibility that the observed pK is that of the introduced thiol, while a second argued that Cys32 is ionized throughout the investigated pH range. We disagree with these alternate interpretations for the reasons stated in the text.

The different values reflect specific effects of the individual viscosogens (*52*). The D32C mutation results in an only 8-fold reduction in k_{cat}/K_m for the reaction of the SBzl ester with the mutant subtilisin. This reaction rate is also partially diffusion controlled, 76% in glycerol and 29% in sucrose-containing buffers (Table 2).

Conclusions. The experiments described herein show that the attributed LBHB of the serine protease family can be replaced with what is at best a normal strength hydrogen bond between the active site histidine and what is most likely a neutral cysteine side chain. The defining LBHB NMR signal is absent in the mutant, which is at worst 2% as active as the WT enzyme. This value, corresponding to a 2.2 kcal/mol reduction of activity, is far short of the 3000-fold rate enhancement calculated from a LBHB with a ΔG of formation of −5 kcal/mol.

ACKNOWLEDGMENT

We thank Dr. James A. Wells for the generous gift of the *Bacillus* strain and the plasmid containing the wild-type subtilisin and Drs. Dan Herschlag and Judith Klinman for helpful comments on the manuscript.

NOTE ADDED AFTER ASAP POSTING

This article was inadvertently released ASAP on 8/08/01 before final corrections were made. Footnote 2 was omitted. The correct version was posted 08/13/01.

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BI015542N