Direct proton magnetic resonance determination of the pK_a of the active center histidine in thiolsubtilisin

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Abstract

The serine proteases constitute a group of endopeptidases whose members owe their catalytic activity to the presence of a catalytic triad of amino acids consisting of a serine, a histidine and an aspartate. The pK_a values for this histidine have been determined for several cases in which there is a negative charge installed at the serine to mimic the oxyanionic intermediate and related transition state for the catalytic pathway. Instances from this laboratory include (1) replacement of the serine by a cysteine in subtilisin to create a thiolate; (2) formation of monoisopropylphosphoryl-Ser 195 monoanionic phosphodiester (in trypsin and chymotrypsin, Ser 221 in subtilisins); and (3) tetrahedral boronates formed with peptide boronic acids. The nuclear magnetic resonance (NMR) signals pertinent to this histidine, or signals indirectly reflecting the state of ionization of this histidine, have been used effectively to monitor changes in the active center ionization state. In every case studied, there is elevation of the pK_a at the histidine when the negative charge is installed at the serine position. Herein is reported the first NMR measurement of the active center His 63 pK_a in thiolsubtilisin Carlsberg; it is elevated by 3 units compared with the parent enzyme. Using a numerical solution (finite difference) of the Poisson-Boltzmann equation, a protein dielectric constant of 4 provides a good estimate of the experimentally observed pK_a elevations. Very significantly, a very low protein dielectric constant ($\epsilon_p = 3-5$) is required in all of the comparisons, and for all three enzymes used (chymotrypsin, trypsin, and subtilisin). Finally, we discuss why the electrostatic perturbation sensed at His of the active center is more amplified by a negative charge on the Ser side than the same charge on the Asp side. A plausible explanation is that the positive charge on the imidazolium ring of the His is localized, with the N^{δ1} carrying a smaller fraction, the N^{ε2} carrying the bulk of the positive charge.

Keywords: Thiolsubtilisin; protein dielectric constant; serine protease; catalytic triad

Of the four principal classes of proteases, there is perhaps more known about the structure and function of the serine

protease class than about any other class. Serine proteases exist in both mammalian and bacterial cells and have diverse functions, such as blood clotting, development, fibrinolysis, fertilization, zymogen activation, digestion, and tissue destruction.

Supported by the results of several decades of chemical model studies, it is generally accepted that the hydrolysis of peptides/proteins by serine proteases proceeds via two oxyanionic tetrahedral intermediates: the first one results from nucleophilic addition of the serine to the carbonyl carbon of the substrate and is converted to a Ser-acylated covalent intermediate with the release of the first product with the newly generated N terminus; the second one results from nucleophilic addition of water to the carbonyl carbon of the

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Abbreviations: DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; KPi, inorganic phosphate, potassium salt; MIP, monoisopropylphosphoryl-Ser 195 (trypsin and chymotrypsin) or monoisopropylphosphoryl-Ser 221 (subtilisins) derivative of the respective serine protease; NMR, nuclear magnetic resonance; *p*-NPA, *p*-nitrophenylacetate; *s*-AAPF-*p*NA, succinyl-(L)Ala-(L)Ala-(L)Pro-(L)Phe-*p*-nitroanilide.

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acyl enzyme and leads to regeneration of free serine and the second product with the new C terminus. The pioneering studies of Blow (for a historical account, see Blow 1997) identified the components of the catalytic triad in mammalian serine proteases. Kraut and coworkers identified a similar triad in the bacterial serine protease subtilisin (Alden et al. 1971) and also suggested the existence of an oxyanion hole whose function is to stabilize the rate-limiting transition state, resembling the tetrahedral intermediate both in structure and charge distribution.

For 2 decades, in a series of reports from this laboratory, several nuclear magnetic resonance (NMR) methods have been used to identify the charge distribution at the active center of a variety of serine proteases both in the absence and in the presence of inhibitors that form a covalent bond at the active center (Jordan and Polgár 1981; Jordan et al. 1985; Adebodun and Jordan 1989; Zhong et al. 1991, 1995; Bao et al. 1998, 1999). A significant database at our disposal has allowed us to conclude that in the tetrahedral oxyanionic intermediates, and in the preceding transition states, there is a significant perturbation of the pK_a of the active center histidine, not apparent from steady-state or pre-steady-state kinetic studies. In this article, we use proton NMR to determine the pK_a of the active center histidine in the chemically substituted Ser 221 Cys variant of subtilisin Carlsberg, adding to this database. Correlations between calculated and measured pK_a shifts of the active center histidines of three of the most famous serine proteases, chymotrypsin, trypsin, and subtilisin concomitant with the installation of a negative charge at the active center serine also are reported. These estimates were obtained from a numerical solution (finite difference) of the Poisson-Boltzmann equation and asking the theory to provide the magnitude of the protein dielectric constant that would be consistent with the experimentally observed pK_a perturbations found in this laboratory. The theoretical results, although admittedly crude, uniformly point to the use of a low protein dielectric constant with this very important class of enzymes.

Results and Discussion

Experimental determination of the active center His 63 pK_a of thiolsubtilisin Carlsberg

Subtilisins have neither cysteines nor cystines in their structures; therefore, their active site Ser 221 can be converted to a cysteine chemically (Polgár and Bender 1966). The chemical substitution avoids the difficulties encountered because of folding problems when Cys 221 thiolsubtilisin was created by genetic means (Li and Inouye 1994). This single substitution does not alter subtilisin's active site conformation, but its catalytic power is greatly diminished (Polgár and Bender 1966). The novel active site triad has a different charge distribution than the wild-type subtilisin, because the

cysteine residue forms a mercaptide-imidazolium ion pair with the catalytically active histidine. Therefore, the charge distribution at the active site of thiolsubtilisin is similar to that formed during catalysis by the serine enzyme that proceeds via a negatively charged tetrahedral adduct forming an ion pair with the imidazolium ion ($\text{Asp}^- - \text{His}^+ - \text{Oxyanion}^-$) (Jordan and Polgár 1981). The active center charge distribution in thiolsubtilisin ($\text{Asp}^- \text{His}^+ \text{Cys}^-$) is more similar to that of thiol proteases, such as papain, except that papain has an uncharged asparagine in place of the aspartate in the catalytic triad ($\text{Asn}^0 \text{His}^+ \text{Cys}^-$). However, thiolsubtilisin is a very ineffective protease; it can only catalyze the hydrolysis of activated ester substrates with good leaving groups that do not require general acid catalysis for their reactions to form acyl enzymes. This property has been used effectively, and thiolsubtilisin has been used as a catalyst for the synthesis of peptides by segment condensation (Nakatsuka et al. 1987). Both enzymes, the wild-type and the Cys-substituted one, have a similar level of activity toward nonspecific substrates and active esters (Phillip and Bender 1983). In earlier work, it was claimed (Phillip and Bender 1983) that the lack of activity of thiolsubtilisin toward specific, nonactivated substrates was not caused by an impairment in protonation of the leaving group; rather, it could be the indirect result of the larger sulfur atom compared with oxygen weakening the hydrogen bonds in the transition state. Also, boronic acid inhibitors (transition state analogs) were bound poorly to thiolsubtilisin compared with subtilisin, suggesting that thiolsubtilisin may not be able to stabilize the transition state during acylation (Phillip and Bender 1983).

Only a few attempts have been made so far to determine the X-ray structure of thiolsubtilisin. The X-ray structure of thiolsubtilisin displayed few changes compared with that of the wild-type enzyme, but the thiol group of Cys 221 may have been oxidized (Alden et al. 1970). A 1.5-Å X-ray crystal structure of an oxidized derivative of the double mutant (Ser 221 Cys/Pro 225 Ala), which turned out to be a better ligase than subtilisin or thiolsubtilisin, also was reported (Abrahmsen et al. 1991). The Pro 225 Ala substitution relieved the steric crowding caused by proline. Polgár and Halász (1973) showed that even though the tertiary structure was maintained during the chemical conversion of subtilisin to thiolsubtilisin, the reactivities of these two enzymes were considerably different. The X-ray crystal structure revealed that in subtilisin, the $\text{N}^{\delta 1}$ of His 64 faces Asp 32 with $\text{N}^{\epsilon 2}$ facing Ser 221. This spatial configuration is different in the case of papain in which $\text{N}^{\delta 1}$ of His 159 at the active center faces the Cys 25 nucleophile, that is, the active center His imidazolium ring in papain (and in other cysteine proteases) is flipped over compared with the same ring in serine proteases. There is only rare precedence for this imidazole conformation in serine proteases, for example, in the double mutant (Asp 102 Ser/Ser 214 Asp) of trypsin, in

which the imidazole of His 57 was rotated 180° (χ^2 from -102° to 91°) relative to the wild-type trypsin (Corey et al. 1992).

The ¹H NMR spectra of nitrogen bound protons of His 63 at the active center of thiolsubtilisin Carlsberg (in this enzyme, it is His 63, whereas in subtilisin BPN' or subtilisin E it is His 64) at 10°C between pH 6.5 and 10.5 are shown in Figure 1. (It must be emphasized that similar pH titrations of the carbon-bound proton resonances on the imidazole ring at the active site histidine appear to be more difficult than assumed for 2 decades; see Bao et al. 1998, 1999). The chemical shift of the resonance at 18 ppm is pH independent up to pH 10.0. This low field NMR signal was assigned to the proton of N^{δ1} of His 63, which is hydrogen bonded to the active site aspartate (Asp 32 in subtilisin numbering; Jordan and Polgár 1981). This resonance is believed by

Fig. 1. Proton NMR spectra of thiolsubtilisin at the indicated pH values. Experimental conditions were 10°C, 10 mM KPi, 70 mM KCl, 1 mM EDTA; pH 7.01 and 9.15 contained 0.4 mM S₂O₃²⁻. Other parameters are given in Materials and Methods.

some to be the result of a low-barrier hydrogen bond between the aspartate and the imidazolium ring (Frey et al. 1994). Our ability to observe this resonance provides additional proof for the conservation of the enzyme active site during modification (cf. results in Zhong et al. 1995; Bao et al. 1998, 1999). There is recent evidence that the chemical shift of the N^{ε2} proton is 13.2 ppm for chymotrypsin at low pH when His 57 is protonated (Bao et al. 1998, 1999). The absence of a signal in this region in thiolsubtilisin signifies very weak hydrogen bonding between N^{ε2} and S^γ, resulting in rapid exchange with the solvent. Once the pH was raised to above 10.0, the histidine is neutralized (loss of an imidazolium proton at N^{ε2}), because a new resonance appears at 14.1 ppm, assigned to N^{δ1}H of unprotonated histidine. The areas under the peaks at 18 and 14 ppm at pH 10.25 were nearly equal indicating an ~50% deprotonation of the imidazolium ion. On the basis of this behavior, a pK_a of 10.25 ± 0.1 is deduced for the active site His 63 (the error is because of the limitations imposed on the integration of signals with such large line widths).

The pK_a of 10.25 determined for His 63 in thiolsubtilisin Carlsberg by this direct method is in good agreement with results of indirect determinations, a kinetically determined value of 10.12 (Polgár and Halász 1973), and a more recent value of 10.2 obtained by titration with thiol-specific time-dependent inhibitors such as 2,2'-dipyrimidyl disulfide (Plou et al. 1996).

Theoretical studies on pK_a perturbations at serine protease active centers

To date, all X-ray crystal structures available for thiolsubtilisin have their Cys 221 oxidized. Wells and coworkers were able to obtain 1.5 Å resolution crystal structure of the double mutant Ser 221 Cys/Pro 225 Ala subtilisin where S^γ also was oxidized to the sulfenic acid (Abrahmsen et al. 1991). Therefore, we constructed Ser 221 Cys subtilisin, added the hydrogen atoms, and minimized a subset of residues within a 5-Å radius of the active center protonated histidine using the Tripos force field, Pullman charges, and a conjugate gradient algorithm and a distance-dependent dielectric of 2. After 100 iterations, the negatively charged S^γ atom was found to move 0.5 Å from its initial position to reduce steric crowding in the active site because of the size of the sulfur atom (larger covalent radius of sulfur [1.04 Å]) compared with that of oxygen [0.66 Å]. In the X-ray crystal structure, the distance between Ser O^γ and His N^{ε2} was 2.8 Å, but in an unfavorable orientation for formation of a hydrogen bond (pH 5.6; Petsko and Neidhart 1988). Next, the entire structure was minimized using the same minimization parameters to relax the entire system to a RMS force less than 0.5 kcal/mole Å. The final optimized distance of N^{ε2} to S^γ was 3.66 ± 0.5 Å. A superposition of the active site residues of subtilisin and thiolsubtilisin is shown in Figure 2, in

Fig. 2. Superposition of the active center residues of subtilisin (cyan) and thiolsubtilisin (colored by atom type) where the sulfur atom is shown in yellow.

which the yellow sulfur atom moved to adopt a different dihedral angle to eliminate steric crowding. Using the finite difference solution to the Poisson-Boltzmann equation, we calculated the pK_a shifts (ΔpK_a) of the active site His in each case. The ΔpK_a of the active center His 63 in thiolsubtilisin Carlsberg is shown in Table 1.

Table 1. Calculated pK shifts of thiolsubtilisin for different assumed dielectric constants

Protein dielectric constant	$\phi_{N\delta 1}$	$\phi_{N\epsilon 2}$	ΔpK_a
2	-8.90	-13.40	4.84
4	-5.63	-8.30	3.02
6	-4.45	-6.50	2.35
8	-3.81	-5.53	2.03
10	-3.40	-4.91	1.80
12	-3.09	-4.47	1.64
14	-2.86	-4.14	1.52
16	-2.67	-3.87	1.42
20	-2.39	-3.46	1.27
40	-1.66	-2.42	0.88

The experimental pK_a is 10.25 (this study). The pK_a of the wild-type enzyme is 7.0–7.2 (by NMR) (Jordan et al. 1985) and 7.17 (kinetic) (Russell et al. 1985). $\Delta pK_a = 3$. Grid spacing is 1.034 Å. The distance Cys 221 : S γ – His 64 : N ϵ^2 = 3.61 Å and Asp 32 : O δ^1 – His 64 : N δ^1 = 2.88 Å.

We then used a different set of experimental data to check the correlation between the experimental and calculated ΔpK_a values using a continuum model. The ΔpK_a in this second case is pK_a of the active enzyme compared to the pK_a of the monoisopropylphosphoryl-Ser 195 chymotrypsin or trypsin, or monoisopropylphosphoryl-Ser 221 subtilisin with a single negative charge at the phosphodiester oxyanion. In this case, the experimental values were determined by ^{31}P NMR (Adebodun and Jordan 1989) after aging of the diisopropylphosphorylserine enzymes, resulting from inactivation by diisopropylfluorophosphate. As shown in Tables 2–4 for monoisopropyl (MIP)-inhibited subtilisin, chymotrypsin, and trypsin, we were able to predict the pK_a shifts on installation of the negative charge at the catalytic serine in the correct order (for any dielectric constant selected):

$$\begin{aligned} \text{His63(64)} \Delta pK_a \text{ Subtilisin} &> \text{His57} \Delta pK_a \text{ Chymotrypsin} \\ &> \text{His57} \Delta pK_a \text{ Trypsin} \end{aligned} \quad (1)$$

Cederholm et al. (1991) estimated the pK_a shift of the three histidines of bovine pancreatic ribonuclease using a protein dielectric of 2 and compared the theoretical results to the values observed by NMR. The calculated values were higher than the experimental ones. We repeated those calculations and found that a protein dielectric of 4–6 gives satisfactory results, especially when the water molecules were deleted (data not shown). Some studies used higher dielectric constants to predict the correct pK_a s and pK_a shifts of the titratable residues. An $\epsilon_p = 10$ –15 (Demchuk and Wade 1996) was suggested, especially when OPLS charges and radii were used (Jørgensen and Tirado-Rives 1988). Other models are available where the enzyme is modeled with a higher dielectric constant (Antosiewicz et al. 1994; Cannon et al. 1997). However, in our case, the distance between charged groups was short, and the two-site model worked well for estimating the pK_a shifts. Surprisingly, in all three models the pK_a shifts were well correlated

Table 2. Calculated pK_a shifts of MIP-inhibited subtilisin for different assumed dielectric constants

Protein dielectric assumed	$\phi_{N\delta 1}$	$\phi_{N\epsilon 2}$	ΔpK_a
2	-7.88	-21.01	6.27
4	-4.95	-11.80	3.64
6	-3.89	-8.60	2.71
8	-3.32	-5.95	2.23
10	-2.94	-5.93	1.92
20	-2.04	-3.71	1.25

The Ser 221-bound monoisopropylphosphoryl group was built using TRIPOS Sybyl, and the model was minimized as mentioned in the case of thiolsubtilisin to an RMS value of 0.44. The distances were His 64 : N δ^1 – ISP : PO1 = 5.10 Å, His 64 : N δ^2 – ISP : PO1 = 3.07 Å, His 64 : N δ^1 – ISP : PO2 = 5.71 Å and His 64 : N ϵ^2 – ISP : PO1 = 6.94 Å. The experimental ΔpK_a value was 4.0 (Adebodun and Jordan 1989).

Table 3. Calculated pK_a shifts of MIP-inhibited chymotrypsin for different assumed dielectric constants

Protein dielectric assumed	$\phi_{N\delta 1}$	$\phi_{N\epsilon 2}$	ΔpK_a
2	-7.62	-18.60	5.69
4	-4.72	-10.70	3.35
6	-3.71	-8.03	2.55
8	-3.17	-6.64	2.13
10	-2.82	-5.78	1.87
20	-2.01	-3.87	1.27

The experimental value of ΔpK_a was 3.0 (Adebodun and Jordan 1989).

using a relatively low protein dielectric, that is, a continuum dielectric over the entire protein.

It is noteworthy that Fersht and coworkers have probed the dielectric constant in the active cleft of subtilisin BPN' by mutating the negatively charged residues to neutral ones on the surface of the enzyme. The Asp 99 Ser (12–13 Å from His 64) and Asp 156 Ser (14–15 Å from His 64) mutations lowered the pK_a of the active site His 64 by 0.4 ± 0.02 unit in each case. This pK_a alteration could be mimicked by using a protein dielectric constant of 2 with the DELPHI program (Russell et al. 1987; Sternberg et al. 1987).

This single point mutation of Ser 221 to cysteine results in a dispersed negative potential on the enzyme surface. A similar phenomenon was first described and modeled using GRASP on trypsin isozymes (Nicholls and Honig 1995; Nichols et al. 1991). Figures 3A and 3B show (the color-coded model, blue for positive and red for negative potential) the change in electrostatic potential due to this active site modification, illustrating the difference between the parent enzyme and its thiolsubtilisin variant. This finding

Table 4. Calculated pK_a shifts of MIP-inhibited trypsin for different assumed dielectric constants

Protein dielectric assumed	$\phi_{N\delta 1}$	$\phi_{N\epsilon 2}$	ΔpK_a
2	-6.66	-13.90	4.46
4	-4.18	-8.36	2.72
6	-3.31	-6.43	2.11
8	-2.85	-5.43	1.80
10	-2.55	-4.79	1.59
20	-1.84	-3.35	1.13

The experimental value of ΔpK_a was 2.4 (Adebodun and Jordan 1989).

suggests that a negatively charged substrate would be repelled by the presence of an additional negative charge at the active center. Russell and Fersht (1987) had shown that the specificity of subtilisin was different toward negatively charged suc-AAPF-pNA than toward the positively charged Bz-VGR-p-NA, and that the change was in the direction expected from the effect of charges on the enzyme on binding. According to theoretical studies by Hwang and Warshel (1988), simply reversing the charge at a single enzyme residue cannot be compensated for by reversal of charge on the substrate. Therefore, the electrostatic potential maps in Figures 3A and 3B are particularly useful for a fuller appreciation of the changes induced by the Ser 221 Cys substitution.

Evidence for charge localization at His 63(64) when protonated

An interesting challenge to the authors' interpretation was raised by an anonymous referee: does installation of a negative charge between Asp 32 and His 63(64) (corresponding

Fig. 3. Surface potential representations displayed with GRASP (Nicholls et al. 1991). The molecular surface is color-coded by electrostatic potential, as calculated with DELPHI (Honig et al. 1993) for (A) subtilisin and (B) thiolsubtilisin. Potentials less than -5 kT are red, those greater than 5 kT are blue, and neutral potentials (0 kT) are white. The color in between is produced by linear extrapolation.

to Asp 102 and His 57 in the trypsin/chymotrypsin class) lead to a similar pK_a elevation to that found by installation of the negative charge on the Ser side of the triad? Remarkably, the answer is that even today the experimental evidence is sparse on this issue, because there is no direct pK_a determination for the Asp 32 (Asp 102 in the trypsin class) in any of these well-studied proteases. We can make some reasonable arguments as follow. The pK_a of His in aqueous buffer is 6.0; of His 57 in chymotrypsin it is 7.0 (direct NMR measurements of the $N^{\delta 1}H$ resonance by Zhong et al. 1995). In a recent report on the Asp 32 Cys variant of subtilisin BPN', the pK_a deduced from the pH dependence of k_{cat}/K_m was 6.53 compared to 7.01 in the wild-type enzyme (Stratton et al. 2001). Because this pK_a is assigned to His 64, the result is consistent with the negative charge at the thiolate being further from the histidinium ring than is the negative charge of the aspartate in the wild-type enzyme, thus leading to a smaller pK_a elevation in the variant. Although the numbers on both the wild-type and Asp 32 Cys variant are consistent with an electrostatic pK_a elevation at the active center His, these perturbations are very much smaller than those discussed in this article at the Ser side. We could offer three possible scenarios for explaining this anomaly: (1) the effective dielectric constant is much higher on the Asp side than on the Ser side of His, thereby attenuating the electrostatic effects on the Asp side; (2) the Asp has an unusually high pK_a , that is, greater than 7.0, and it exists in its conjugate acid form (AspCOOH) providing little electrostatic imperative to alter the pK_a of the adjacent His; and (3) the Asp has a low pK_a , but it shares a proton with the His, so that the effective charge at Asp and His N^{δ} is much less than unity, resulting in a much smaller ΔpK_a produced at His on the Asp side. We tend to favor the third explanation, which is also in accord with the conclusions reached by Kuhn et al. (1998) from a 0.78-Å structure of *Bacillus lentus* subtilisin. That study reported observation of the proton between His 64 and Asp 32 with a proton to $N^{\delta 1}$ distance of 1.2 Å and a proton to carboxyl oxygen distance of 1.5 Å and concluded that there was relatively little negative charge density at the carboxyl group.

We suggest that a simple explanation of all of these observations is that the His imidazolium ring carries a highly localized charge distribution, with the $N^{\delta 1}$ carrying little and the $N^{\epsilon 2}$ carrying the bulk of the positive charge. Indeed, even simple Pullman charges comparing the $N^{\delta 1}H$ tautomer of His to the $N^{\epsilon 2}$ -protonated His H^+ form are in accord with this idea (Kahyaoglu 1998). In this fashion, there is an asymmetry in the ΔpK_a induced at the His in the catalytic triad by adjacent negative charges, smaller on the Asp side, and rather large on the Ser side induced by the various anionic tetrahedral intermediate mimics. Additional evidence that installation of the strong anionic perturbation on the Ser side results in little if any change on the hydrogen bond between the catalytic Asp and His was reported from

this laboratory using a very sensitive and highly regiospecific H/D fractionation factor study of His (Bao et al. 1999). We determined H/D fractionation factors at both $N^{\delta 1}H$ and $N^{\epsilon 2}H$ for different ionization states of His 57 in chymotrypsin, finding that with the His H^+ form, the fractionation factor at $N^{\delta 1}H$ was 0.64 ± 0.02 at pH 3.0 for uncomplexed enzyme and 0.65 ± 0.01 at pH 6.5 for enzyme complexed to a nanomolar K_i peptide boronic acid class inhibitor. In fact, the fractionation factor on the $N^{\epsilon 2}H$ side was near 1.0 in both the absence and presence of the inhibitor. These results are certainly consistent with no significant change in hydrogen bonding between the Asp and His on introduction of the negative charge on the Ser side, which is only likely if the charges between Asp and His are highly localized and small. The results also suggested the strengthening of this hydrogen bond between Asp and His by protonation of the imidazole side chain; the fractionation factor for $N^{\delta 1}H$ for uncomplexed chymotrypsin decreased from 0.82 ± 0.02 at pH 10.0 to 0.64 ± 0.02 at pH 3.0.

Conclusions

It has been amply shown that installation of a negative charge onto the active center serine of serine proteases (in complexes with peptide boronic acids [Zhong et al. 1995; Bao et al. 1998, 1999]; in the covalently modified monoisopropylphosphoryl enzymes [Adebodun and Jordan 1989]; when it is replaced by a cysteine thiolate [Jordan and Polgár 1981; this study]; or addition of peptide trifluoromethylketones [Liang and Abeles 1987; Lin et al. 1998]) induces a substantial increase in basicity of the active center histidine. The calculations reported here use a protein dielectric ϵ_p for the continuum model in the DELPHI approach, which represents an average value across the entire protein, rather than of the active center per se. The calculations here presented suggest that the active centers of both the chymotrypsin and subtilisin class of serine proteases also may have a low protein dielectric constant (as also suggested by Gunner and Honig 1991) to account for the observed pK_a changes in going from the ground state to the transition state analog structures, and indeed to the transition states themselves. In turn, the results suggest that a low protein dielectric constant may be an important contributor to the catalytic rate acceleration in this important class of enzymes. Finally, we note that recently we found evidence for the importance of a low effective dielectric constant on a totally different system, the thiamin diphosphate-dependent yeast pyruvate decarboxylase (Jordan et al. 1999). Although enzymologists have long suggested that a low effective dielectric constant indeed could contribute to the observed catalytic rate accelerations in enzymes, seldom have experimentally observed pK_a s been used to support this hypothesis in the fashion outlined here.

Materials and methods

Phenylmethanesulfonyl fluoride (PMSF), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), potassium thiolacetate, subtilisin Carlsberg, Sephadex G25, imidazole and succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (s-AAPF-*p*NA) were purchased from Sigma Chemicals. Affi-Gel 501 mercurial agarose gel was from Bio-Rad. Thiolacetate was dissolved in excess methanol/water (1 : 1) and the insoluble portion was discarded. The solvent was evaporated under vacuum, in the dark. The purified thiolacetate was kept under vacuum at 5°C. All other chemicals were the purest grade available.

Preparation of thiolsubtilisin

The C221-thiolsubtilisin was prepared from subtilisin in three steps according to early reports of Polgár (1976) with very few modifications. First, the enzyme was totally inhibited with excess PMSF:

Next, the phenylmethanesulfonyl group was displaced by thiolacetate in an S_N2 type reaction at 30 °C for 48 hours in an incubator.

Finally, in a step assisted by the enzyme, spontaneous hydrolysis of the S-acylated thiolenzyme led formation of thiolsubtilisin:

Subtilisin Carlsberg (500 mg crystallized) was dissolved in 5 mL of 50 mM imidazole buffer (pH 7.4), containing 50 mM CaCl₂ and subjected to an additional purification on a Sephadex G25 column (1.8 × 40 cm) eluted at a flow rate of 30–40 mL/h in a cold room to remove small peptides resulting from autolysis. The activity of the eluate was assayed in 980 µL of Tris buffer (pH 7.0), containing 1 mM CaCl₂ and 20 µL substrate (20 mM stock solution of s-AAPF-*p*NA, MW = 642.5, dissolved in dimethyl sulfoxide). The release of *p*-nitroaniline was monitored at 410 nm. Fractions with greater than 1.5 AUFS at 280 nm were collected and concentrated using an Amicon concentrator at 4°C and 5000 rpm. The total yield of enzyme was estimated at 90 mg (A_{280 nm}^{0.1%} = 0.86; Gill and von Hippel 1989; L. Polgár pers. comm.) in 8 mL of solution after concentration. To this solution, 150 µL of PMSF (20 mg/mL in acetonitrile) was cautiously added, and the reaction mixture was incubated at room temperature for 1 h. Greater than 99.9% of the activity was lost at the end of the incubation period as assayed under identical conditions to those used above. The phenylmethanesulfonyl enzyme (PMS) enzyme was treated with 425 mg of thiolacetate and incubated at 30°C for 48 h in an incubator. After the incubation period, the reaction mixture (pH 5.9–6.1) was subjected to gel filtration on a Sephadex G25 column equilibrated with 10 mM phosphate buffer (pH 7.0), containing 1 mM EDTA and 70 mM KCl. The protein fraction collected from the column was treated with an additional 5 µL of freshly prepared PMSF solution to inactivate the residual parent enzyme that could degrade the thiol enzyme. The protein was stored under nitrogen at 5°C and taken quickly to the next step.

Purification

The thiolsubtilisin Carlsberg (70 mg) from the previous step was applied to a 1.2 × 5-cm mercurial agarose AffiGel 501 column under Ar. The column was washed with 15 mL of 10 mM phosphate buffer (pH 7.0), containing 1 mM EDTA and 70 mM KCl, and then eluted with the same mixture at a flow rate of 10 mL/h. The eluate showed no thiol content in the assay with Ellman's reagent. The thiol enzyme retained in the column was eluted with either 0.5 mM HgCl₂ (for extended storage period) or 12 mM of DTT in 10 mM phosphate buffer containing 70 mM KCl.

Activation of thiolsubtilisin

The mercury derivative of thiolsubtilisin was activated by treatment with 12 mM of DTT for 5 min at room temperature. The thiol enzyme free of mercury was isolated on a Sephadex G25 column equilibrated with 10 mM KP_i (pH 7.0), containing 70 mM KCl and 1 mM EDTA under Ar. The eluate was subjected to chromatographic purification to eliminate excess DTT and then concentrated with an Amicon Centricon-10 concentrator at 4°C and 5000 rpm. The concentrated enzyme solution was dialyzed by adding fresh and deoxygenated buffer until the eluate showed no further evidence of DTT with Ellman's reagent. ¹H NMR spectra showed no evidence of residual DTT. The purity of the enzyme was determined using 12% SDS-PAGE electrophoresis. A single large band was observed corresponding to a molecular weight of 27,000.

Colorimetric determination of the cysteine content of thiolsubtilisin

Because DTT had been used to activate the enzyme, it had to be removed subsequently to prevent its reaction with thiol-specific reagents. The standard buffer was 1 mL of KP_i (pH 7.0), containing 70 mM KCl and 1 mM EDTA. Addition of 50 µL of 2.5 mM DTNB gave an absorbance of 0.016. However, addition of 50 µL of concentrated NaOH to this solution gave a final absorbance of 1.615 (total hydrolysis of DTNB). This corresponded to ε = 14,070 (molar extinction coefficient M/cm) that correlated well with Ellman's reported value of 13,600 M/cm (Ellman 1959). The enzyme concentration was estimated based on the extinction coefficient of subtilisin Carlsberg at 280 nm (ε = 23,478). To 2 mL of the same buffer solution, 10 µL of the enzyme solution was added, and the absorbance was recorded as 0.09, which corresponded to an enzyme concentration of 6.81 × 10⁻⁶ M. Separately, the Cys content was estimated as follows: to 1 mL of the same buffer at pH 7.0, 10 µL of thiolenzyme and 50 µL of 2.5 mM of DTNB solution were added, and the absorbance was recorded (A_{412 nm} = 0.081). This value was not corrected for the DTNB blank because the reference and the sample cuvettes both contained the same concentration of DTNB. This absorbance corresponded to 6.3 × 10⁻⁶ M Cys from the standard curve, indicating ~92% thiol content and confirming that the active site Ser 221 was successfully transformed to Cys. This enzyme did not show appreciable activity (<1%) against s-AAPF-*p*NA. However, it hydrolyzed the active ester *p*-nitrophenyl acetate and was inhibited by *p*-chloromercuribenzoate. (*p*-NPA [63 mg] recrystallized from ethanol was dissolved in 10 mL/methanol. This solution [1 mL] was slowly added to 100 mL of distilled water with strong agitation to prevent precipitation).

¹H NMR spectroscopy of thiolsubtilisin

Proton NMR experiments were conducted on a Varian Inova 500-MHz instrument using a 5-mm triple resonance ¹⁵N/¹³C/¹H probe

and the VNMR software package supplied by the manufacturer. The 1–1 binomial water suppression pulse sequence (Hore 1983) was used. In addition, the free induction decays were zero-filled to 64K points. Typical instrument parameters used for acquisition and processing were as follows: acquisition time, 0.7 sec; number of points, 35,008–40,000; spectral width, 25,000 Hz; number of transients, 400–4000; pulse width, $p1 = p2 = 6.0 \mu\text{sec}$; temperature, 10°C ; line broadening, 50 Hz; delay time, 0.2 sec; gain, 20. Backward linear prediction was used to eliminate truncation ringing in the FT spectra (Magnetic Moments 1992). Chemical shifts are reported in ppm downfield from internal 4,4-dimethyl-4-silapentane-1-sulfonic acid, sodium salt (DSS). The pH of the solutions was measured at room temperature with an Orion pH meter using a 2-mm microelectrode and was not corrected for the isotope effect. For NMR analysis, the samples contained 1.0–1.2 mM enzyme (90% $\text{H}_2\text{O}/10\% \text{D}_2\text{O}$) in 10 mM KPi , 70 mM KCl, and 1 mM EDTA at pH ranging from 6.5 to 10.50. The pH was rechecked after each experiment and adjusted using K_3PO_4 solutions in D_2O .

Input and programs used for the theoretical calculations

The atomic coordinates of protein crystal structures were downloaded from the Brookhaven Protein Data Bank. The pdb entries were 1sbc for subtilisin (Petsko and Neidhart 1988), 1sbt for subtilisin (Alden et al. 1971) where the MIP group was attached and the structure was refined to a RMS value of 0.44, 1gmh for MIP-inhibited chymotrypsin (Harel et al. 1991), and 1trn for MIP-inhibited trypsin (Gaboriaud et al. 1996). The computer platform was a SGI Indigo2 (Silicon Graphics Inc.). The molecular modeling software package from BIOSYM Technologies was used for visualization of macromolecules. The DISCOVER or TRIPOS SYBYL (version 6.3) compute/minimize module was used for minimization of the geometries. The BIOPOLYMER module was used to build or mutate the protein structures. The DELPHI module developed by Honig and coworkers (Gilson and Honig 1987; Nicholls and Honig 1991; Honig et al. 1993) was used to calculate the electrostatic potentials. Other parameters were as follow: $I = 0.1$ (ionic strength); $D = 80$ (solvent dielectric); ϵ_p (protein dielectric) was a variable parameter; border space = $10 \text{ \AA}$; grid size = $65 \times 65 \times 65 \text{ \AA}^3$; grid point spacing assigned automatically (usually $1.0\text{--}1.1 \text{ \AA}$); ionic radius = $2.0 \text{ \AA}$; solvent radius = $1.4 \text{ \AA}$ (corresponding to accessible surface of the molecule). The capping mode was turned off, and pH was not specified during the calculations. Also, the water molecules found in the crystal structures were deleted. DELPHI defaults were used for atomic radii to provide the united-atom model. A different charge file was edited for every calculation by assigning a partial or formal charge(s) to specific atoms. The DELPHI calculations were run once for each site of interest on the assumption that the unit charge was located at that site. Because in the FDPB equation the commutative operation was valid, $q_i\phi_j = q_j\phi_i$, the effect of each charged atom was determined by obtaining the potential induced by the site of interest at the charged atom and then multiplying this potential by the appropriate charge. The potential induced by the Ser 221 Cys mutation in the case of thiolsubtilisin was obtained by calculating the potential due to the partial positive charges of the active site histidine nitrogen ($\text{N}^{\delta 1}$ and $\text{N}^{\delta 2}$) atoms and multiplying this value by the charge assigned to the S^γ atom (-1 in this case) of Cys 221. In the case of the monoisopropylphosphoryl-inhibited serine proteases, the two oxygens bonded to the P atom each carried -0.5 partial charges. A superposition of MIP-derivatized trypsin and

chymotrypsin showed structural similarity. This model was used for subsequent finite differences Poisson-Boltzmann calculations in which the output of the DELPHI program for calculating the electrostatic potential is in kT/e units, and the ΔpK_a is given by:

$$\Delta pK_a = \gamma_i (\phi_o - \phi_m)/2.303 \quad (5)$$

where ϕ_o and ϕ_m are the potentials at site i caused by the original and the mutated groups, respectively; γ_i is a constant, and it is -1 for acidic and $+1$ for basic groups. In the case of histidine, ΔpK_a was calculated by averaging the potentials at the two imidazole nitrogens atoms:

$$\Delta pK_a = [(\Phi_{\text{N}\delta 1} + \Phi_{\text{N}\delta 2})]/2 (2.303) \quad (6)$$

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