NMR studies of the hydrogen bonds involving the catalytic triad of *Escherichia coli* thioesterase/protease I

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Abstract ***Escherichia coli*** **thioesterase/protease I (TEP-I) is a lipolytic enzyme of the serine protease superfamily with Ser^{10}, Asp^{154} and His^{157} as the catalytic triad residues. Based on comparison of the low-field ^{1}H nuclear magnetic resonance spectra of two mutants (S10G and S12G) and two transition state analogue complexes we have assigned the exchangeable proton resonances at 16.3 ppm, 14.3 ppm, and 12.8 ppm at pH 3.5 to His^{157}-N$^{\delta1}$H, Ser^{10}-O$^{\gamma}$H and His^{157}-N$^{\varepsilon2}$H, respectively. Thus, the presence of a strong Asp^{154}–His^{157} hydrogen bond in free TEP-I was observed. However, Ser^{10}-O$^{\gamma}$H was shown to form a H-bond with a residue other than His^{157}-N$^{\varepsilon2}$.** © **2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

Key words: Serine protease; Low barrier hydrogen bond; Nuclear magnetic resonance; Lipolytic enzyme; Thioesterase

1. Introduction

Escherichia coli thioesterase/protease I (TEP-I) has been classified as a member of a new subclass of lipolytic enzymes with diverse substrate specificity and regiospecificity [1]. This class of enzymes possesses a distinctive GDSLS sequence motif, instead of the GxSxG motif found in common lipases. We have employed heteronuclear multi-dimensional nuclear magnetic resonance (NMR) techniques to determine the structure and dynamics of TEP-I [2,3]. Our results showed that TEP-I adapted the α/β hydrolase-like structure [4]. The active site environment of TEP-I is found to be highly flexible [3]. A recent 2.3 Å resolution crystal structure confirmed our NMR results and identified Ser^{10}, Asp^{154} and His^{157} as the catalytic residues (Liaw, unpublished results). Thus this subclass of enzymes belongs to the serine protease superfamily.

Many enzymes use a Ser-His-Asp catalytic triad for enhancing the catalytic activities. What are the properties that render this apparatus work is a question central to the understanding of the catalytic mechanism of these serine enzymes. A great deal of attention has been devoted to the understanding of the catalytic triad for the past more than 30 years (for recent reviews see Bachovchin [5] and Frey [6]). Several theories have been proposed to explain how the triad works, including the charge-relay model [7–9], 'low barrier' hydrogen bond theory [10–13], and the 'imidazole ring flip' [14]. In order to understand the catalytic mechanism it is essential to understand the hydrogen-bonding network of the catalytic triad. An ultrahigh-resolution (0.78 Å) X-ray crystal structure of subtilisin at pH 5.9 resolved hydrogen bonds in the serine protease catalytic triad (Ser-His-Asp) [15]. Electron density was observed for an unusual short hydrogen bond between aspartic acid and histidine in the catalytic triad, confirming the existence of a special hydrogen bond that had been previously identified by NMR [16]. However, no hydrogen bond between the catalytic Ser^{221}-O$^{\gamma}$H to His^{64}-N$^{\varepsilon2}$ was found. Steitz and Shulman reviewed X-ray crystal structures and NMR data on serine protease and confirmed that the His^{57}–Ser^{195} H-bond does not exist in resting enzymes, but does form on complexation with substrates [17]. ^{15}N NMR results demonstrated the existence of an $>$N:----H-O type H-bond between His^{57} and Ser^{195} in the resting enzyme when His^{57} is in neutral state [18]. Markley and Westler also reported observing the N$^{\varepsilon2}$H proton of His^{57} in chymotrypsin at 13.1 ppm (pH 3.5) and at 12.6 ppm (pH 1.0), which they interpreted as due to the formation of a strong His^{57}–Ser^{195} H-bond [19]. Thus the details of the question regarding serine protease H-bonding, proton abstraction from Ser to His, and nucleophilic attack of Ser on substrate are complicated and not solved. In this letter we report the detection, assignments and characterization of the low-field ^{1}H NMR resonances of TEP-I.

2. Materials and methods

2.1. Protein expression and purification

The procedures for expressing and purifying *E. coli* TEP-I have been described previously [20,21]. The uniform ^{15}N-labeling was achieved by growing the *E. coli* cells in M9 medium with $^{15}NH_4Cl$ as the only nitrogen source [2]. The protein concentration was determined spectrophotometrically, using a molar extinction coefficient, $\varepsilon_{280} = 34\,850\ M^{-1}\ cm^{-1}$ [22]. The pH of the samples was varied by careful addition of a small amount of 0.2 M NaOH or HCl. All reported pH values were direct readings from a pH meter without correction for isotope effect.

2.2. Enzyme assays

The esterase activity of TEP-I was assayed spectrophotometrically at 37°C, using *p*-nitrophenyl butyrate as the substrate. The assay mixture (1 ml) contains $1.3–7.9\times10^{-4}$ M of *p*-nitrophenyl butyrate, 2.1% Triton X-100, pH 7.0. The reaction was started by addition of the enzyme or its inhibitor complex and stopped by addition of acetone (0.4 ml). Initial rates were estimated by measuring of *p*-nitro-

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phenol formation at 346 nm (ε_{max} = 925 M^{-1} cm^{-1}) [23]. For protease activity the assay mixture contained the enzyme, freshly made dioxane solution of the substrate, *N*-carbobenzoxy-L-tyrosine *p*-nitrophenyl ester. The enzymatic activity was estimated at 25°C spectrophotometrically, using a molar absorption coefficient of 3454 M^{-1} cm^{-1} at 400 nm [24]. For thioesterase activity the assay is based on the release of coenzyme A (CoA) by enzymatic reduction of 5,5-dithiobis (2-nitrobenzoic acid) (DTNB). The reduction of DTNB was measured by following the increase in absorbance at 412 nm, using a molar extinction coefficient of 13 600 M^{-1} cm^{-1}. The assay mixture contained DTNB, 80 μg/ml bovine serum albumin and 14 μM palmitoyl-CoA [25]. All assays were carried out in 50 mM phosphate buffer, pH 7.0. The enzyme concentrations range from 1.64 to 3.28×10^{-8} M.

2.3. NMR spectroscopy

Proton NMR spectra were obtained at 500 MHz with a Bruker AMX500 spectrometer using a 5 mm triple resonance probe. Spectra were recorded at 2°C using the 1331 pulse sequence [26] with a recycle delay of 2 S, 8K data points for 1–2K scans. The delays between the pulses were adjusted for maximum excitation at 14.7 ppm and minimum excitation at 4.7 ppm. Data were processed using XWINNMR software package (Bruker AG, Karlsruhe, Germany) on UNIX-based Silicon Graphics O2 workstations. The proton chemical shift values were indirectly referenced to DSS at 0 ppm [27].

3. Results and discussion

3.1. Detection and assignment of the low-field resonances

Shown in Fig. 1 are low-field ^{1}H NMR spectra of various protein samples. Spectra were obtained at 2°C in 50 mM phosphate buffer, pH 6.1. Under the experimental conditions

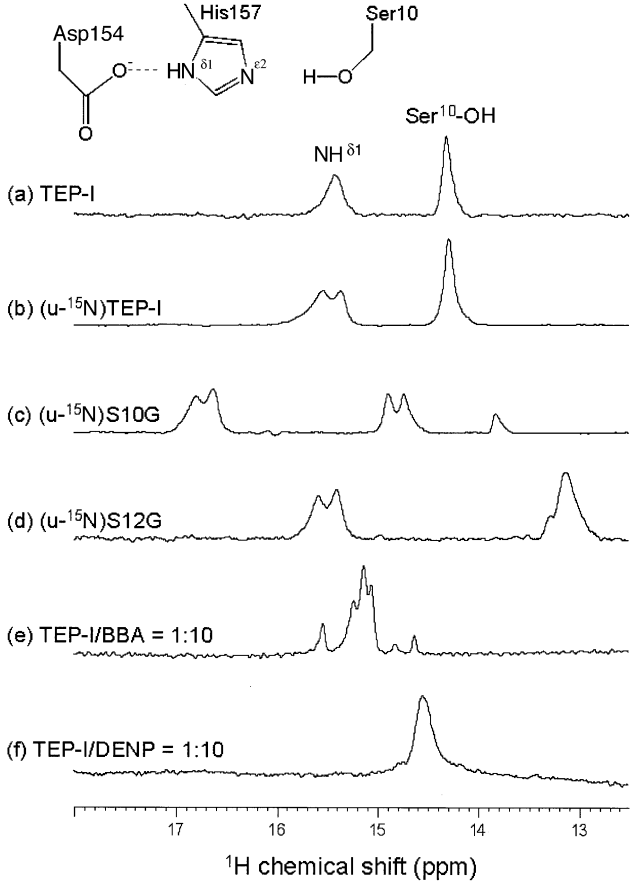

Fig. 1. ^{1}H NMR spectra of various TEP-I samples (0.5–2.2 mM) in 50 mM phosphate buffer, pH 6.1, 10% D_2O. NMR spectra were obtained at 2°C with the 1331 pulse sequence with carrier frequency set at 4.7 ppm using a Bruker AMX500 spectrometer.

two resonances at 15.4 ppm and 14.3 ppm for the wild-type protein were observed (Fig. 1a). In the free enzyme these two resonances can be observed only at low temperatures in H_2O, thus are due to exchangeable protons. These extremely low-field resonances have been observed in several serine proteases [12,16,17]. Resonances at 15 ppm or lower field have been assigned to the $N^{\delta1}H$ of the catalytic His residues. The detection of this low-field resonance provided a strong evidence supporting the formation of a strong hydrogen bond between His-$N^{\delta1}H$ and the carboxyl oxygen of the catalytic Asp. Following this argument we can tentatively assign this resonance to His^{157}-$N^{\delta1}H$. This assignment is supported by the splitting of this resonance into a doublet of $^1J_{NH}$ = 90 Hz in the spectrum obtained with (u-^{15}N)TEP-I (Fig. 1b) [28]. However, strong hydrogen bonds arising from non-catalytic histidine residues have been observed in other proteins [29]. There are three histidine residues in TEP-I, His^{61}, His^{157}, and the C-terminal His^{180}. The assignment of the 15.4 ppm resonance to either His^{61} or His^{180} can be ruled out by the following arguments. (i) Both His^{61} and His^{180} are far from the active site and have been shown to be unaffected by inhibitor binding (Tyukhtenko et al., unpublished results), in contrast to the drastic effect of inhibitor binding on this resonance (Fig. 1e,f). (ii) NMR relaxation studies showed that the backbone of the His^{180} residue is very flexible, thus is unlikely to be involved in H-bonding. The absence of splitting of the 14.3 ppm resonance into a doublet in the spectrum obtained with (u-^{15}N)TEP-I sample suggests that it is not a nitrogen proton. To further assign this resonance we have prepared two mutants, the S10G mutant and the S12G mutant. The S10G mutant is inactive while the S12G mutant retains roughly 30% activity (unpublished observation). We observed three major resonances at 16.7, 14.9 and 14.7 ppm in the spectrum of theS10G mutant (Fig. 1c). The 16.7 ppm resonance become a doublet in the spectrum obtained with (u-^{15}N)S10G. Thus, this resonance can be assigned to $N^{\delta1}H$ of His^{157}. The two higher field resonances at 14.9 and 14.7 ppm did not change in u-^{15}N-labeled protein, suggesting that they are not from His^{157}. Furthermore, these two resonances did not broaden at higher temperatures, suggesting that they are not from protons in the active site, which has been shown to be flexible and solvent-exposed. The origins of these two resonances as well as the minor resonance (intensity ~0.25 proton) are not clear. For the (u-^{15}N)S12G mutant we observed a doublet at 15.5 ppm and a singlet at 13.2 ppm (Fig. 1d). The 15.5 ppm resonance can be readily assigned to the His^{157}-$N^{\delta1}H$. We assign the latter resonance to the same resonance at 14.3 ppm in free enzyme. The large upfield shift of the 14.3 ppm resonance to 13.2 ppm in S12G suggests that the 14.3 ppm resonance is from a proton near Ser^{12} residues. Thus, we tentatively assign the 14.3 ppm resonance to Ser^{10}-$O^{\gamma}H$.

Boronic acid derivatives, such as benzyl boronic acid (BBA), have been shown to bind to the active site serine and form reversible complexes with proteases [30,31]. Similarly, phosphorus-containing compounds, such as diethyl *p*-nitrophenyl phosphate (DENP), also react with the active site serine to form stable transition state-like complexes [32]. We found that both BBA and DENP are good inhibitors of TEP-I. However, the kinetics of inhibition of these two inhibitors are very different. BBA inactivates TEP-I rapidly and reversibly whilst DENP inhibits the activity of TEP-I slowly,

but irreversibly (unpublished observation). Fig. 1e shows the low-field ^{1}H spectrum of TEP-I in 10 times excess of BBA. Fig. 1f shows the corresponding low-field ^{1}H spectrum of TEP-I in the presence 10 times excess of DENP. We have shown that only residues located near the active site were perturbed by both BBA and DENP (unpublished results). The disappearance of the 14.3 ppm resonance in these two inhibitor complexes strongly supports our assignment of this resonance to Ser^{10}-O$^{\gamma}$H.

It is interesting to note that the spectrum in Fig. 1e was obtained immediately after addition of BBA whilst the spectrum in Fig. 1f was obtained 2 days after the addition of DENP at room temperature. In both cases, the N$^{\delta1}$H resonance at 15.4 ppm disappeared soon after the addition of the inhibitors. However, the effect of the two inhibitors on the resonance at 14.3 ppm is very different. Addition of BBA to TEP-I at pH 6.1, 2°C caused the immediate disappearance of the 14.3 ppm resonance. On the other hand, the time it takes for the 14.3 ppm resonance to disappear after the addition of DENP is highly dependent on pH and temperature. At pH 6.1, 2°C it takes weeks for the complete disappearance of the 14.3 ppm resonance after the addition of DENP. This result indicates the presence of long-lived intermediate species in TEP-I/DENP complex, the Michaelis complex. Transformation from the TEP-I/DENP Michaelis complex to the tetrahedral transition state adduct is a slow process at pH 6.1. Furthermore, in addition to difference in binding kinetics the active site structures of the two enzyme/inhibitor complexes are also different, as evident from the different patterns of the low-field spectra between 14 and 16 ppm. The nature of these new peaks and the structure of the Michaelis complex are currently under investigation in our laboratory. For the present study the disappearance of the 14.3 ppm resonance upon binding of the two transition state inhibitors provides additional strong support to our assignment of the 14.3 ppm resonance to Ser^{10}-O$^{\gamma}$H.

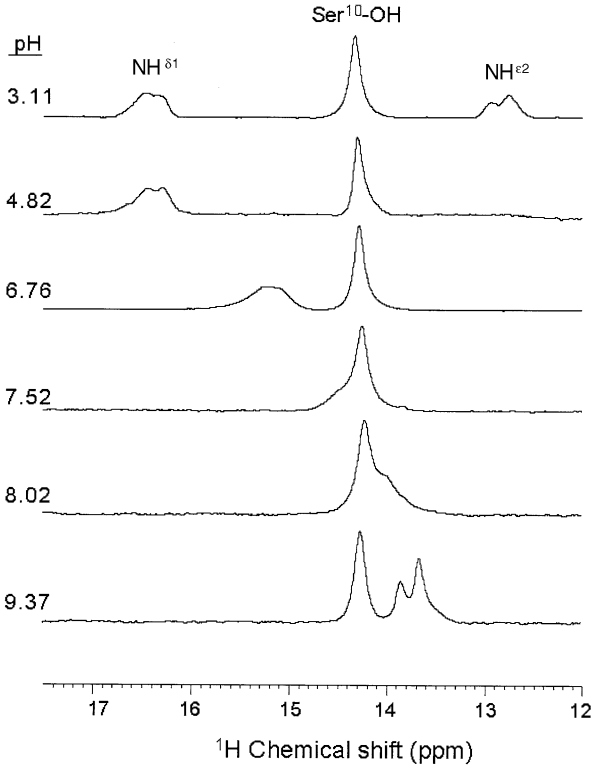

Fig. 2. ^{1}H NMR spectra of TEP-I, obtained at various pHs. Sample and NMR conditions are the same as in Fig. 1.

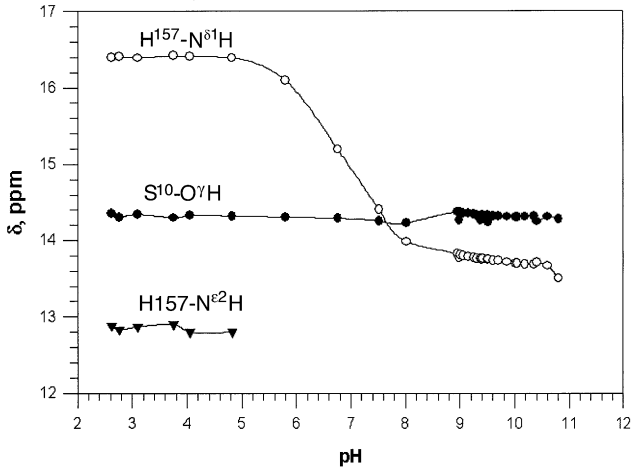

Fig. 3. Variation with pH of the chemical shifts of the low-field ^{1}H resonances of free TEP-I. The data points were obtained from the respective resonances in Fig. 2.

3.2. pH titration

To investigate the response of the low-field ^{1}H resonances to pH variation we have obtained a set of low-field ^{1}H spectra over the pH range of 3.0–11. Within this pH range the protein is very stable. The results are shown in Fig. 2. At pH 3.11 three well-resolved resonances near 16.4, 14.3 and 12.8 ppm were observed. The two resonances at 16.4 and 12.8 ppm, which become doublets ($^1J_{NH}\sim$90 Hz) in (u-^{15}N)TEP-I sample, were assigned to N$^{\delta1}$H and N$^{\varepsilon2}$H of His^{157}, respectively [19]. The Ser^{10}-O$^{\gamma}$H resonance at 14.3 ppm remains as a singlet at all pHs in ^{15}N-labeled protein, as expected. Increasing pH caused the upfield shift of the N$^{\delta1}$H resonance from 16.4 ppm at pH 3.11 to 13.8 ppm at pH 9.37, similar to that observed previously in other serine proteases [12,17,19]. Increasing pH also caused a drastic broadening of the N$^{\varepsilon2}$H resonance. This resonance became barely observable at pH 4.8 and disappeared at higher pH. In contrast, the Ser^{10}-O$^{\gamma}$H resonance at 14.3 ppm hardly changes over the pH range of 3–11. The variation of chemical shift with pH is more clearly shown in Fig. 3. The pK_a of His^{157} deduced from the titration curve of N$^{\delta1}$H is $\sim$6.8. The near invariance of the Ser^{10}-O$^{\gamma}$H resonance with the titration of the His^{157}-N$^{\varepsilon2}$ nitrogen suggests that the Ser^{10}-O$^{\gamma}$H is not hydrogen-bonded to His^{157}-N$^{\varepsilon2}$. In the X-ray crystal structure Ser^{10}-O$^{\gamma}$ was found to be 3.6 Å from His^{157}-N$^{\varepsilon2}$, too far to form a hydrogen bond, in agreement with our results. Interestingly, we found that Ser^{10}-O$^{\gamma}$ is 2.58 Å from the backbone carbonyl group of Ile^{42}, well within the distance for forming a hydrogen bond. Thus, we propose that in free enzyme Ser^{10}-O$^{\gamma}$H is hydrogen-bonded to the carbonyl group of Ile^{42}, causing it to resonate at low field.

In summary, we have detected several low-field, exchangeable ^{1}H resonances in TEP-I. By comparing the spectra obtained with free TEP-I, (u^{15}N)TEP-I, (u-^{15}N)S10G, (u-^{15}N)S12G, TEP-I/BBA (1:10) and TEP-I/DENP (1:10) we assign the 16.3 ppm, 14.3 ppm and 12.8 ppm resonances at pH 3.5 to the active sites His^{157}-N$^{\delta1}$H, Ser^{10}-O$^{\gamma}$H, and His^{157}-N$^{\varepsilon2}$H, respectively. We have shown that in free TEP-

I only the His^{157}–Asp^{154} H-bond is present in the catalytic triad. We propose that Ser^{10}-$O^{\gamma}H$ is H-bonded to the carbonyl group of Ile^{42}. Binding of transition state inhibitors, BBA and DENP, perturbed the hydrogen bond network.

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