Determination of the Ionization State and Catalytic Function of Glu-133 in Peptide Deformylase by Difference FTIR Spectroscopy†

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ABSTRACT: Peptide deformylase (PDF) catalyzes the hydrolytic removal of the N-terminal formyl group from newly synthesized polypeptides in eubacteria and the organelles of certain eukaryotes. PDF is a novel class of amide hydrolase, which utilizes an Fe^{2+} ion to effect the hydrolysis of an amide bond. The ferrous ion is tetrahedrally coordinated by two histidines from a conserved HEXXH motif, a cysteine, and a water molecule. In this work, the function of the conserved glutamate (Glu-133 in *Escherichia coli* PDF) is evaluated by difference FTIR spectroscopic analysis of a Co(II)-substituted *E. coli* wild-type and E133D mutant PDF. At pH <6, the wild-type enzyme exhibited a relatively sharp C═O stretch band at 1742 cm^{-1}, which is assigned to the COOH group of Glu-133. The pH titration study and curve fitting to the data revealed a pK_a of 6.0 for Glu-133 (in the presence of 500 mM NaCl). For the E133D mutant, which is only ∼10-fold less active than the wild-type enzyme, a similar pH titration study of the Asp-133 C═O stretch band at 1740 cm^{-1} revealed a pK_a of 10.1. This unusually high pK_a for a carboxyl group is likely due to its hydrophobic environment and electrostatic repulsion from the metal-bound hydroxide. These results argue that in the active form of E133D PDF, Asp-133 is protonated and therefore acts as a general acid during the decomposition of the tetrahedral intermediate by donating a proton to the leaving amide ion perhaps through a water molecule in the cavity created by the E133D mutation. In contrast, Glu-133 is deprotonated in the active form of wild-type PDF. We propose that Glu-133 acts as a proton shuttle accepting a proton from the metal-bound water and subsequently acts as a general acid during the decomposition of the tetrahedral intermediate.

Peptide deformylase (PDF)[1] catalyzes the hydrolytic removal of the N-terminal formyl group from nascent ribosome-synthesized polypeptides in eubacteria and organelles of certain eukaryotes. PDF represents a novel class of amide hydrolase, which utilizes an Fe^{2+} ion to catalyze the hydrolysis of an amide bond (*1*, *2*). This Fe^{2+} enzyme is, however, extremely labile, undergoing rapid inactivation upon exposure to molecular oxygen (*1*, *3*). Recent studies have shown that a number of other metal ions can be used to form active PDF. It has been discovered that Ni^{2+} and Co^{2+} can be incorporated into PDF to produce highly active and stable enzymes (*2*, *4*, *5*). In addition, Zn-PDF is also highly stable, although its activity is ∼2 orders of magnitude lower than that of the native Fe-PDF (*1*, *2*, *5*, *6*).

X-ray crystallographic studies of PDF in various metal ion forms have revealed that the metal ion is coordinated with two histidines, a cysteine, and one or two water molecules (*2*, *5*, *7*, *8*). The two histidines are derived from a helical HEXXH motif, which is the signature motif found in many zinc metalloenzymes (for a recent review, see ref *9*). In the X-ray structure of PDF, the glutamate in the HEXXH motif is hydrogen bonded to the metal-bound water. This glutamate (Glu-133 in PDF) plays an important role in PDF catalysis, as is the case in all other HEXXH-containing zinc metalloenzymes (*9*). Mutation of Glu-133 to an alanine completely abolishes the catalytic activity, whereas mutation to an aspartate results in an only ∼10-fold reduction in activity (*4*). On the basis of steady state kinetic measurements and analysis of the electronic absorption spectra of Co^{2+}-substituted PDF (Co-PDF) variants under various pH conditions, it has been suggested that the function of the active site metal is to ionize the bound water molecule, whereas Glu-133 acts primarily as a general acid, donating a proton to the leaving amide ion during the decomposition of the tetrahedral intermediate (*4*). Furthermore, on the basis of the pH profile of E133D PDF activity, it has been proposed that the pK_a of Asp-133 is ∼9.5, more than 5 pH units higher than that in aqueous solution. However, this pK_a could not be independently verified by absorption spectroscopy in the previous study, as ionization of Asp-133 caused no detectable changes to the Co-PDF spectrum. Since the corresponding Glu in other zinc metallopeptidases is often viewed as an essential general base during catalysis, we felt that a more in-depth study of the ionization state and catalytic function of Glu-133 or Asp-133 in PDF is warranted.

The vibrational spectrum of a molecule reports on the bond orders of molecular bonds and is very sensitive to the small changes in the distribution of electrons within bonds that come about when the environment of the molecule is changed. Using sensitive FTIR spectroscopy to determine a C═O stretch band at >1710 cm^{-1} in proteins was pioneered

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[1] Abbreviation: PDF, peptide deformylase.

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by Knowles's group in the 1980s on TIM (*10*). Since then, numerous papers have been published using this technique on a number of enzyme systems, such as acyl-proteases (*11*−*16*), yeast aldolase (*17*), phospholipase A_2 (*18*), citrate synthase (*19*), lactate dehydrogenase (*20*, *21*), liver alcohol dehydrogenase (*22*, *23*), 4-chlorobenzoyl-CoA dehalogenase (*24*), and acyl cysteine proteases (*25*). In this study, FTIR difference spectroscopic techniques have been used to determine the protonation state of Glu-133 in WT PDF and Asp-133 in the E133D mutant under a variety of pH and salt conditions. Since the C═O stretching frequency of a carboxyl group is typically above 1700 cm^{-1} when protonated but is around 1600 cm^{-1} (the IR active asymmetric C═O stretch mode of COO^-) when deprotonated, the protonation state of the carboxyl group can be determined on the basis of the observed C═O frequency. Our current results have not only confirmed our earlier mechanistic proposals but also provided new insights into the PDF mechanism.

MATERIALS AND METHODS

Escherichia coli Co-PDF and the E133D and E133A mutants were prepared according to previously published procedures (*4*) and stored at 193 K until they were used. For pH-dependent IR studies, the protein samples were washed with D_2O buffers four times (each with 40-fold dilution) in a Centricon concentrator, and the pH was then measured with a microelectrode, without deuterium correction on the reported pH values. The following buffers were used: buffers with 0.5 M NaCl at pH 5−5.5, 50 mM acetate; pH 5.5−6, 20 mM MES; pH 6−6.5, 20 mM bis-tris; pH 6.5−7.5, 20 mM phosphate; pH 7.5−8.5, 20 mM Tris; pH 9, 20 mM CHES; pH 9.5−10, 20 mM pyrophosphate; and pH 10.5−11, 20 mM CAPS. The Cl^- free buffers at pH 6 and 7.5 were prepared with 20 mM phosphate. The final concentrations of the protein sample were typically 3−5 mM. The Cl^- free samples were washed with Cl^- free buffer four times with at least 40-fold volume expansion each time. The final concentration of free Cl^- ion in the sample should be <1 μM. The spectra that are reported are the difference spectra between WT or E133D PDF and E133A PDF under identical pH conditions, except for those at pH <6 where E133A PDF is unstable. In such cases, the E133A spectrum at pH 6 was used for subtraction.

FTIR spectroscopy was performed on a Magna 760 Fourier transform spectrometer (Nicolet Instrument Corp.) using a MCT detector. The procedure for obtaining the difference FTIR spectra is described in detail in the Appendix. We used a two-position sample shuttle to alternate between the main sample and reference sample positions; this procedure substantially decreased the spectral contribution of residual water vapor after subtraction. A water vapor spectrum was also taken and was subtracted from any spectra presented in this paper. The water vapor peaks leftover in the presented spectra are easily identified by their narrow bandwidths and known positions. Both WT/E133D and E133A sample solutions were simultaneously loaded into a dual-cell shuttle accessory. CaF_2 windows with 25 μm Teflon spacers were used. Spectra were collected in the range of 1100−4000 cm^{-1} with 2 cm^{-1} resolution. A Blackman−Harris three-term apodization and a Happ−Genzel apodization were applied, respectively. Omnic 4.1a (Nicolet Instruments, Corp.) software was used for data collection and analysis.

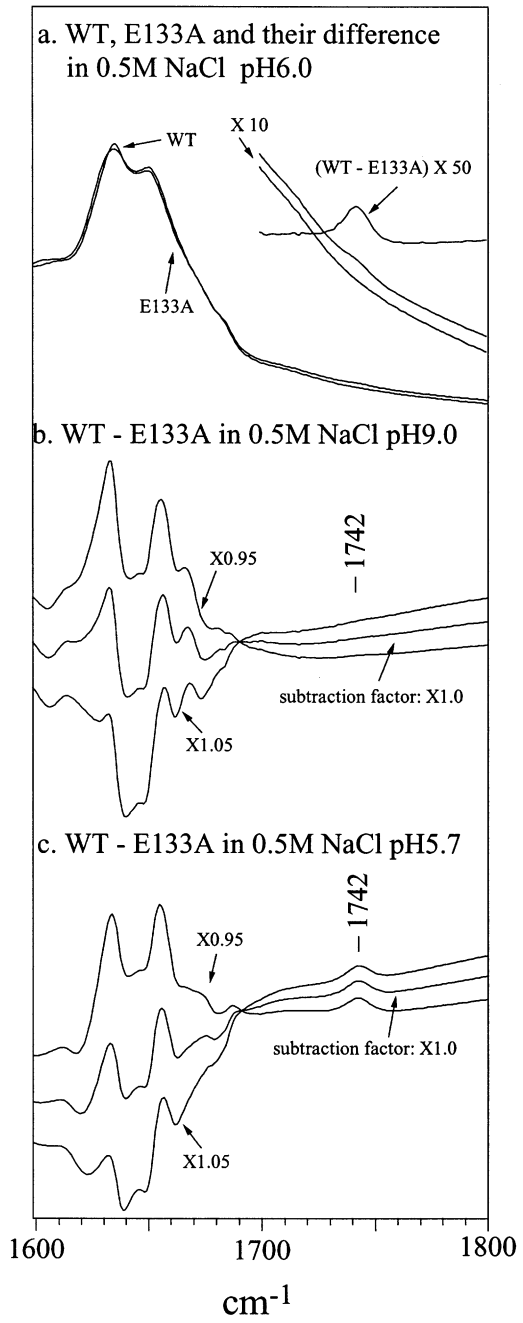


FIGURE 1: FTIR difference spectra of WT PDF and E133A in the 1600−1800 cm^{-1} region. (a) FTIR spectra of WT PDF and E133A. The inset shows the 10-fold amplification of the two spectra in the 1700−1800 cm^{-1} region. It also shows the difference spectrum between the two with a multiplication factor of 50. The enzyme concentration was ~3 mM in 20 mM MES buffer (pH 6) containing 0.5 M NaCl. (b) FTIR difference spectra between WT PDF and E133A at pH 9.0 with three different subtraction factors of 0.95, 1.0, and 1.05. The enzyme concentration was ~3 mM in 20 mM CHES buffer (pH 9) containing 0.5 M NaCl. (c) FTIR difference spectra between WT PDF and E133A at pH 5.7 with three different subtraction factors of 0.95, 1.0, and 1.05. The enzyme concentration was ~3 mM in 20 mM MES buffer (pH 5.7) containing 0.5 M NaCl.

RESULTS

Identification of Glu-133 Absorption. Figure 1a shows the FTIR spectra of Co(II)-substituted WT and E133A PDF from

E. coli at pH 6.0 in the presence of 0.5 M NaCl. We have previously shown that EcPDF undergoes denaturation at pH <5.5 and high ionic strength can significantly improve its stability (*4*). The major bands between 1600 and 1700 cm^{-1} (~0.8 OD unit) are due to absorption by the many amide carbonyl groups in the protein. Amplification of the two spectra in the 1700−1800 cm^{-1} region by a factor of 10 (in the *y*-axis) reveals a small difference in intensity at ~1740 cm^{-1} (Figure 1a inset). The difference spectrum (multiplied by a factor of 50) shows an extra band at 1742 cm^{-1} in the spectrum of WT PDF on top of an otherwise flat background. No baseline correction was performed on this difference spectrum. The intensity of the peak is extremely small, only ~0.3% of the major protein band intensity in the original spectrum. To rule out the possibility that this band is generated by artifacts that might take place by subtraction, additional experiments were performed.

Artifacts can arise from a small mismatch in sample concentration between the two cells and slight misregistration in wavelength between the two runs, which can generate sloping backgrounds and/or features which look like peakes in the difference spectrum. To test for these, Figure 1b shows the difference spectra between WT PDF and the E133A mutant at pH 9.0 with three different subtraction factors of 0.95, 1.0, and 1.05. These difference spectra contain both positive and negative bands. The intensities of the major bands in the 1600−1700 cm^{-1} region are ~2% of that of the amide I band in the original spectrum. The criterion used in the subtraction procedure to produce a proper difference spectrum is to make the IR intensity of the amide I band as small as possible. In this case, the two samples are well matched so that a subtraction factor of 1.0 yields the proper difference spectrum with a reasonably flat baseline. Under- or oversubtraction by 5% greatly changes the appearance in the 1600−1700 cm^{-1} spectral region. However, all major bands remain in place despite quite different subtraction factors. This indicates that the bands in the difference spectra are due to protein vibrations instead of noise (*26*, *27*). They appear in the difference spectrum because of frequency changes of the protein bands that are induced by the mutation of Glu-133 to Ala-133. The mutation causes local and nonlocal environmental changes of the backbone and side chain C═O bonds in the enzyme through a network of hydrogen bonding interactions. These difference spectra also show that even with an over- or undersubtraction factor, the slope of the baseline in the spectral region above 1710 cm^{-1} will only change marginally but not significant enough to generate artificial peaks in this region.

Figure 1c shows the difference spectra between WT PDF and E133A mutants at pH 5.7 with three different subtraction factors of 0.95, 1.0, and 1.05. In this case, the two samples are not well matched in concentration; one has slightly higher water content than the other. This water generates a broad band near 1400 cm^{-1} which tails off at ~1700 cm^{-1}. With a subtraction factor of 1.0 to minimize the protein amide I band intensity, a slight slope in the baseline can be observed in the difference spectrum. The band at 1742 cm^{-1}, however, appears clearly in the difference spectrum, with a well-defined baseline. Under- or oversubtraction by 5% changes the baseline slope and the spectral appearance in the 1600−1700 cm^{-1} spectral region to some extent. However, the baseline in the spectral region above 1710 cm^{-1} is still clearly well-defined despite the small slope change. Thus, our results show that the intensity of the 1742 cm^{-1} band does not change with different subtraction factors and is not affected by a small imbalance in the concentration of the two samples.

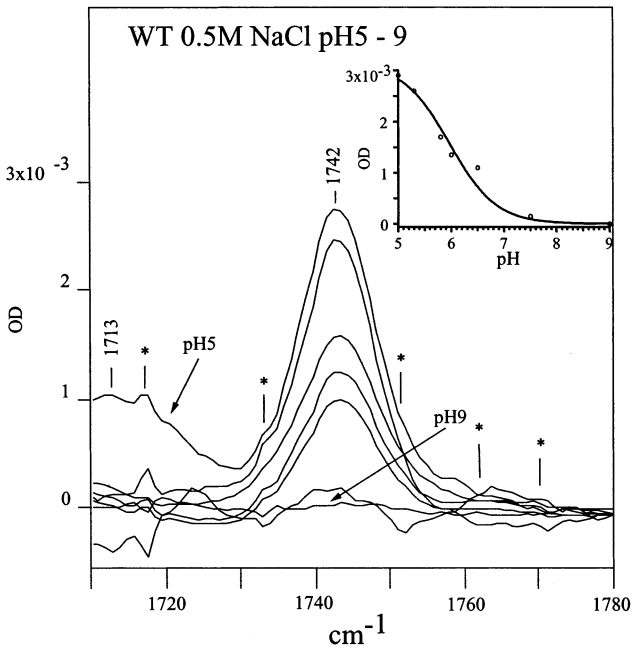


FIGURE 2: Normalized C═O stretch bands of the Glu-133 COOH group (relative to the amide I band) at pH 5−9. The spectra were obtained from difference FTIR spectra between WT and E133A PDF at the same pH except those samples with pH values of <6, where the E133A sample at pH 6 was used in the subtraction. The enzyme concentration was 3−5 mM. The buffer contained 0.5 M NaCl. The inset shows the plot of the C═O stretch band intensity (at 1742 cm^{-1}) vs pH. The solid line is the result of curve fitting to the equation $I = I_0/(1 + 10^{pH-pK_a})$, where I_0 and pK_a are curve fitting parameters. The artifacts generated by water vapor are marked with asterisks.

On the basis of the data analysis described above, it is clear that the 1742 cm^{-1} band in the difference spectrum between WT PDF and E133A mutants at acidic pHs is real. This band is assigned to the C═O stretch mode of the COOH group of Glu-133 in WT PDF. This band intensity is very weak, about 0.003 OD unit at pH5.7 in a 0.8 OD sample. However, the signal-to-noise ratio is quite high, more than 40:1 in this case. The major noise in the difference spectrum is due to imperfect subtraction of the water vapor bands, which could be easily identified from their known positions and very narrow bandwidths (~2 cm^{-1}). Thus, it is apparent that our difference FTIR procedure can produce very good spectra in the region above 1700 cm^{-1}, suitable for a pH titration study of the COOH group in WT and E133D PDF.

Apparent pK_a of Glu-133. Figure 2 shows the pH titration study of WT PDF in the presence of 0.5 M NaCl by FTIR difference spectroscopic measurements (pH 5−9). The intensity of the 1742 cm^{-1} band decreased as the pH increased and eventually disappeared at pH 9. The artifacts generated by imperfect subtraction of the water vapor spectrum are marked with asterisks. They are easily identified since their bandwidths are only ~2 cm^{-1}, compared with the ~20 cm^{-1} bandwidth of the C═O stretch mode. Another band at 1713 cm^{-1} appeared at pH 5.0, and this band is likely due to the C═O stretch of other carboxyl groups in PDF. The frequency of this band is characteristic of the acetic acid

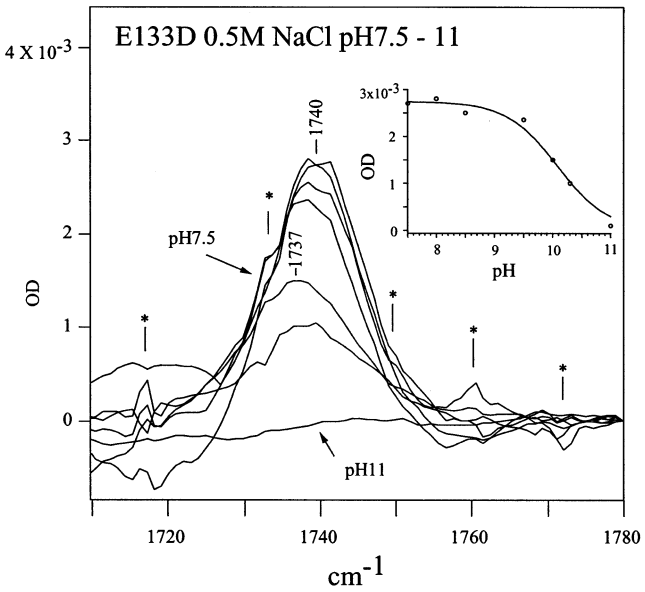


FIGURE 3: Normalized C═O stretch bands of the Asp-133 COOH group (relative to the amide I band) at pH 7.5−11. The spectra that are shown are the difference FTIR spectra between E133D and E133A mutants recorded under identical conditions. The enzyme concentration was 3−5 mM. The buffer contained 0.5 M NaCl. The inset is the plot of the C═O stretch band intensity (at 1740 cm^{-1}) vs pH. The solid line is the result of curve fitting to the equation $I = I_0/(1 + 10^{pH-pK_a})$, where I_0 and pK_a are curve fitting parameters. The artifacts generated by water vapor are marked with asterisks.

in aqueous solution, suggesting that it is due to the carboxyl groups of solvent accessible glutamic and/or aspartic acid residues. The elevated pK_a and the higher C═O stretch frequency of Glu-133 relative to those of other carboxyl groups in the protein suggest that the environment surrounding Glu-133 is quite hydrophobic. We also performed subtractions between WT PDF spectra at low pH values and that at pH 9. The resulting difference spectra are very similar to those in Figure 2 in the region above 1700 cm^{-1} (data not shown). These results indicate that there is no other carboxyl group in WT PDF with an elevated pK_a.

A plot of the normalized intensities of the 1742 cm^{-1} band (relative to the protein amide I band intensity) against pH produced a sigmoidal curve (Figure 2 inset). Curve fitting of these data points to the equation

$$I(\mathrm{pH}) = I_0/(1 + 10^{\mathrm{pH}-pK_a}) \quad (1)$$

gave an apparent pK_a value of 6.0. In our previous study of the pH dependence of WT PDF catalytic activity and electronic absorption spectral properties, we observed a single ionization event in the pH range of 5−12, with an apparent pK_a of 5.7 in the presence of 2 M NaCl (*4*). This pK_a was assigned to the active site Glu-133. The close agreement among the results obtained by three different techniques (catalytic activity, UV−vis absorption spectroscopy, and FTIR spectroscopy), in our opinion, builds a convincing case that the observed pK_a (∼6) is due to ionization of Glu-133. Since WT PDF is most active at pH 6−12 (*4*), Glu-133 must be deprotonated in the active enzyme form.

pK_a Value of Asp-133. Figure 3 shows the pH titration study of the E133D mutant in the presence of 0.5 M NaCl by FTIR difference spectroscopic measurements (from pH 7.5 to 11). The activity profile of E133D PDF is bell-shaped with a maximum at pH 8.5 (*4*). Therefore, the present experiment monitors the second ionization event. At near-neutral pH, the difference spectra between E133D and E133A PDF showed a band at 1740 cm^{-1}. As the pH increased, the intensity of this band decreased and practically disappeared at pH 11. The plot of the normalized intensities of the 1740 cm^{-1} band (relative to the protein amide I band intensity) against pH again generated a sigmoidal curve, which indicates the ionization of a single functional group with a pK_a value of 10.1 (Figure 3 inset). We assign this pK_a to the ionization of Asp-133 and the 1740 cm^{-1} band to the stretch vibration of the Asp-133 carboxyl group, on the basis of the frequency of the absorption band being similar to that of Glu-133 in WT PDF and the similar catalytic function of Glu-133 and Asp-133 in the two enzymes. In a previous study, a pK_a value of 9.5 was predicted for Asp-133 in the E133D mutant based on the pH dependence of its catalytic activity (*4*). Thus, our current result agrees very well with this earlier prediction. Since E133D PDF is most active in the pH range of 8−9, our data suggest that Asp-133 is protonated in the active enzyme form. In principle, the observed pH profiles (both activity and FTIR) could be caused by pH-dependent protein denaturation. However, this possibility can be ruled out by our earlier observation that the electronic absorption spectra of WT, E133D, or E133A PDF did not undergo any significant change as the pH was increased from 9 to 11.6. Besides, at the sample concentrations used in this work (3−5 mM), a denatured sample would produce large amounts of precipitate visible to the naked eye, as is the case under acidic conditions (pH <5). We noted that the 1740 cm^{-1} band underwent a small red shift (∼3 cm^{-1}) at high pH. The cause of this red shift is not yet clear, but could be due to changes of the hydrogen bonding network in the active site at high pH.

Protonation State of Glu-133 or Asp-133 in Chloride Free Buffer. Since the pK_a value of an active site residue can be perturbed by the presence of Cl^- ions (*4*), we have also performed similar difference FTIR measurements on WT and E133D PDF in Cl^- free buffers. These results are presented in Figure 4. Spectrum a is the difference spectrum between WT and E133A PDF at pH 6 in the presence of 0.5 M NaCl. This spectrum is taken from Figure 1 for comparison. Spectrum b is the same difference spectrum between WT and E133A PDF at pH 6 in a Cl^- free buffer. The absence of any C═O stretch band at 1742 cm^{-1} indicates that Glu-133 is completely deprotonated under such conditions. Because PDF is unstable at acidic pH and in the absence of high concentrations of Cl^- ion, it was not possible to obtain the spectrum at pH <6. However, our data suggest that the intrinsic pK_a of Glu-133 should be no higher than 5.5 under Cl^- free conditions. A pK_a of ∼5.2 was previously predicted for Glu-133 based on the activity of WT PDF as a function of pH (*4*).

Spectra c and d are the difference spectra between E133D and E133A PDF at pH 7.5 in the presence and absence of 0.5 M NaCl, respectively. The major difference in these two spectra is that the C═O stretch band of Asp-133 under Cl^- free condition is red shifted, from 1740 to 1730 cm^{-1}, and becomes broader. This heterogeneous band broadening indicates that the environment of Asp-133 in the absence of Cl^- ions is no longer homogeneous. One possible explanation

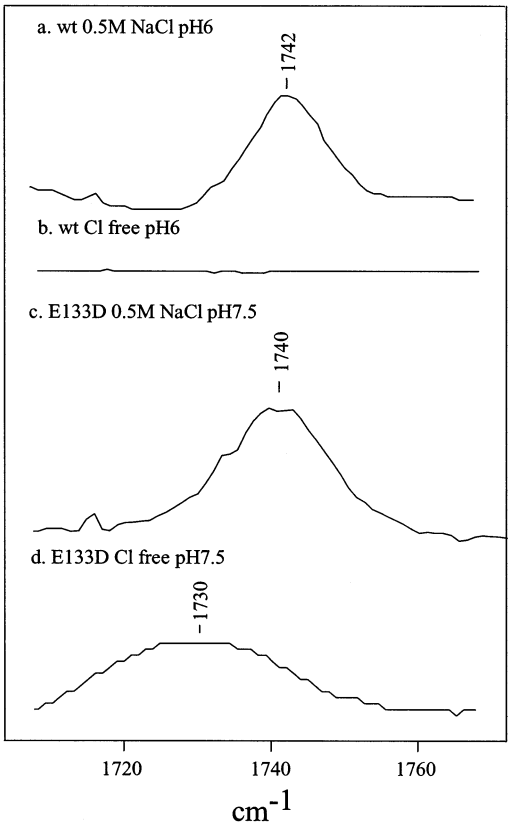


FIGURE 4: FTIR difference spectra showing the C=O stretch band for the side chain of residue 133 in WT and E133D PDF under various conditions. (a) WT PDF at pH 6.0 and in the presence of 0.5 M NaCl. (b) WT PDF in a Cl^- free buffer (pH 6.0). (c) E133D PDF in 20 mM phosphate buffer (pH 7.5) and 0.5 M NaCl. (d) E133D PDF in 20 mM phosphate buffer (pH 7.5) that is Cl^- free.

is that the cavity created by the E133D mutation is partially occupied by a water molecule(s) (see the Discussion) in the absence of Cl^- ions. Most importantly, unlike that of WT PDF, Asp-133 of the mutant is protonated regardless of the presence or absence of Cl^- ions in its catalytically active form.

DISCUSSION

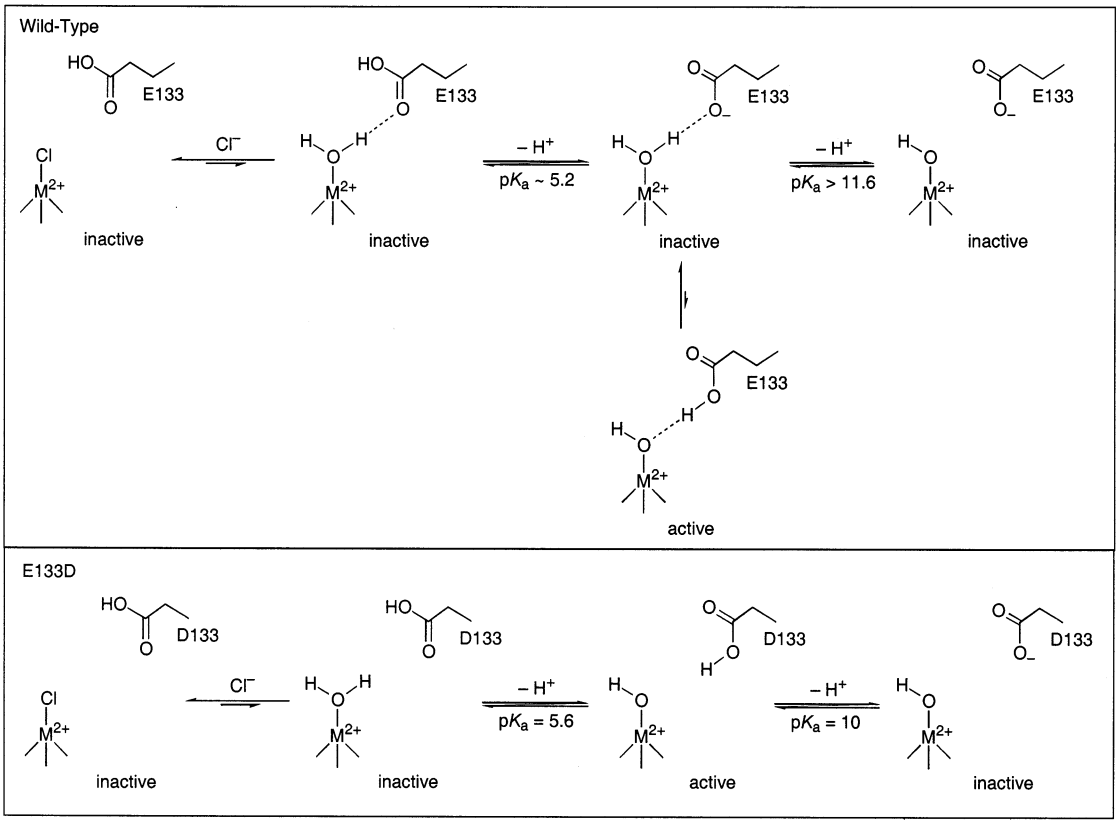
A large number of zinc metallopeptidases contain the HEXXH signature motif and have active site structures very similar to that of PDF (*9*). The catalytic metal is usually tetrahedrally coordinated with the two histidines in the signature motif, a water molecule, and another residue such as glutamate, histidine, or cysteine. The Glu in the HEXXH motif is hydrogen bonded to the metal-bound water molecule and plays a crucial role in catalysis. It is frequently postulated in the literature that this Glu helps ionize the metal-bound water by abstracting one of the water protons (as a general base) and/or facilitate product formation by protonating the leaving −NHR group (as a general acid). Thus, it is of great interest to determine the protonation state of the Glu residue under various conditions to understand the reaction mechanisms of these enzymes.

We have previously proposed that the intrinsic pK_a of Glu-133 in WT PDF is ~5.2, based on an activity profile constructed under low-salt conditions (*4*). We further reasoned that in the active form of the enzyme (at pH >5.2), Glu-133 is deprotonated and hydrogen bonded to the metal-bound water molecule. The absence of a C=O stretch signal at >1700 cm^{-1} for WT PDF at pH 6.0 in Cl^- free buffer therefore provides direct evidence for our earlier proposals (Figure 4, spectrum b). At pH <6 and in the presence of 0.5 M NaCl, our FITR data indicated that the carboxyl group of Glu-133 is protonated and has an apparent pK_a of 6.0. This value is very similar to the apparent pK_a (5.7) estimated from its activity profile determined in 2 M NaCl (*4*). As we have shown in previous UV−vis studies of Co-PDF, Cl^- ions can significantly increase the apparent pK_a of the metal-bound water, due to the much higher affinity of Cl^- ion for the metal than water (*4*). We believe that the elevated pK_a of Glu-133 in the presence of 0.5 M NaCl is also due to direct binding of the Cl^- ion to the metal center. This is because in the presence of high Cl^- concentrations, the protonated enzyme (Co-H_2O/Glu-CO_2H) is quantitatively converted into the more stable metal−chloride form (Scheme 1). This effectively shifts the ionization equilibrium of Glu-133 toward the free acid side. We have previously shown that Cl^- ion does not displace the metal-bound water once Glu-133 is deprotonated, most likely due to the strong hydrogen bonding interaction between the bound water and the carboxylate of Glu-133 (*4*). Another way to view the system is that when water is bound to the metal ion, the carboxylate ion of Glu-133 is stabilized by the formation of a hydrogen bond to the metal-bound water, whereas this is not possible when a Cl^- ion is bound to the metal center.

Unlike WT PDF, the E133D mutant exhibits two ionization events in its activity profile with pK_a values of 5.6 and 9.5 (*4*). The pK_a of 5.6 was assigned to the metal-bound water, whereas the pK_a of 9.5 was tentatively assigned to Asp-133, implying that in the active form, the carboxyl group of Asp-133 is protonated. This is highly unusual, as this pK_a value is approximately 5 pH units higher than that of a typical solvent-exposed carboxyl group and its protonation state is the opposite of that of Glu-133 in the WT enzyme. It is thus gratifying that the FTIR data from this work have now confirmed both the pK_a value (10.1) and the protonation state (free carboxylic acid) of Asp-133 in the active enzyme form.

On the basis of the apparent dissociation constant for dissociation of Cl^- ion from E133D PDF (88 mM at pH 6.7) obtained from the UV−vis studies (*4*), we have calculated that at pH 7.5 and in the presence of 0.5 M NaCl, approximately 50% of the E133D enzyme is in the metal−chloride form, whereas the other 50% is in the metal−hydroxide form. As the pH increases, the metal−chloride form is converted into the metal−hydroxide form. Since the population of the metal−chloride form is insignificant at pH >8.5, the Cl^- ion should not significantly perturb the pK_a value of Asp-133. Therefore, the measured pK_a of 9.5−10 represents the intrinsic pK_a of Asp-133. A pK_a value of 10 for a carboxyl group in a protein is quite unusual but not without precedence. For example, the pK_a of Asp-96 in bacteriorhodopsin is believed to be >12 (*28*), and a pK_a of 11.1 has been reported for a carboxyl group in an enzyme model (*29*). The unusually high pK_a of Asp-133 is likely due to a combination of two factors. First, the carboxyl group of Asp-133 is separated from the metal-bound hydroxide by ~4 Å. Electrostatic repulsion between the negative charges would destabilize the carboxylate ion. Second, the carboxyl group is situated in a hydrophobic environment, which further enhances the destabilization effect caused by the charged

Scheme 1



groups (*2*, *5*, *7*, *8*). The degree of hydrophobicity near a carboxyl group is reflected in its C═O stretch frequency, which is very sensitive to the environment. For example, our model study shows that the C═O stretch frequency of an acetic acid monomer is at 1721 cm^{-1} in D_2O but shifts to 1775 cm^{-1} in hexane. The observed C═O frequency for Asp-133 (1740 cm^{-1}) indicates a relatively hydrophobic environment. The above argument also applies to Glu-133 in WT PDF. In this case, Glu-133 is hydrogen bonded to the metal-bound water. Therefore, the Co-H_2O/Glu-133 should be viewed as an integral unit. The first pK_a of this unit is 5.2, which is very similar to that of E133D PDF (pK_{a1} = 5.6). Since WT PDF does not undergo a second ionization event up to pH 11.6, the second pK_a of the Co-H_2O/Glu-133 unit must be >12. The still higher second pK_a for WT PDF can be explained by the fact that ionization would break a stable hydrogen bond and generate a pair of negative charges at an even closer distance (~3 Å).

Since Asp-133 is protonated in the catalytically active form of E133D PDF, it cannot act as a general base during catalysis. However, because the E133A mutant is catalytically inactive, Asp-133 must be required as a general acid, donating a proton to the leaving amide ion during the decomposition of the tetrahedral intermediate. The high catalytic activity of the E133D mutant implies that Asp-133 is very effective in proton donation despite the fact that it is moved away from the reaction center by ~1.5 Å. It is possible that the efficient proton transfer is assisted by a water molecule(s). This suggestion is based on the observation that at pH 7.5 and in the absence of Cl^- ions, the C═O stretch band of Asp-133 becomes heterogeneously broadened and the average frequency of the C═O stretch mode shifts down by 10 cm^{-1} (compare spectrum d with spectrum c in Figure 4). Such a change may be explained by a partial occupation of a water molecule(s) in the cavity created by the E133D mutation. Any hydrogen bond interactions with the carbonyl of Asp-133 would reduce its bond strength, resulting in a red shift in the FTIR spectrum.

Despite the opposite protonation states of Glu-133 and Asp-133 in the "active" enzyme form, we conclude that Glu-133 in WT PDF also acts as a general acid. In the E133A and E133D mutants, where the carboxyl group is either absent or in the opposite protonation state to act as a general base, the metal-bound water has pK_a values well below 7 and readily ionizes to form the metal−hydroxide species at physiological pH. There is, therefore, no need for a general base to ionize the metal-bound water. The presence of Glu-133 actually increases the pK_a of the metal-bound water from 6.5 (as determined for E133A PDF) to >11.6 (in WT PDF) (*4*). How can one, then, reconcile the fact that Glu-133 is apparently in the wrong protonation state to act as a general acid? Actually, in both WT and E133D PDF, the active enzyme form is the protein that has undergone the first ionization event (pK_a = 5−6). The difference is in the identity of the ionizing group. In WT PDF, the first proton is lost from Glu-133, whereas the metal-bound water is the first group ionized in the E133D mutant (Scheme 1). The different order of deprotonation is most likely caused by the reversed relative acidity between the metal-bound water and the carboxyl group of Glu-133 or Asp-133 in the two enzymes. This is reasonable, as the shorter side chain of Asp-133 would place the carboxyl group more deeply into the hydrophobic pocket resulting in its more elevated pK_a. In the active form of WT PDF, a proton is shared between the metal-bound water and the carboxyl of Glu-133 via a hydrogen bond. Both FTIR and UV−vis spectroscopic data

indicate that the thermodynamically more stable state has the proton located on the water molecule. We believe that this is actually the inactive enzyme form, whereas the active form is the less stable state in which the proton is associated with the carboxyl group of Glu-133. Therefore, for WT PDF, the active enzyme form only constitutes a small fraction of the total proteins under physiological conditions, although substrate binding might shift the position of this equilibrium. This scenario offers a possible explanation for the surprisingly small reduction in activity when Glu-133 is mutated to Asp-133 (<10-fold), as the catalytically active form of the latter enzyme is the predominant species at physiological pH. Our mechanism also predicts that the Fe^{2+}- and Co^{2+}-bound PDF forms (wild type) should have very similar pH profiles, even though water molecules bound to these two metal ions often have significantly different pK_a values. This is indeed the case (*4*), because the only observable ionization event in the pH 5−12 range is the deprotonation of Glu-133 and does not directly involve the metal ion.

In conclusion, the FTIR studies in this work have now provided direct evidence for our earlier mechanistic proposal. Most importantly, the unusually high pK_a of 9.5−10 in the activity profile is now assigned to Asp-133 in E133D PDF. This finding solidifies our proposal that Asp-133 and the corresponding Glu-133 in WT PDF act as general acids during catalysis. The corresponding glutamate in other zinc metallopeptidases has frequently been viewed as a general base in the literature, helping ionize the metal-bound water. While the conclusions from PDF may not be directly applied to these other enzymes, a re-evaluation of the function of the conserved glutamate in these enzymes should be considered.

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