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Aspartic proteinases – Fourier transform IR studies of the aspartic carboxylic groups in the active site of pepsin

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Abstract Fourier transform (FTIR) difference spectra of pepsin minus diazoacetylnorleucine methyl ester (DAN) or minus diazoacetyl-L-phenylalanine methyl ester (DAP) modified pepsin, respectively, demonstrated that Asp-215 is not deprotonated in pepsin. The FTIR difference spectrum of pepsin minus 1,2-epoxyparanitrophenoxypropane (EPNP) modified pepsin demonstrates that Asp-32 is present in pepsin as CO_2^- anion. The position of the ν(C = O) vibration demonstrates that no $(O\cdots H\cdots O)^-$ hydrogen bond between Asp-215 and Asp-32 is formed. Furthermore, no H_3O^+ is present in the active center. Studies of the complex of pepsin with the inhibitor pepstatin prove that the inhibitor removes the water from the active site and Asp-32 becomes protonated.

Key words: Aspartic proteinases; Enzymatic mechanism of pepsin; FTIR difference spectra; Modifications of pepsin; Proteinases

1. Introduction

The structures of many aspartic proteinases are known from X-ray structural analysis with an accuracy better than 2.2 Å [1–6]. In some cases a strong homoconjugated $(O\cdots H\cdots O)^-$ hydrogen bond between Asp-215 and Asp-32 (pepsin numbering) has been postulated [7,8]. Usually, base catalysis by a water molecule present in the active site is discussed [8,9], but acid catalysis due to the presence of a H_3O^+ cation has also been suggested [8].

Carboxylic acid groups which are involved in an $OH\cdots N \leftrightharpoons O\cdots H^+N$ hydrogen bond with a N-base cause a ν(C = O) vibration of the non-polar structure in the region 1710–1725 cm^{-1}. In polar structure an antisymmetrical stretching vibration, $\nu_{as}(CO_2^-)$, may be found in the region 1625–1550 cm^{-1} as well as a symmetrical vibration $\nu_s(CO_2^-)$ near 1400 cm^{-1} [10–11]. Many acidic salts of carboxylic or dicarboxylic acids containing crystallographically symmetrical hydrogen bonds were studied by Hadzi and Nowak who found that very strong $(O\cdots H\cdots O)^-$ bonds give only one band at an intermediate position, instead of the ν(C = O) vibration of COOH groups or the stretching vibrations of (CO_2^-) [12]. With aqueous acetic acid–acetate solutions a strong $(O\cdots H\cdots O)^-$ hydrogen bond is present. With this strong hydrogen bond the ν(C = O) band is observed at 1685 cm^{-1}[13]. If this hydrogen bond is broken a ν(C = O) vibration of $COOH\cdots OH_2$ groups is found at 1710 cm^{-1} [13].

In this paper the aspartate proteinase of porcine pepsin A (EC 3.4.23.1) is studied by Fourier transform (FTIR) difference spectroscopy.

2. Experimental

Pepsin and pepstatin were purchased from Boehringer Mannheim GmbH, Germany. Pepsin was purified by column chromatography and lyophilised. DAN and EPNP was purchased from Sigma Chemie, Munich. DAP was synthesised according to a procedure given in [15].

The samples were prepared as films on germanium ATR crystals from 0.6 mmol solutions with pH 3.6. The ATR spectra were taken from the samples in equilibrium with nitrogen atmosphere with a relative humidity of 47%.

The FTIR spectra were recorded at 293 K with a Bruker IFS 113 v spectrometer (detector DTGS and resolution 2 cm^{-1}).

3. Results and discussion

Fig. 1 shows the FTIR difference spectrum of pepsin at pH = 2 minus pepsin at pH = 6. This spectrum shows an intense positive ν(C = O) band at 1723 cm^{-1} demonstrating that at pH = 2 the carboxylic groups of pepsin are not deprotonated. The negative bands at 1582 and 1395 cm^{-1} (ν_{as} and ν_s vibrations of CO_2^- groups, respectively), demonstrate that at pH = 6 the carboxylic groups are deprotonated. Furthermore, in the region of the Amide I band (1700–1600 cm^{-1}) only small changes are observed in the difference spectrum, demonstrating that the conformation of pepsin changes only slightly if the carboxylic groups are deprotonated or the carboxylate groups are protonated, respectively.

To obtain information about the carboxylic groups of the essential pepsin residues Asp-215 and Asp-32, respectively, it is necessary to distinguish them from the other carboxylic groups. This can be performed by specific modifications of the pepsin molecules. The carboxylic group of Asp-215 can be selectively modified by esterification with diazoacetyl-L-norleucine methyl ester (DAN) or with diazoacetyl-L-phenylalanin methyl ester (DAP), respectively [14–15].

The FTIR difference spectrum of pepsin minus DAN modified pepsin is shown in Fig. 2a and that of pepsin minus DAP modified pepsin in Fig. 2b. In these spectra the bands of pepsin are positive and those of the modified pepsin are negative. In both cases the carboxylic group of Asp-215 should be selectively modified [16–17]. The ν(C = O) band of the ester group is found as a broad negative feature respectively at 1740 cm^{-1} or at 1739 cm^{-1} with the DAN and DAP modified pepsins. Thus, the positive band observed in the difference spectra at 1715 cm^{-1} is the ν(C = O) vibration of Asp-215 in pepsin. This result demonstrates that the carboxylic group of Asp-215 is not deprotonated. Furthermore, the position of the ν(C = O) vibration demonstrates that Asp-215 is not involved in a strong

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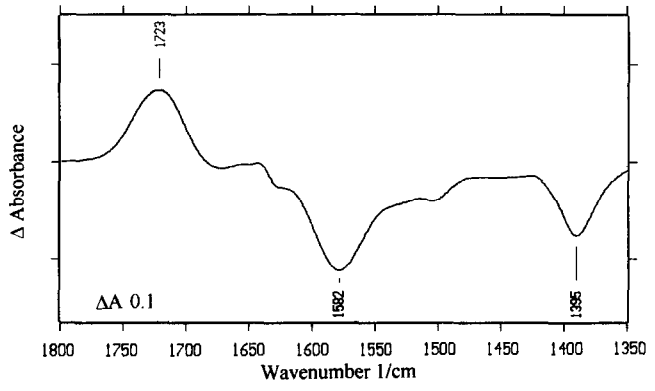

Fig. 1. FTIR difference spectrum of pepsin at pH = 2 minus pepsin at pH = 6.

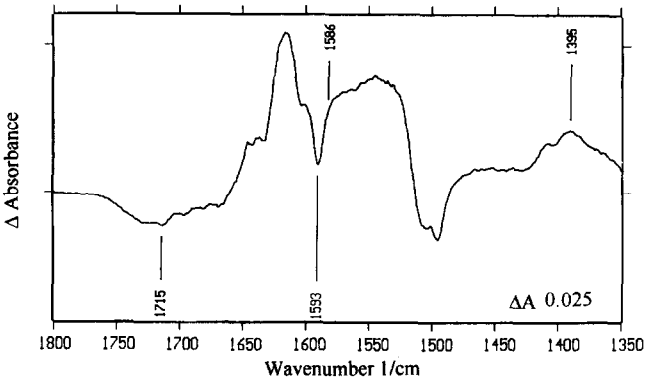

Fig. 3. FTIR difference spectrum of pepsin at pH = 3.6 minus EPNP modified pepsin.

homoconjugated hydrogen bond with the carboxylate group of Asp-32. These results are in good agreement with recently published X-ray data [16–17]. In the case of such a strong homoconjugated hydrogen bond the ν(C = O) vibration should be observed at lower wavenumbers [10–13].

Asp-32 is specifically modified by esterification with 1,2-epoxyparanitrophenoxypropane, (EPNP) [18–19]. Fig. 3 shows the difference spectrum of pepsin minus EPNP modified pepsin. In this spectrum the ν(C = O) vibration of the ester group is observed as a broad negative band at 1715 cm^{-1}, indicating that the carboxylic group of Asp-32 is modified by esterification. The finding that this ester ν(C = O) band is observed at slightly lower wavenumbers than with the other modifications may be due to hydrogen bond formation of the glycol OH group of the EPNP rest with the O atom of the ester carbonyl group. Particularly important, however, is the result that no positive ν(C = O) band is observed in the region 1725–1700 cm^{-1}. Furthermore, new positive bands are found, a shoulder at 1586 cm^{-1} ($\nu_{as}CO_2^-$) and a relatively intense band at 1395 cm^{-1} ($\nu_s CO_2^-$). This result demonstrates that in pepsin the carboxylic group of Asp-32 is deprotonated and hence present as a CO_2^- anion.

In the difference spectrum of deuterated pepsin minus deuterated DAN modified enzyme no change in the region 1750–1700 is observed. In this region $\nu_{as}(H_3O)^+$ should vanish with deuteration if a H_3O^+ species would be present [21–22]. This result demonstrates that no H_3O^+ is present in the active center of pepsin. Thus, the catalytic mechanism of pepsin cannot be an acid catalysis.

Pepsin can be inhibited by pepstatin [20]. Fig. 4a shows the FTIR difference spectrum of pepsin + pepstatin minus DAN modified pepsin + pepstatin and Fig. 4b that of pepsin + pepstatin minus DAP modified pepsin + pepstatin. In both spectra the ν(C = O) vibration of the carboxylic groups is shifted slightly toward higher wavenumbers. With the DAN modified pepsin it shifts from 1715 to 1719 cm^{-1} and with the DAP modified from 1715 to 1724 cm^{-1}. Both shifts of the bands in the presence of the inhibitor pepstatin show that, the interaction of the O atom of the C = O groups of pepsin is slightly less, indicating that water molecules are removed from the active center by the inhibitor.

Fig. 5 shows the difference spectrum of pepsin + pepstatin minus EPNP modified pepsin + pepstatin. If we compare this difference spectrum with that in Fig. 3 the following results are

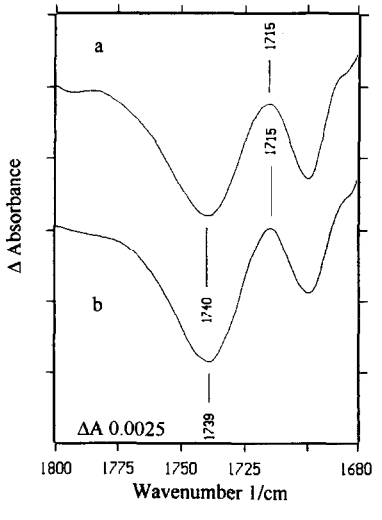

Fig. 2. FTIR difference spectra: (a) of pepsin at pH = 3.6 minus DAN modified pepsin at pH = 3.6; (b) of pepsin at pH = 3.6 minus DAP modified pepsin.

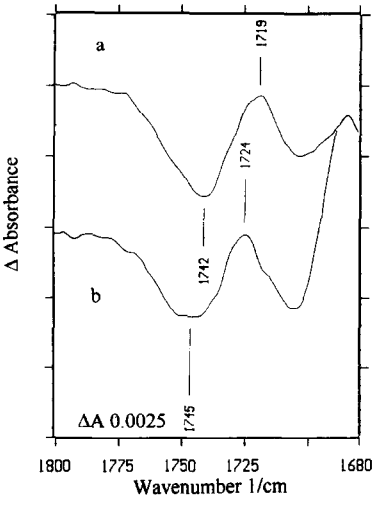

Fig. 4. FTIR difference spectra of pepsin+inhibitor at pH 3.6: (a) minus DAN modified pepsin + inhibitor; (b) minus DAP modified pepsin + inhibitor

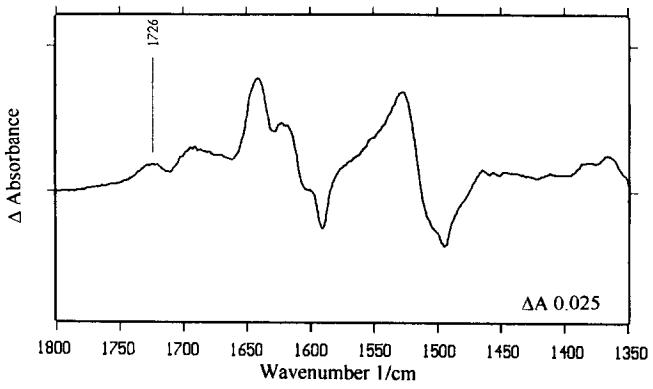

Fig. 5. FTIR difference spectrum of pepsin+inhibitor minus EPNP modified pepsin+inhibitor.

observed: the $\nu_s(CO_2^-$ band near 1400 cm^{-1} has vanished completely. The shoulder at 1586 $cm^{-1}\nu_{as}$ (CO_2^- has also been disappeared. The strong negative band at 1593 cm^{-1} is a ring vibration of the EPNP residue. Instead of the bands of $\nu_s(CO_2^-)$ and $\nu_{as}(CO_2^-)$, a ν(C = O) stretching vibration band at 1726 cm^{-1} appears. Thus, if the water is removed by pepstatin Asp-32 becomes protonated. This result demonstrates that a water molecule is split in the catalytic mechanism of pepsin. Thus, an OH^- must be present and this is the precondition for nucleophilic attack on the carbon atom of the peptide group.

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