Cryoenzymology of Porcine Pepsin†

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ABSTRACT: Physical and kinetic properties of porcine pepsin have been examined in aqueous methanol solvents at temperatures below ambient to seek evidence for covalent intermediates in the catalyzed hydrolysis of good substrates. It was first demonstrated that aqueous methanol cryosolvents have no significant deleterious effects upon this protein. The addition of methanol does lead to a drastic reduction in the midpoint of the thermal melting curve of pepsin. This could account for rate reductions previously observed in catalysis by this enzyme. This effect is lessened by the addition of active-site ligands including substrates and is fully reversible upon dilution into aqueous solution. Two substrates were chosen which have chromophoric groups on opposite sides of the scissile peptide bond. The UV spectral changes from hydrolysis of Pro-Thr-Glu-Phe-(NO_2)Phe-Arg-Leu and the fluorescence spectral changes from hydrolysis of DNS-Ala-Ala-Phe-Phe-OP4P$^+$-CH_3 were studied at temperatures down to −60 °C. The resulting Arrhenius plots were linear in the region where pepsin exists in the native state with downward curvature exhibited at higher temperatures where the reversible denaturation occurs. No "burst" reactions were observed with either substrate. In addition, efforts at trapping intermediates by low-temperature denaturation and precipitation have provided no evidence for covalent intermediates on the reaction pathway. Although this evidence is negative, we cannot rule out the possibility of the formation of covalent intermediates following an initial rate-limiting step.

It is generally agreed that the mechanism of action of porcine pepsin and other members of the aspartyl protease class of enzymes involves the participation of two aspartyl residues (32 and 215) (Clement, 1973). These have been located in the active-site pocket by chemical modification (Chen & Tang, 1972) and more recently by X-ray crystallographic studies (Bott et al., 1982; James et al., 1982). Unfortunately, the general agreement on structural data does not extend to the detailed mechanism.

There are two major alternatives for peptide bond cleavage catalyzed by carboxyl groups. First, nucleophilic attack to give a tetrahedral intermediate followed by expulsion of the amine component would yield an anhydride intermediate. The second alternative is the general base assisted attack of water upon the carbonyl, followed by prototrophic shifts and direct elimination to give the product acid and amine.

These mechanisms have one major difference: the inclusion of a covalent intermediate from direct nucleophilic attack and the lack of such an intermediate enzyme–substrate complex in the general base assisted mechanism. The distinction between these two possibilities forms the basis of the current report.

Much previous effort has been expended to obtain data on the mechanism of pepsin action. A great deal of the thinking on the mechanism has been guided by a series of observations on the products generated in pepsin-catalyzed reactions. Products arising from apparent "acyl" fragment (Takahashi et al., 1974; Takahashi & Hofmann, 1975) as well as apparent "amino" fragment transfer reactions (Neumann et al., 1959) have been isolated. These observations were originally interpreted to mean that covalent intermediates, such as the anhydride described above, are obligatory for pepsin-catalyzed reactions. It is more difficult to suggest reasonable mechanisms in which covalent intermediates could give rise to amino fragment transfer, although such mechanisms have been proposed (Kluger & Chin, 1982; Knowles, 1970).

We have employed two new substrates for pepsin that possess chromophoric groups on opposite sides of the peptide bond that is cleaved. This provides the potential to look for evidence for intermediate formation of either type through the observation of "burst" reactions (see Scheme I).

In each case, the bond cleavage results in a change in the spectra of the respective chromophore, and thus, intermediate formation potentially could be observed. To slow down the overall reaction so that turnover is stopped and thereby permit an increase in the concentration of enzyme sufficient to observe the formation of a stoichiometric burst of products, we have employed cryoenzymology. This paper reports our initial studies on the solvent system and experimental conditions necessary to observe pepsin reaction at temperatures below 0 °C and describes our initial efforts to observe intermediate formation.

Experimental Procedures

Materials

Pepsin, crystallized and lyophilized, of specific activity 2500 units/mg or higher, was from Sigma (lots 60F-8056 and 128C 8110). This material was dissolved in 0.1 M acetic acid, passed though a Sephadex G-25 column, and lyophilized. Some experiments were also performed by using pepsin that had been prepared from pepsinogen (Sigma, lot 037C0029) according to the method of Rajagopolan et al. (1966). No differences were observed between the two samples of pepsin. Peptide substrates employed in this study were prepared previously (Deyrup & Dunn, 1983; Dunn et al., 1984). Pro-Thr-Glu-Phe-(NO_2)Phe-Arg-Leu (substrate I) had a K_m of 0.13 mM and a k_{cat} of 94 s^{-1} at pH 3.0 in water (Dunn et al., 1984), and DNS-Ala-Ala-Phe-Phe-OP4P$^+$-CH_3[1] (substrate II) had a K_m of 0.039 mM and a k_{cat} of 288 s^{-1} at pH 3.1 in water (Deyrup & Dunn, 1983).

Methods

The activity of pepsin was measured by (1) following the decrease in absorbance at 310 nm during the hydrolysis of substrate I (Dunn et al., 1984) or by (2) following the increase in fluorescence of the dansyl chromophore in substrate II with excitation at 300 nm and emission at 525 nm (Deyrup &

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[1] Abbreviations: DNS, 5-(dimethylamino)-1-naphthalenesulfonyl; OP4P$^+$-CH_3, 3-(*N*-methylpyridinium-4-yl)propyl ester; Cl_3CCOOH, trichloroacetic acid.

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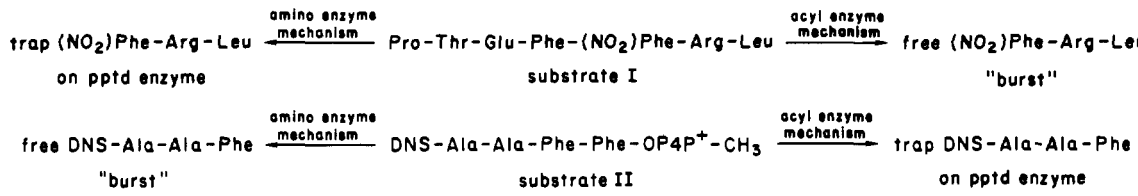
Scheme I



Dunn, 1983). As described later, the parameters for fluorescence detection were somewhat different under various conditions.

Effect of Methanol on the Activity of Pepsin. A series of solutions were prepared containing 16.2 μM substrate I, 0.02 M sodium formate buffer, and mixtures of water and methanol from 0 to 50% methanol. The apparent pH (pH*) of all solutions was adjusted to a value of 2.89. These samples were then equilibrated in quartz cuvettes in the sample compartment of a Cary 219 spectrophotometer at 1 °C. When equilibrated, an aliquot of pepsin was added to initiate the reaction. The absorbance was recorded for the complete reaction and used to calculate a pseudo-first-order rate constant for the reaction.

Stability of Pepsin in 50% Methanol. A solution of 50 μg/mL pepsin was prepared in 50% methanol containing 0.1 M sodium formate buffer at a pH* of 2.90. This was incubated at 0 °C, and 20-μL aliquots were removed at time intervals up to 2 h. These aliquots were added to a solution of substrate I in water at 25 °C to measure the activity remaining. Over the 2-h interval, there was no measurable decrease in pepsin activity.

Fluorescence of Pepsin as a Function of the Concentration of Methanol. Solutions of pepsin in varying concentrations of methanol were scanned in a Perkin-Elmer MPF-4 fluorometer with excitation at 260 nm over the emission range 300–380 nm at 0 °C at pH* 2.80 and 3.96. Over the range 0–50% methanol, there was a gradual linear increase in fluorescence at both pH* values as seen in Table I.

UV Melting Curves of Pepsin in Solutions of Varying Methanol Content. Sixty micrograms of pepsin was added to a quartz cuvette containing 3 mL of a buffered solution that was adjusted to a specific pH* value. The solution was placed in the sample compartment of a Perkin-Elmer Model 320 spectrophotometer and cooled down to the desired starting temperatures by circulating ethanol using a Neslab ULT-80 cryostat. Absorbances were measured at three wavelengths (350, 276, and 256 nm), and the temperature of the circulating fluid was then changed. When the temperature reequilibrated, as measured in the cuvette with a Teflon-coated thermocouple, the absorbance was measured again. This was repeated at intervals of 3–8 °C until no further change was noted. Experiments were performed with 10% methanol at pH* 3.3, 20% methanol at pH* 3.31, 30% methanol at pH* 3.43, and 50% methanol at three pH* values, 2.2, 3.22, and 5.2.

In addition, a mixture of pepsin (3 μM) and pepsinogen-(1–12) (Dunn et al., 1983) (10 μM) was prepared in H_2O and incubated at 30 °C for 30 min. This was then added to a solution of 50% methanol at pH* 2.6 at 0 °C in the Perkin-Elmer Model 320 spectrophotometer. This was cooled down to −30 °C and then the melting curve determined by the same process. Since the added peptide has no aromatic amino acids, the absorbance change observed again reports on the pepsin unfolding process.

Rate of Hydrolysis of Substrate I as a Function of Temperature in 50% Methanol. Sodium formate buffers of pH* 3.45 and 5.15 were prepared containing 50% methanol. These were used to measure the rate of 20 μM substrate cleavage at temperatures from +39.4 °C to −33.0 °C to construct an Arrhenius plot. At temperatures below −10 °C, the reaction progress curve shows a pronounced lag phase. This is temperature dependent and was assumed to result from the substrate-induced renaturation (see Results). Control experiments demonstrated the same UV changes when enzyme at 0 °C was added to buffer at reduced temperature. In cases where this phenomenon was observed, the linear portion of the reaction curve was employed to derive the initial rate of substrate cleavage (ΔA per minute).

Reactions at Higher Enzyme Concentration at Low Temperatures To Observe Transient Formation. Solutions were mixed at −35 °C and below to give 10–20 μM pepsin and 65 μM substrate. Some increase in absorbance at 300 nm could be observed, but control experiments demonstrated that this arises from refolding of the enzyme. Reactions were followed for 3–12 h or until any precipitation occurred. When precipitation was observed, the precipitate was allowed to settle to the bottom of the cuvette and collected. It was then redissolved at 0 °C, and the supernatant and precipitate were examined for enzymatic activity and spectral properties. Experiments were done at pH* 3.45 and 5.15.

Rate Assays with Substrate II in 50% Methanol. Reactions were carried out in solutions of 0, 10, and 20% methanol at pH* 3.16 and 0 °C with a concentration range of 13–99 μM substrate II in the Perkin-Elmer MPF-4 with excitation at 300 nm following emission at 525 nm. Enzyme concentration was 2.89 nM in H_2O, 5.71 nM in 10% methanol, and 11.43 nM in 20% methanol to correct for the rate depression observed in methanol solutions. Initial velocities were measured from the linear increase in fluorescence observed for hydrolysis of substrate II under these conditions. Plots of 1/initial velocity vs. 1/[S] were linear and were used to derive values of K_m and k_{cat}.

Temperature Dependence of the Fluorescence Spectra of Substrate II and of the Hydrolysis Product, DNS-Ala-Ala-Phe, in 50% Methanol. A 104 μM solution of substrate II in 50% methanol–sodium formate, pH* 4.0, was cooled to −42.8 °C in the sample compartment of the Perkin Elmer MPF-4. The excitation spectrum was scanned from 220 to 400 nm while the resulting emission at 540 nm was recorded. The temperature of the circulating bath was increased in increments of 5–10 °C and the excitation spectrum recorded until the temperature reached 0 °C. At that point, 50 μg of pepsin was added, and the reaction was followed to completion by following the fluorescence increase. This solution was cooled to −43.2 °C and the excitation spectrum recorded as before. The temperature setting of the circulating bath was increased in increments and the excitation spectrum recorded after equilibrium was established until the temperature was again near 0 °C. These experiments were also carried out for a solution of 50% methanol–sodium formate at pH* 2.65.

pH Dependence of the Spectrum of Substrate II and of Its Hydrolysis Product, DNS-Ala-Ala-Phe, in 50% Methanol. A 104 μM solution of substrate II in 50% methanol–water adjusted to pH* 1.97 with HCl was cooled to 0 °C in a glass titration cell fitted with a glass electrode connected to a Radiometer pH meter. When equilibrated, a 2-mL sample was taken out and transferred to a quartz cuvette in the fluorom-

eter, also at 0 °C, for measurement of the excitation spectrum from 220 to 400 nm by measuring the emission at 540 nm. The sample was then returned to the titration cell, and a small volume of 3 M NaOH in 50% methanol was added to change the pH. After equilibration of the pH* reading, an aliquot was again transferred for measurement of the excitation spectra. This was repeated at pH* intervals up to 5.03. the pH* was then adjusted back to 3.82 with 3 M HCl in 50% methanol, and an aliquot of pepsin was added sufficient to completely hydrolyze the substrate in 40 min at 0 °C. After that time, excitation spectra were again recorded at pH* intervals from 2.11 up to 5.58.

Observation of Hydrolysis of Substrate II in 50% Methanol at Subzero Temperatures. The hydrolysis reaction can best be followed at pHs below the pK_a of the substrate (3.25) with excitation at 300 nm and emission at 540 nm. Solutions of substrate of 65–150 μM were prepared in 50% methanol–sodium formate buffers and cooled to the desired temperature in the fluorometer. A second cell, connected in series with the fluid circulating through the instrument but outside the sample compartment, was used to cool down a stock solution of pepsin of 12.5 mg/mL in 50% methanol–water. To initiate the hydrolysis reaction, an 80-μL aliquot was transferred to the sample cell and mixed with a precooled plastic mixing stick. Reactions were followed both by continuous fluorescence readings and by repetitive scanning of the excitation spectrum at timed intervals. Some reactions were also carried out at temperatures down to −60 °C and with 60% methanol.

Trapping Experiments. Samples of substrate I (193 μM) or substrate II (208 μM) were prepared in 60% methanol–sodium formate buffer at pH* 3.0 or pH* 5.0 and cooled to −55 °C. Approximately 1 mg of pepsin in 60% methanol was added, and the sample was incubated for 0.5–2 h. The solution was then transferred rapidly to a 3-mL cell in a second block that was also cooled to −55 °C. This cell contained 1.5 mL of 10% Cl_3CCOOH in 70% methanol. The solution was allowed to sit for 1 h and then centrifuged in a cooled centrifuge (−20 °C) for 30 min. The resulting precipitate was washed with 10% Cl_3CCOOH solution 3 times and then with acetone twice. The precipitate was dried with a gentle stream of N_2 and then redissolved in 1 mL of NH_4HCO_3 at pH 8.0. The resulting solution was then scanned in the Perkin-Elmer 320 for UV absorbance and in the Perkin-Elmer MPF-4 for fluorescence. Amino acid composition for the precipitate obtained in the reaction between substrate I and pepsin was determined on the short column of a Beckman 120C amino acid analyzer.

Results

Suitability of Methanol as the Organic Component of the Cryosolvent. The low freezing points and viscosities of methanol cryosolvents make them the prime choice for subzero temperature investigations. Previous workers have reported on the inhibition of pepsin action by the addition of alcohols to a reaction mixture (Tang, 1965; Zeffren & Kaiser, 1967). Decreases in k_{cat}/K_m are expected in the presence of organic cosolvent due to hydrophobic partitioning (Maurel, 1978; Fink & Geeves, 1979). We have observed that at 1 °C, pH* 2.89, the reaction rate is decreased 57-fold for substrate II and 675-fold for substrate I in 50% methanol compared to aqueous solution. We have also found, however, that the full activity of the enzyme is restored upon dilution into 100% aqueous solution and does not diminish with time of incubation in 50% methanol. Therefore, the enzyme suffers no irreversible harm in 50% methanol solution. In addition, solutions of pepsin, up to 20 mg/mL in 50% methanol, retain full activity when stored in a freezer at approximately −20 °C for at least 2 weeks. Furthermore, no major changes in enzyme structure are indicated by fluorescence upon going from 0 to 50% methanol under the above conditions since only a linear increase in fluorescence is observed (Table I) which can be attributed to solvent effects on exposed aromatic residues (Fink, 1979). However, as illustrated in Figure 1, where the rate of hydrolysis of substrate I as a function of the concentration of methanol is plotted, there is a nonlinear response of rate to methanol concentration. This suggested either a methanol-induced structural change or a perturbation due to a change in the pH*–rate profile and led to the studies of UV melting curves reported next.

Table I: Effect of Methanol on the Fluorescence of Porcine Pepsin[a]

	pH 2.80		pH 3.97	
% MeOH[b]	λ_{max} emission (nm)[c]	ΔF^d (%)	λ_{max} emission (nm)[c]	ΔF^d (%)
0	336		336	
10	336	6.1	336	13.9
20	336	10.5	335	60.1
30	335	12.2	334	81.8
40	334	22.8	334	112.1
50	334	26.3	333	106.9

[a] 1.43 μM porcine pepsin in 0.02 M sodium formate buffer of the indicated pH. [b] Volume per volume. [c] Excitation was at 260 nm in all cases. [d] Increase in fluorescence intensity relative to fluorescence intensity in aqueous solution.

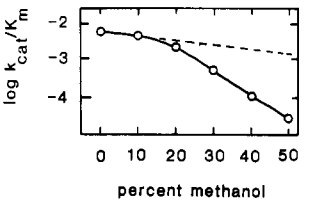


FIGURE 1: Pseudo-first-order rate of hydrolysis of 16.2 μM substrate I as a function of percent methanol at 1 °C. The observed rate of hydrolysis was divided by the concentration of enzyme used to give the ratio k_{cat}/K_m. Assays were done at pH* 2.89 in 0.02 M formate buffer.

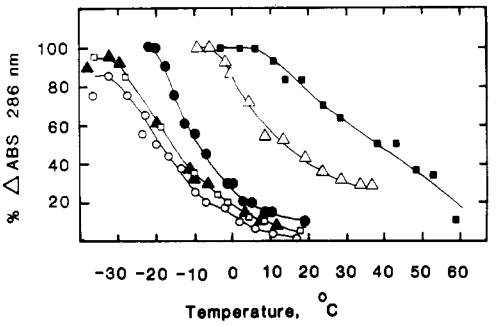


FIGURE 2: Plot of the percent of the total observed change in absorbance at 286 nm vs. the temperature of the solution for the following: 10% methanol–water, pH* 3.30 (■); 20% methanol–water, pH* 3.31 (△); 30% methanol–water, pH* 3.43 (●); 50% methanol–water at pH* 2.2 (○), pH* 3.22 (□), and pH* 5.20 (▲).

Influence of Methanol on the Melting Transition of Pepsin. As shown in Figure 2, the midpoint of the thermal melting curve of pepsin is displaced to progressively lower temperature as increasing amounts of methanol are added. It is obvious from Figure 2 that at 0 °C the amount of fully "native" pepsin in the presence of >20% methanol would be quite low, thus accounting for the low activity observed. However, we were also able to show that the addition of a high-affinity ligand can partially increase the enzyme's resistance to this effect of

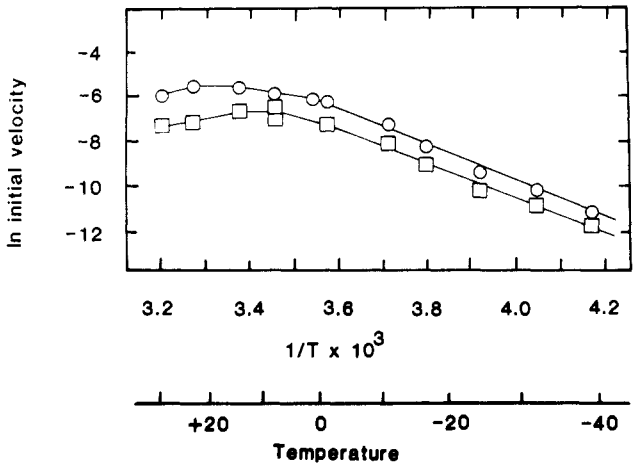


FIGURE 3: Arrhenius plot of natural log of the initial velocity for substrate I hydrolysis in 50% methanol–water at pH* 3.45 (□) and pH* 5.15 (○) vs. the reciprocal of the absolute temperature (in degrees kelvin). A second temperature scale in degrees centigrade is provided for convenience.

organic solvent. When a complex was formed between pepsin and pepsinogen-(1–12) peptide (Dunn et al., 1983), the midpoint of the melting curve in 50% methanol, pH 5.2, increased from −16 to −6 °C.

Hydrolysis of Substrate I as a Function of Temperature. Since we were confident that methanol was a reasonable cryosolvent for pepsin, we carried out a series of assays over the temperature range from +39.4 to −40 °C. At 0 °C, we examined the response of rate to substrate concentrations from 12 to 110 μM. The response was essentially linear, thus demonstrating that under these conditions [S] ≪ K_m. Therefore, $v = (k_{cat}E_0/K_m)[S]$, and we can use the initial velocity as a measure of k_{cat}/K_m. At no point did the change in absorbance deviate from a pseudo-first-order curve; i.e., no burst reactions were observed. A plot of ln initial velocity vs. $1/T$ is given in Figure 3 for substrate I hydrolysis at pH* 3.45 and 5.15. The implication of this plot will be discussed below.

We attempted to study the reaction at even lower temperatures but encountered difficulties in the form of precipitation when the substrate concentration was increased. A reaction was allowed to proceed overnight in 50% methanol at −40 °C. Enzyme was added first to pH* 3.0 buffer, and the renaturation reaction was allowed to proceed to completion. At that point, addition of substrate caused a small additional increase which was followed by a 3-h period without any absorbance changes. At the end of that time, an increase in the "noise" of the recording was followed by a rapid decrease in absorbance as precipitation occurred. An aliquot of the supernatant was assayed with a fresh solution of substrate I and shown to contain all the original pepsin activity. The precipitate was redissolved in buffer at room temperature and shown by spectra to consist of unreacted substrate. When enzyme was added to this solution at room temperature, hydrolysis proceeded smoothly.

Hydrolysis of Substrate II in Aqueous Methanol. Utilizing the parameters for fluorescence that we have established for substrate II, we studied the pepsin-catalyzed hydrolysis at 1.3 °C in methanol–water mixtures. Up to 20% methanol, where the rates are still fast enough and the spectral change large enough for accurate kinetics, the resulting Lineweaver–Burk plots indicate that the K_m value (0.032 ± 0.005 mM) remains relatively constant from 0 to 20% MeOH while the value of k_{cat} decreases from 127 s^{-1} in water to 20 s^{-1} in 20% methanol. The decrease in k_{cat} cannot be attributed to the effect of methanol on the pK_a of catalytic groups since the pH dependence of k_{cat} and K_m for both substrates I and II is flat between pH 2 and 5 (Deyrup & Dunn, 1983; Dunn et al., 1984).

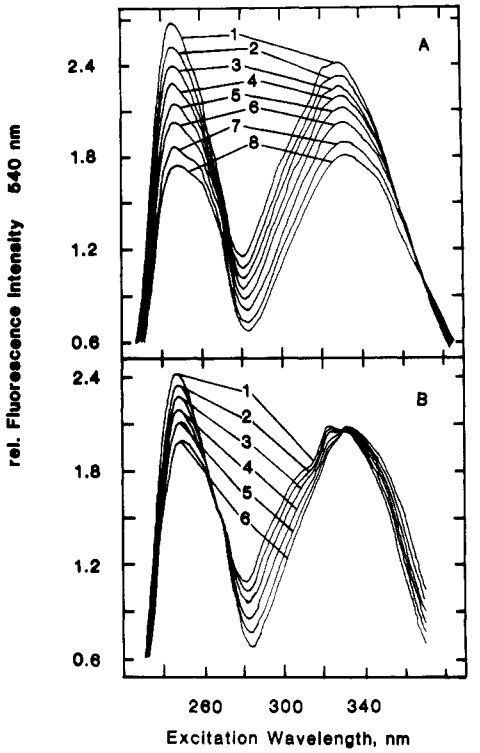


FIGURE 4: Plots of relative fluorescence intensity vs. excitation wavelength (in nanometers) for substrate II (A) and the product DNS-Ala-Ala-Phe (B) at various temperatures in 50% methanol–water. (A) curve 1, −42.8 °C; curve 2, −38.0 °C; curve 3, −32.8 °C; curve 4, −27.3 °C; curve 5, −21.2 °C; curve 6, −13.4 °C; curve 7, −5.4 °C; curve 8, 0.0 °C. (B) Curve 1, −43.2 °C; curve 2, −38.2 °C; curve 3, −31.2 °C; curve 4, −21.8 °C; curve 5, −11.8 °C; curve 6, −0.6 °C.

We then went to 50% methanol–water mixtures to permit the study of these reactions at lower temperature. In order to compensate for the rate depression by methanol and by the lower temperature, we increased the concentration of enzyme from 0.4 μg/mL to several hundred micrograms per milliliter. The increased pepsin concentration becomes a problem with respect to inner filter effects on the fluorescence emission of substrate II. For this reason, we switched the wavelength of excitation for kinetic runs from 300 to 320 nm. However, as the temperature of reaction was decreased, we observed a smaller and smaller change in fluorescence upon hydrolysis. The origin of this decrease can be observed in the temperature dependence of the fluorescence of the substrate and product shown in Figure 4, where the product has an isosbestic point at 320 nm. To further characterize the spectral changes, we titrated the substrate and product with the results given in Figure 5. From these data, pK's were calculated of 3.25 for substrate II and 3.65 for the product DNS-Ala-Ala-Phe. In both cases, this pK_a refers to the dimethylamino function of the dansyl group. Since the imposition of spectrally sensitive protonation as well as temperature-dependent spectra causes considerable variability in the observed spectral changes at fixed excitation and emission wavelengths, we have followed the low-temperature hydrolysis of substrate II by scanning the excitation spectra at timed intervals. From the resulting data, appropriate choices of data sets could be replotted to give kinetic curves. Despite the influence of added pepsin upon the fluorescence spectrum, seen most clearly in Figure 6 as a decrease from curve 0 to curve 1 in the 250–280-nm region, the hydrolysis reaction can be conveniently observed by the change in spectra with 300-nm excitation and 540-nm emission

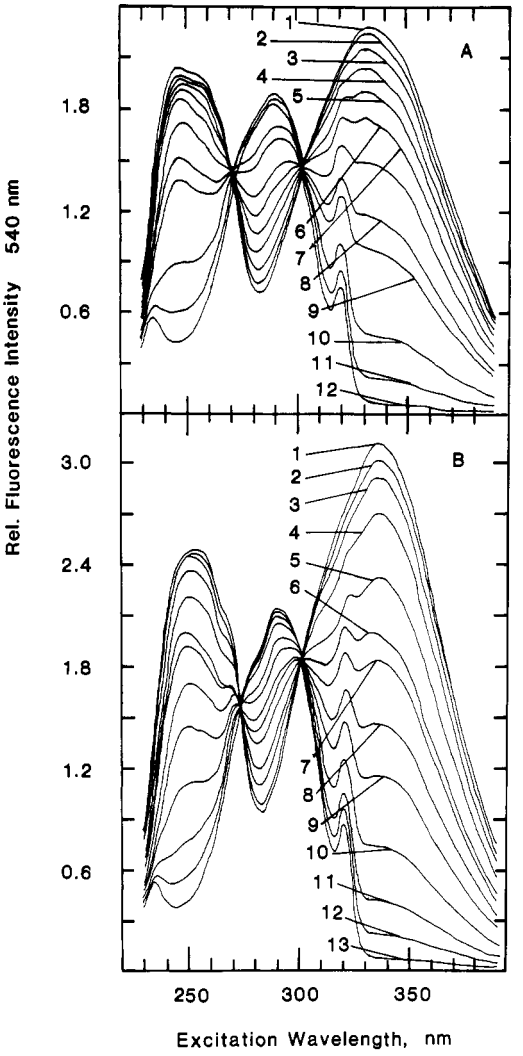


FIGURE 5: Plots of relative fluorescence intensity vs. excitation wavelength (in nanometers) for substrate II (A) and the product DNS-Ala-Ala-Phe (B) at various apparent pHs in 50% methanol–water. (A) Curve 1, pH* 5.03; curve 2, pH* 4.61; curve 3, pH* 4.28; curve 4, pH* 4.03; curve 5, pH* 3.83; curve 6, pH* 3.67; curve 7, pH* 3.48; curve 8, pH* 3.22; curve 9, pH* 2.85; curve 10, pH* 2.57; curve 11, pH* 2.29; curve 12, pH* 1.97. (B) Curve 1, pH* 5.58; curve 2, pH* 5.04; curve 3, pH* 4.63; curve 4, pH* 4.36; curve 5, pH* 4.05; curve 6, pH* 3.82; curve 7, pH* 3.75; curve 8, pH* 3.54; curve 9, pH* 3.36; curve 10, pH* 3.08; curve 11, pH* 2.78; curve 12, pH* 2.49; curve 13, pH* 2.11.

for reactions carried out at pH* 3.1. An Arrhenius plot derived from resulting kinetic curves is shown in Figure 7.

Trapping Experiments. Conditions were found that would lead to the denaturation and precipitation of the protein from reaction solutions at subzero temperatures. Experiments were set up to permit the trapping of significant amounts of substrate or substrate fragments involved in covalent interactions with the enzyme. The results of such an experiment are shown in Figure 8, where the fluorescence due to the redissolved pepsin is readily observable, but where there is no apparent fluorescence due to dansyl-substituted components in the precipitate. When the precipitate from the reaction between substrate I and pepsin was examined, the amount of *p*-nitrophenylalanine observed (<2 nmol) was the same as that seen in control experiments where the precipitated enzyme is mixed with substrate after denaturation.

Discussion

The goal of these experiments was to determine whether or not we could observe evidence for a covalent acyl or amino

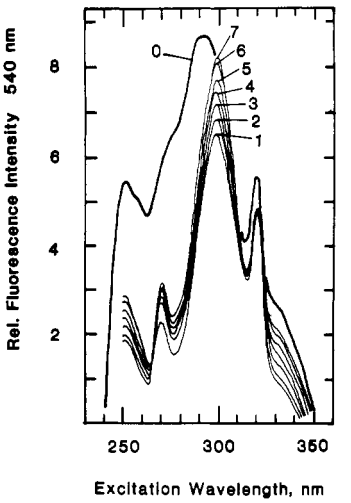


FIGURE 6: Scans of relative fluorescence intensity vs. wavelength (in nanometers) for substrate II in 50% methanol at −28.6 °C in the absence of pepsin (curve 0) and after adding pepsin at 0 (curve 1), 3 (curve 2), 6 (curve 3), 11 (curve 4), 18 (curve 5), 36 (curve 6), and 48 min (curve 7).

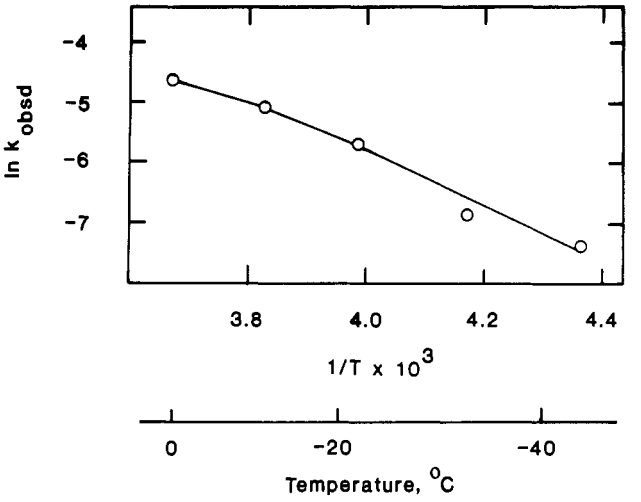


FIGURE 7: Arrhenius plot of ln k_{obsd} for hydrolysis of substrate II in 50% methanol vs. reciprocal temperature in degrees kelvin. A temperature scale in degrees centigrade is provided for convenience.

intermediate in pepsin catalysis at subzero temperatures. The first step in the investigation was to find a suitable cryosolvent. It has been known for some time that the activity of pepsin is reduced in the presence of alcohols. This has been attributed to a competitive effect of binding at the active site. The results in this paper show that there is a readily observable shift in the midpoint of the thermal melting transition of pepsin with the presence of methanol. The midpoint in a 100% aqueous solution is at approximately 61 °C. In 10% methanol–water, the midpoint has been reduced to about 36 °C. Thus, assays carried out in the presence of alcohols at physiological temperatures are likely to include a significant proportion of denatured pepsin. Thus, an apparent rate reduction would be seen due to this effect. Thus, the nonlinearity of the data in Figure 1 could be attributed to the presence of denatured enzyme at the higher methanol concentration.

Of more importance to the present study is the observation that this transition is fully reversible, does not lead to inactivation of the protein, and is partly reversed by the addition of ligands. This can be seen in the Arrhenius plot for substrate I in 50% methanol (Figure 3), which is linear from −40 °C up to approximately 0 °C even though the midpoint of the thermal transition is −16 °C (see Figure 2). Apparently the

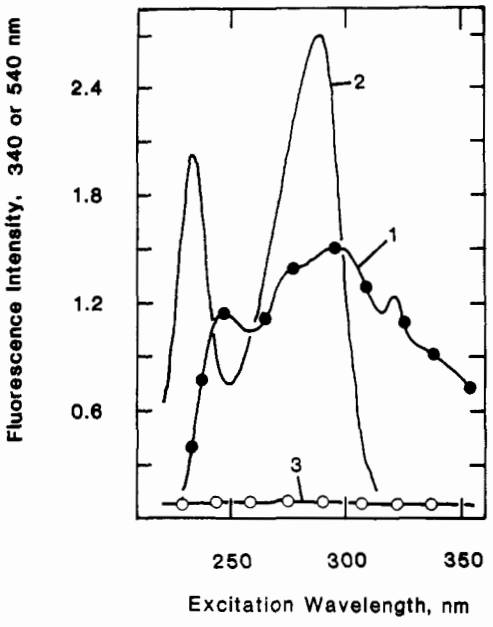


FIGURE 8: Plots of relative fluorescence intensity at either 340 or 540 nm vs. excitation wavelength. Scan 1, excitation spectrum (emission at 540 nm) of substrate II at −56 °C in 60% methanol at pH* 3.1. Scan 2, excitation spectrum (emission at 340 nm) of pepsin in redissolved precipitate from the trapping experiment. Scan 3, excitation spectrum with emission at 540 nm of redissolved precipitate from trapping experiments, illustrating the lack of presence of the dansyl component.

binding of substrate I causes a shift in the thermal transition of as much as 30 °C. At temperatures above 0 °C, curvature in the Arrhenius plot is apparent. We ascribe this to the unfolding of pepsin in this solvent. This downward curvature is seen at both pH* 5.15 and pH* 3.45, which agrees with the insensitivity of the thermal transition to pH* shown in Figure 2 for 50% methanol.

The linearity of the Arrhenius plot for substrate I can be taken as evidence that there is neither a change in the rate-limiting step for catalysis nor a change in the temperature-induced structure change over the temperature interval observed. In addition, the shift in the thermal transition alluded to above almost certainly implies that considerable substrate binding occurs. Thus, it is reasonable to assume that the rate-limiting step we observe under these conditions occurs after substrate binding.

Although the effects of methanol and subzero temperatures on the catalytic and structural properties of the enzyme were probed with a minimum of experiments, the results suggest that they do not cause any adverse effects which would affect the catalytic mechanism at subzero temperatures. In the various pepsin reactions we have observed at subzero temperatures, no evidence was obtained for any burst of product formation. Experiments were performed at very high concentrations of enzyme (ca. 20 μM) such that a single turnover would yield a readily observable spectral shift. In no case was such a burst seen. The implication of this is that no stable acyl intermediate is formed under these conditions.

Similar observations were obtained in studies of substrate II. Due to the complex nature of the dansyl chromophore and the pH dependence of the spectra, the direct observation of substrate II hydrolysis was not as straightforward as substrate I hydrolysis. We believe the origin of the difficulty lies in the complex temperature and pH dependence of the spectra. The low-pH form of the dansyl group has an excitation maximum between 290 and 300 nm while the high-pH form has excitation maxima at 240–250 and 330–340 nm. As the temperature drops in a buffered solution, however, the contribution to the spectra from the acid form increases greatly. Compounding this complexity is the difference in pK_a of the substrate dansyl group and the product dansyl group. Taken together, these results lead to the observation that at certain pHs and temperatures, the conversion from substrate to product does not give a shift in spectra that can be observed.

Despite these problems, we have found conditions, pH* 3.1 in 50% methanol, where the hydrolysis can be followed down to −40 °C (Figure 6). In Figure 6, the large decrease in fluorescence intensity observed at shorter excitation wavelengths is due to inner filter effects from the high levels of pepsin utilized. The changes shown in Figure 6 at 300 nm give the same kinetic curves as those shown at 330–340 nm; therefore, the influence of the pepsin absorbance does not affect the subsequent spectral shifts.

Such data have been used to construct the Arrhenius plot in Figure 7. The plot is basically linear with leveling out seen at the higher temperatures. This decrease in rate at higher temperatures is presumably due to the onset of thermal denaturation.

The hydrolysis of substrate II provided no evidence for any burst reactions under the conditions we have employed. Again, the implication is that no stable amino intermediate is formed in this reaction.

In addition, attempts at trapping intermediate enzyme complexes have been unsuccessful. As can be seen most directly in Figure 8, substrate II offers an advantage in that the fluorescence can be observed independently of the protein absorbance or fluorescence. Thus, any dansylated fragments would be observable. However, no fluorescence due to dansyl is observed when the precipitate is redissolved. The precipitate from reaction mixtures with substrate I yielded the same amount of the *p*-nitrophenylalanine group in control experiments as in the test experiment. Thus, the two results for these different substrates again argue that no stable intermediates are formed in the hydrolysis reaction of these substrates by pepsin.

Despite the large number of publications on various experimental approaches, there are few unambiguous studies of the pepsin mechanism. The reasons for this are numerous, including the fact that most early work was carried out with acylated dipeptides that are now recognized as very poor substrates. In addition, the imposition of transpeptidation reactions, which are of major significance for some substrates, has also complicated mechanistic assessment.

Unfortunately, the absence of a product burst or trapped enzyme-substrate moiety is negative evidence in the sense that we cannot eliminate the possibility that covalent intermediates are on the productive catalytic pathway, but are not detected for kinetic reasons. Thus, the results reported in this paper are consistent with the conclusion either that pepsin utilizes a general base mechanism to hydrolyze good substrates or that the rate-limiting step precedes the formation of any covalent intermediates. In addition, as reported in the following paper (Hofmann & Fink, 1984), similar results have been obtained for the closely related enzyme penicillopepsin. The implications of our findings and the limitations of our conclusions are discussed more fully in the Appendix accompanying the following paper (Hofmann & Fink, 1984).

Registry No. I, 90331-82-1; II, 85353-23-7; methanol, 67-56-1; pepsin, 9001-75-6.

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Cryoenzymology of Penicillopepsin†

Theo Hofmann* and Anthony L. Fink

Appendix: Mechanism of Action of Aspartyl Proteinases‡

Theo Hofmann, Ben M. Dunn,§ and Anthony L. Fink

ABSTRACT: Intrinsic spectral and kinetic properties of penicillopepsin and its action on *N*-acetylalanylalanyllysyl-*p*-nitrophenylalanylalanylalanine amide have been investigated at subzero temperatures in aqueous methanol and dimethyl sulfoxide solutions in an attempt to find evidence for or against a covalent mechanism in the catalyzed hydrolysis of peptide bonds. The study of fluorescence and circular dichroism spectra as a function of solvent concentrations gave no evidence for any solvent-induced structural effects at temperatures below the thermal denaturation transition. The effect of temperature on the intrinsic fluorescence of penicillopepsin in either 60% (v/v) methanol or 50% (v/v) dimethyl sulfoxide did not indicate any temperature-induced structural changes. On the other hand, Arrhenius plots for the hydrolysis reaction over the range 0 to −50 °C showed downward curvature. A probable explanation for this phenomenon is that the reduction in flexibility of the enzyme due to thermal and viscosity factors leads to the stabilization of a nonproductive conformation. The pH optima of k_{cat}/K_m are shifted from 5.1 in aqueous solvents to 5.6 in 60% methanol and to 6.6 in 50% dimethyl sulfoxide. Aqueous methanol caused small decreases of K_m and of k_{cat}; the decrease in the latter was greater than that brought about by the decrease in the water concentration. In aqueous dimethyl sulfoxide, there was no detectable change in k_{cat} up to 15%, but K_m increased by more than an order of magnitude. Above 15%, only k_{cat}/K_m could be measured. No evidence for the accumulation of either covalent amino or covalent acyl intermediates was obtained when penicillopepsin was incubated at −70 °C in 67% methanol with several substrates. Although negative, these experiments do not rule out conclusively the involvement of covalent intermediates in penicillopepsin-catalyzed reactions.

Penicillopepsin is a member of the aspartyl proteinases, enzymes which are characterized by the involvement of two aspartyl residues in the hydrolysis of peptide bonds. Three-dimensional structures have been obtained by X-ray analysis for four aspartyl proteinases, penicillopepsin at 1.8-Å resolution (James & Sielecki, 1983), pig pepsin at 2.7 Å (Andreeva et al., 1978), *Rhizopus* pepsin at 2.5 Å (Bott et al., 1982), and *Endothia* pepsin at 2.7 Å (Jenkins et al., 1977). They show a remarkable similarity of folding of the peptide backbone. In spite of the detailed knowledge of the structure, there are at present no proposals for the mechanism of action that can account satisfactorily for *all* the observed hydrolytic and transpeptidation reactions catalyzed by these enzymes. Above all, the question as to whether the peptide bond hydrolysis proceeds by a noncovalent or by a covalent mechanism has not been answered satisfactorily. We felt that studies at low temperature would help to contribute to our understanding of the fundamental steps of the mechanism of these enzymes. Cryoenzymological studies on penicillopepsin in mixed organic–aqueous solvents were therfore initiated in parallel with similar studies on pig pepsin (Dunn & Fink, 1984). We especially hoped that putative intermediates might accumulate

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