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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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and could be trapped, or at the very least that "burst" reactions would be observed. As a preliminary step toward detailed kinetic studies at low temperature, we now report the characterization of the molecular and enzymic behavior of penicillopepsin in mixed organic–aqueous solvents which allow the study of the enzyme down to −80 °C. Some preliminary experiments designed to trap intermediates are also described.

Experimental Procedures

Materials

Penicillopepsin was prepared as described (Hofmann, 1976). Leu-Ser-(NO_2)Phe-Nle-Ala-Leu-OMe[1] was obtained from Vega Fox Biochemicals (Tucson, AZ); DNS-Ala-Ala-Phe-Phe-OP4P$^+$-CH_3 was a gift from Dr. Ben M. Dunn. The following peptides were synthesized: Ac-Ala-Ala-Lys-(NO_2)Phe-Ala-Ala amide and Ac-Lys-(NO_2)Phe amide (Hofmann & Hodges, 1982), Leu-Ser-Nle-(NO_2)Phe-Ala-Leu amide and Ala-Ala-Nle-(NO_2)Phe-Ala amide (T. Hofmann and R. S. Hodges, unpublished results). Me_2SO (reagent grade) from Mallinckrodt was distilled from calcium hydride under vacuum at 37 °C and stored at 4 °C. Anhydrous methanol (spectral grade) from Mallinckrodt was used without further purification. Cryosolvents were prepared on a volume per volume basis at 0 °C.

Methods

The apparent protonic activity, pH* (the apparent pH in organic–aqueous solvents), was determined at 0 °C with a Radiometer 26 pH meter fitted with a Beckman 39505 combination glass–calomel electrode. Values of pH* at subzero temperatures were obtained from the know temperature dependence of pH* (Hui Bon Hoa & Douzou, 1973). Buffer systems used were 10 mM acetate (unless otherwise noted) made up to an ionic strength of 10 mM with KCl.

Fluorescence spectra were obtained on a Perkin-Elmer MPF-4 spectrofluorometer (Perkin-Elmer, Mountainview, CA) equipped with a special thermostated sample cell holder. Excitation was at 296 nm. No filters were used in the emission light path. Circular dichroism spectra were obtained with a Jasco-Durrum J-41A spectropolarimeter equipped with a thermostated insulated brass block sample cell holder. A 1-cm path-length cell was used. The thermostated cell holders were maintained at constant temperatures (±0.1 °C) by circulating ethanol from a Hetofrig Ultra cryotherm (Heto, Birkerod, Denmark) or from Neslab Models ULT 80, LTE 9, or RTE 8 constant-temperature baths (Neslab, Portsmouth NH). A stream of dry nitrogen over the faces of the quartz cuvettes was used to prevent condensation.

Kinetic experiments with Ac-Ala-Ala-Lys-(NO_2)Phe-Ala-Ala amide as substrate were carried out as described (Hofmann & Hodges, 1982) with a Cary Model 118C or Model 219 spectrophotometer (Varian Instruments, Los Altos, CA) or with a Perkin-Elmer Model 320 spectrophotometer fitted with thermostated cell holders. Because the maximum wavelength of the difference spectrum between the substrate and the protonated form of the product (NO_2)Phe-Ala-Ala amide shifts from 296 nm in aqueous solvents to 298 nm in 60% MeOH and to 302 nm in 50% Me_2SO, assays were performed at these wavelengths or at extrapolated intermediate wavelengths for intermediate concentrations of cryosolvents. At substrate concentrations higher than 0.15 mM, assays were performed at either 306 or 320 nm as described (Hofmann & Hodges, 1982). Corrections were made for the changes in the molecular difference extinction coefficients at the different wavelengths and with the different solvent compositions and changes in temperature. Corrections were also made, where appropriate, for the partial deprotonation of the α-amino group of (NO_2)Phe-Ala-Ala amide which has the following pK_a values: in aqueous solution, pK_a = 6.6 at 25 °C (Hofmann & Hodges, 1982) and 6.75 at 0 °C; in 60% MeOH, pK_a = 6.5 at 0 °C; in 50% Me_2SO, pK_a = 8.1 at 0 °C.

Determination of k_{cat}/K_m. Under conditions where it was not possible to determine K_m because of its high value, initial rates were determined at low substrate concentrations and divided by the latter to give values for k_{cat}/K_m.

Choice of Suitable Cryosolvent. Four criteria were used in the search for suitable solvent mixtures for the low-temperature studies on penicillopepsin. The aqueous cryosolvent must maintain fluidity to as low a temperature as possible, it must not cause aggregation of the protein (which would cause nonspecific absorbance changes), it must not cause unfolding, and the enzyme must retain full activity over prolonged periods of incubation. Fluorescence was the method of choice for testing the aggregation and unfolding. The Rayleigh scattering peak is very sensitive to light scattering in a solution, and any increase in this peak with time was interpreted as indicating protein aggregation. The fluorescence emission spectrum of penicillopepsin is sensitive to unfolding. The X-ray analysis (James et al., 1977; James & Sielecki, 1983) shows that its three tryptophan residues are buried. One of them is located between the two lobes of the molecule; the other two (Trp-40 and Trp-71) are within 10–16 Å of the active-site aspartic acid pair. The ring carbons C^{γ} and C^{δ_2} of Trp-40 are within 3.8 Å of the ring carbon C^{η_2} of Trp-71 and are thus close enough to allow efficient energy transfer. Trp-40 and Trp-71 are close to the flexible Tyr-75 flap which is hinged at the C^{α} atom of Trp-71 (James et al., 1982). Some residues of this flap are involved in binding a pepstatin analogue in the active site and presumably also in substrate binding (James et al., 1982). The average quantum yield of the three tryptophan residues is about 3.1 times as high as that of Ac-Trp amide in aqueous solution (unpublished results). Because of the buried nature of these residues, the maximum fluorescence emission in aqueous media is at 322 nm, blue shifted from 356 nm of the model compound. The fluorescence emission spectrum thus provides a sensitive probe for the loosening of both the Tyr-75 flap and the interaction(s) between the two major lobes of the molecule. A loosening of the structure in either of these two areas would result in a red shift of the emission, in broadening of the fluorescence peak, and in decreases in quantum yields, as shown by the fluorescence spectrum of penicillopepsin denatured for 10 min at pH 7.5 and room temperature which shows a large decrease in intensity and a shift in λ_{max} [Figure 1A (−··−)].

The fluorescence spectra of penicillopepsin in a variety of cryosolvents (MeOH, Me_2SO, EtOH, dimethylformamide, acetonitrile, dioxane, and combinations with ethylene glycol) were examined over a wide range of pH* values, from 2.0 to 8.0. The most suitable solvents found were 60% MeOH–0.01 M acetate, pH* 6.0–6.6, and 50% Me_2SO–0.01 M acetate, pH* 4.0–7.5. In MeOH at higher pH* values, penicillopepsin unfolded slowly; at lower pH* values or higher MeOH concentrations, it aggregated strongly over several hours. This aggregation, however, was insufficient to interfere with the experiments needed to determine the pH profile in 60% MeOH

[1] Abbreviations: Ac, acetyl; Me_2SO (DMSO in figures), dimethyl sulfoxide; DNS, 5-(dimethylamino)-1-naphthalenesulfonyl; EtOH, ethanol; MeOH, methanol; (NO_2)Phe, *p*-nitrophenylalanine; Nle, norleucine; OMe, methyl ester; OP4P$^+$-CH_3, 3-(*N*-methylpyridinium-4-yl)propyl ester; Cbz, benzyloxycarbonyl; RLS, rate-limiting step.

at 0 °C. In Me_2SO at pH* below 4.0 or above 7.5, or at higher Me_2SO concentrations at any pH*, slow unfolding was observed, but no aggregation occurred under any conditions. Penicillopepsin, when stored in 60% MeOH–0.01 M acetate, pH* 6.5 at −50 °C, or in 50% Me_2SO–0.01 M acetate, pH* 5.0–7.3 at 0 °C or at −50 °C, did not lose any activity in 24 h. The enzyme was also stable in 60% MeOH at pH* 6.5, 15 °C, for 3 h. The absence of aggregation in both MeOH and Me_2SO solvents at −50 °C was confirmed by examining UV spectra at that temperature as a function of prolonged incubation.

Trapping Experiments. Penicillopepsin (3 mg, 90 nmol) in 0.7 mL of 50% MeOH–0.01 M acetate, pH* 6.5, was cooled to −76 °C in a thick-walled Pyrex tube (13 × 150 mm) in a dry ice–EtOH bath. A solution of substrate (3 μmol) in 0.5 mL of 60% MeOH–H_2O and 0.4 mL of MeOH, both at −76 °C, were rapidly added and mixed with the enzyme solution by means of a precooled "plumper". The final concentration of MeOH was 67%. The mixture was left at −76 °C for 24–28 h. A solution of 50% trichloroacetic acid in MeOH (w/v) (0.46 mL) at −76 °C was then added and rapidly mixed with the incubate. After 30 min, a mixture of acetone–ether (50% v/v; 3 mL) at −76 °C was added and the precipitate centrifuged in a Sorvall Model RS-2B centrifuge with an SS 20 rotor. The centrifuge compartment was kept at −20 °C. The tube containing the reaction mixture was placed in a 50-mL centrifuge tube and packed with dry ice–EtOH before centrifugation for 10 min at 5000 prm. During this time, the temperature in the reaction tube did not rise above −76 °C as long as dry ice remained in the container. The supernatant was decanted rapidly and concentrated in a rotary evaporator for analysis of products (see below). The precipitate was suspended in 10% trichloroacetic acid–MeOH (3 mL) at −76 °C and centrifuged again. The precipitate was then washed once more with 10% trichloroacetic acid–MeOH (3 mL) and twice with acetone (5 mL). All solvents were kept in a dry ice–EtOH bath. Centrifugation of the washings for 5 min was done at −76 °C. The tubes with the precipitate were drained well. Ammonium bicarbonate buffer (0.1 M, 1 mL) was added to the damp precipitates. The enzyme readily dissolved and was freeze-dried and hydrolyzed with 6 N HCl (0.2 mL). The hydrolysate was analyzed on an "extended" short column (0.9 × 18 cm) of a Beckman-Spinco Model 121C amino acid analyzer with 0.35 M citrate buffer, pH 5.23, used for short-column analyses. This system gives very good separation of *p*-nitrophenylalanine and the basic amino acids. Residual nitrophenylalanine from substrates containing this amino acid was calculated by comparison with the histidine and lysine peaks. Molar ratios were based on the fact that penicillopepsin has five lysines and three histidines.

Controls were carried out in which the enzyme alone was incubated and substrate solution was added immediately after the trichloroacetic acid. Trichloroacetic acid effectively inactivated the enzyme. A sample taken immediately after its addition was assayed with Ac-Ala-Ala-Lys-(NO$_2$)Phe-Ala-Ala amide at 0 °C and was completely inactive.

The supernatants obtained after the concentration procedure (see above) were freeze-dried. The residues were dissolved in 100 μL of electrophoresis buffer at pH 3.6 (200:20:1780 (v/v) acetic acid:pyridine:water; Kurosky & Hofmann, 1976). A portion (10 μL) was analyzed by high-voltage electrophoresis at pH 3.6.

Results and Discussion

Effect of Cryosolvents on Fluorescence and Circular Dichroic Properties of Penicillopepsin. The fluorescence

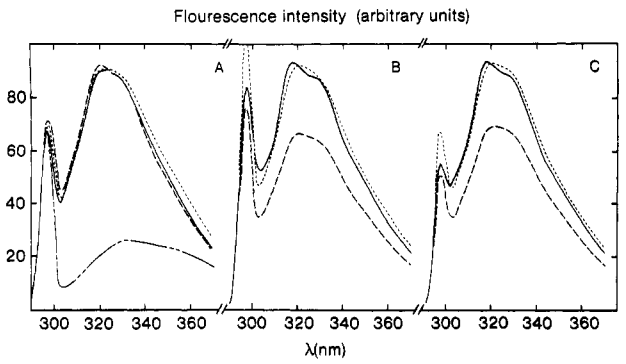

FIGURE 1: Comparison of fluorescence emission spectra of penicillopepsin in cryosolvents and as a function of temperature. (A) Fluorescence spectra of penicillopepsin (3.05 μM) in 0.01 M acetate, pH 5.5 (—); in 60% MeOH–0.01 M acetate, pH* 6.53 (- - -), and in 50% Me_2SO–0.01 M acetate, pH* 7.3 (···), were recorded at +0.2 °C and at the same instrument sensitivity. Penicillopepsin denatured in 0.01 M phosphate, pH 7.5, after 10 min at 22 °C (—·—) was also recorded; for this spectrum, the sensitivity of the fluorometer had been adjusted so that a spectrum of native enzyme at pH 5.5, 22 °C, coincided with the spectrum shown in the solid curve. (B) Fluorescence spectra of penicillopepsin (3.05 μM) in 60% MeOH–0.01 M acetate, pH* 6.53, were recorded at −1.6 (- - -) and −52 °C (—). For comparison of the spectral shape, the 1.6 °C spectrum is also shown after it was normalized at 320 nm with the −52 °C spectrum (···). (C) Fluorescence spectra of penicillopepsin (3.05 μM) in 50% Me_2SO–0.01 M acetate, pH* 7.33, were recorded at +1.2 (- - -) and −53 °C (—). The spectrum at +1.2 °C was also normalized at 320 nm with the −53 °C spectrum (···). Excitation for all spectra was at 296 nm, where the Rayleigh scattering peak is observed.

emission spectrum of penicillopepsin in 0.01 M acetate at pH 5.5 (Figure 1A, solid line) shows a maximum at 322 nm and a distinct shoulder at about 326 nm. It is probable that these represent two emission bands, one originating from Trp-191 and the other from the Trp-40–Trp-71 pair. The spectra in 60% MeOH and in 50% Me_2SO show that the solvents have no effect on the quantum yield of the tryptophan residues whereas the quantum yield of the model compound Ac-Trp amide increases 2-fold in 60% MeOH. This is not surprising in view of the fact that these residues are buried and that their average quantum yield at 25 °C is about 3.1 times that of Ac-Trp amide. The solvents to cause small highly reproducible changes in the shape of the spectra. Thus, in MeOH, the maximum is shifted to 319.5 nm and is more pronounced. The small blue shift of the trailing edge of the spectrum suggests that the 326-nm band is also somewhat narrowed. The most probable explanation is that the motility of the molecule in MeOH is reduced. Although the tryptophan residues are buried in the crystal, as shown by X-ray crystallography, in solution there is limited access of solvent, as shown by solvent perturbation studies in ethylene glycol (unpublished results). The most mobile part of the molecule is the Tyr-75 flap (James et al., 1982) which is hinged at the C^{α} atom of Trp-71 and presumably controls access of solvent to the tryptophan pair. The concept of reduced motility is supported by the spectrum of penicillopepsin in 60% MeOH at −52 °C (Figure 1B, solid line) which shows further sharpening of the spectrum and a blue shift of the maximum to 317.5 nm. (The dotted line in Figure 1B is the spectrum in 60% MeOH at 0 °C, normalized at 320 nm.)

In 50% Me_2SO at 0 °C (Figure 1A, dotted lined, and Figure 1C, dashed and dotted lines), the maximum is within experimental error the same as that in water, but the band represented by the shoulder at 326 nm is shifted to higher wavelength. This suggests that in this solvent the structure of penicillopepsin is somewhat looser and probably results in increased contact with Me_2SO which would shift the emission

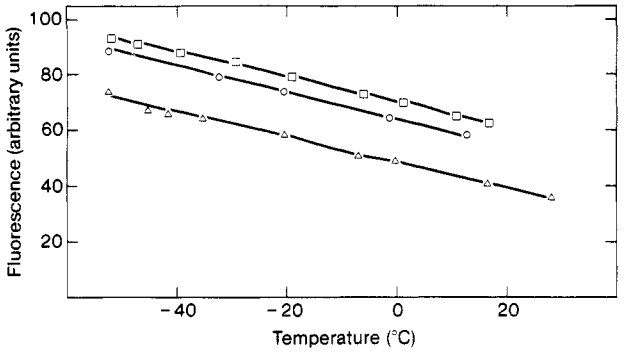

FIGURE 2: Effect of temperature on fluorescence intensity. Emission spectra were recorded at temperatures between +28 and −52 °C. The fluorescence intensities at the maximum emission wavelengths are plotted against temperature: (a) penicillopepsin (62 μg/mL; 1.8 μM) in 60% MeOH–0.01 M acetate, pH* 6.53 (○); (b) penicillopepsin (69 μg/mL; 2.06 μM) in 50% Me_2SO–0.01 M acetate, pH* 7.33 (□); (c) Ac-Trp amide (6.2 μM) in 60% MeOH–0.01 M acetate, pH* 6.53 (△).

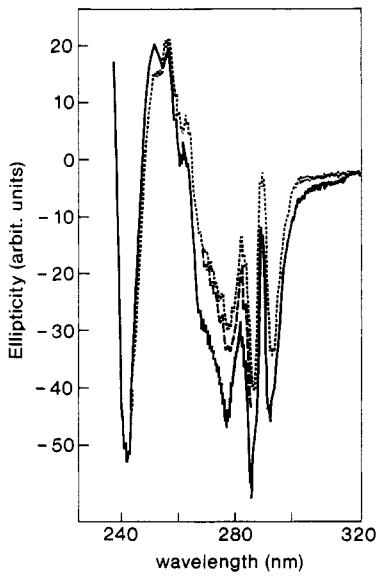

FIGURE 3: Effect of cryosolvents on near-UV circular dichroic spectra of penicillopepsin. Spectra of penicillopepsin (35 μM) in 0.01 M acetate, pH 5.0 (---), in 60% MeOH–0.01 M acetate, pH* 5.6 (—), and in 50% Me_2SO–0.01 M acetate, pH* 6.5 (– –), were recorded at +0.5 °C. A base line obtained with solvent alone was subtracted from the recorded spectra by computer.

to the red, but not decrease the quantum yield. As with MeOH, the spectrum is blue shifted at −53 °C and sharpened (Figure 1C, solid line). The spectrum of mildly denatured penicillopepsin is shown in Figure 1A for comparison.

The temperature dependence of the fluorescence intensity at the maximum is shown in Figure 2 for Ac-Trp amide in 60% MeOH, and for penicillopepsin in 60% MeOH and in 50% Me_2SO. All three curves are linear with comparable slopes, showing that the enzyme does not undergo any major temperature-induced conformational changes between +25 °C and −60 °C.

The near-UV circular dichroism spectra (Figure 3) at 0 °C in water, MeOH, and Me_2SO are very similar. There are no shifts in the positions of the ellipticity bands. The major difference between water and the mixed solvents is that in 60% MeOH the negative bands at 278 and 287 nm and the positive band at 291 nm show increased ellipticity. Minor differences are seen in the 254-nm band which increases in 60% MeOH and in the 278- and 287-nm bands which decrease only slightly in Me_2SO compared to MeOH. The changes in MeOH can

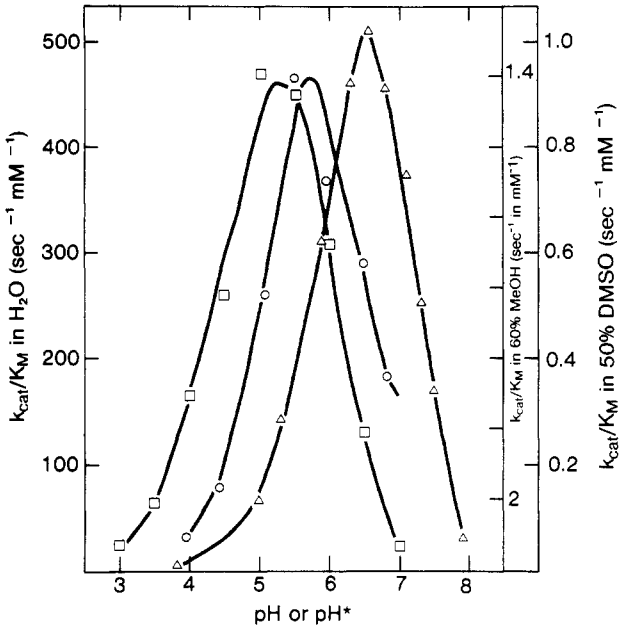

FIGURE 4: Effect of cryosolvents on pH optima of k_{cat}/K_m of penicillopepsin-catalyzed hydrolysis of Ac-Ala-Ala-Lys-(NO_2)Phe-Ala-Ala amide. Initial rates of hydrolysis were determined spectrophotometrically at +0.2 °C, 298 nm, in 60% MeOH–buffer systems (○) or at +0.8 °C, 302 nm, in 50% Me_2SO–buffer systems (△). The buffers used were 0.01 M formate (pH* 3.8–4.5), 0.01 M acetate (pH* 4.8–6.0), or 0.005 M phosphate (pH* 6.0), all adjusted to I = 0.01 M with KCl. For comparison, the pH optimum in aqueous buffers is also included (□) [from Hofmann et al. (1984)]. Corrections for the observed rates were made for the incomplete protonation of the product (NO_2)Phe-Ala-Ala amide at the higher pH values, as described under Methods. k_{cat}/K_m values were calculated by dividing the observed rates by the substrate concentration.

probably also be ascribed to the decreased exposure of the tryptophan (and possibly some tyrosine) residues due to decreased motility, as discussed above. The changes in Me_2SO are so small that they may not be significant although they were reproducible in several independent experiments. No effect of the cryosolvents was observed in the far-UV circular dichroic spectra (not shown).

Effect of Cryosolvents on pH Optima. The effect of 60% MeOH and 50% Me_2SO on the pH optima of k_{cat}/K_m for the penicillopepsin-catalyzed hydrolysis of Ac-Ala-Ala-Lys-(NO_2)Phe-Ala-Ala amide is shown in Figure 4. The previously determined pH optimum in 0.02 M aqueous buffers is shown for comparison. Whereas MeOH causes only a small shift upward of about 0.5 pH unit, the shift in Me_2SO is larger (1.4 pH units). The pH shift in MeOH is of the same order as the pH shift caused by 60% MeOH on carboxylate buffers and presumably represents the shift in ionization of the active-site carboxyl groups. Similarly, the shift in 50% Me_2SO, although larger, corresponds to the effect of that solvent on the ionization of carboxyl groups.

Effect of Cryosolvent Concentration on k_{cat} and K_m. The effects of increasing concentrations of MeOH and Me_2SO on the kinetic parameters of the hydrolysis of Ac-Ala-Ala-Lys-(NO_2)Phe-Ala-Ala amide near 0 °C are shown in Table I and Figure 5. MeOH causes a small increase in K_m (Figure 5A), from 0.052 mM in aqueous solvents to 0.21 mM in 60% MeOH, whereas k_{cat} is decreased to about 15% of its value in aqueous buffer; if the decrease were due solely to the decreased water concentration, a value of 40% would have been expected (Figure 5A, dashed line). The observed values are about half of those expected for the water concentration effect. The effects on both K_m and k_{cat} are compatible with the proposal, based on the fluorescence and circular dichroism

Table I: Effect of Me_2SO on k_{cat} and K_M for the Penicillopepsin-Catalyzed Hydrolysis of Ac-Ala-Ala-Lys-(NO_2)Phe-Ala-Ala Amide[a]

[Me_2SO]		k_{cat}		k_{cat}/K_m
% (v/v)	M	(s^{-1})	K_m (mM)	(mM^{-1} s^{-1})
0	0	9.8	0.052	188.5
2.5	0.32	9.6	0.12	80
5.0	0.64	10	0.22	45
10	1.28	~9	~0.6	~15
15	1.92	≈10	≈1–2	≈5–10

[a] Conditions: 10 mM acetate, pH* 5.05; 0 °C; E_0 = 0.01–0.031 M; S_0 = 0.07–1.2 mM.

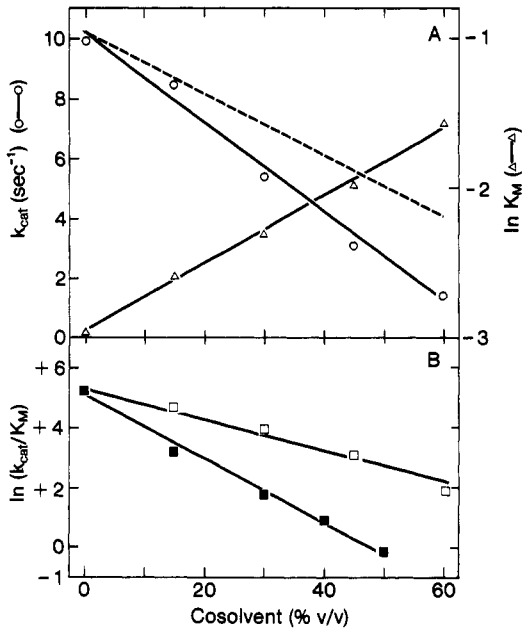

FIGURE 5: Effect of cryosolvents on hydrolysis of Ac-Ala-Ala-Lys-(NO_2)Phe-Ala-Ala amide by penicillopepsin. The kinetic parameters k_{cat}, K_m, or k_{cat}/K_m were determined at different concentrations of cryosolvent under the following conditions. (A) MeOH–0.01 M acetate at +0.8 °C; S_0 = 0.093 mM; E_0 = 0.043–2.18 μM; k_{cat} (○) and ln K_m (△) are plotted. The pH (pH*) values were as follows: 0%, pH 5.1; 15%, pH* 5.2; 30%, pH* 5.3; 45%, pH* 5.45; 60%, pH* 5.6. The dashed line represents the values calculated for the effect of the decreased water concentration. (B) Plot of ln (k_{cat}/K_m) in Me_2SO–0.01 M acetate (■) at 0.6 °C (S_0 = 0.093 mM; E_0 = 0.012–2.18 μM) at the following pH (pH*) values: 0%, pH* 5.05; 15%, pH* 5.4; 30%, pH* 5.75; 40%, pH* 6.2; 50%, pH* 6.6. Also shown in (B) is a plot of ln (k_{cat}/K_m) in MeOH–0.01 M acetate (□); the values were calculated from the separately determined parameters from (A).

studies, that the active-site groove is less flexible in aqueous MeOH and especially exhibits less motility. The fact that the effects are small suggests that MeOH does not cause any changes that affect the overall mechanism significantly. The natural log of K_m has been shown to increase linearly with cryosolvent concentration in nearly all enzymes studied. This increase reflects hydrophobic partitioning of the substrate between solvent and active site (Maurel, 1978; Fink, 1979).

Me_2SO shows a very large effect on K_m (Table I) which could not be measured above 15% cosolvent (K_m ~ 1–2 mM) because the absorbance of the substrate becomes too high in the wavelength range where kinetic measurements can be made. Me_2SO has apparently no effect on k_{cat} (Table I), but at 10% and 15%, the accuracy of the values is such that a small effect due to the lower water concentration would not have been detected. The large effect of Me_2SO on K_m reflects the greater hydrophobicity of this solvent. Except for the experiments with Me_2SO shown in Table I, the kinetic parameters in the mixed solvents were determined at the pH or pH* optima of each solvent concentration in order to have the pH-dependent groups that determine them in the same state of ionization. The actual pH or pH* values used are given in the legend to Figure 5.

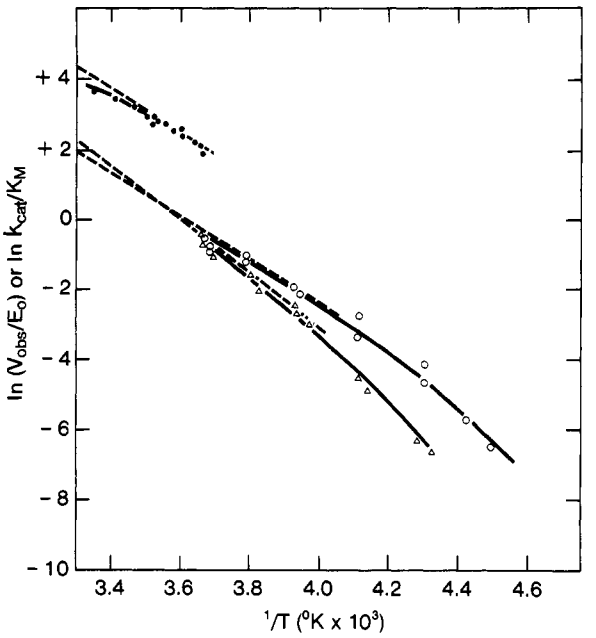

FIGURE 6: Arrhenius plot for the penicillopepsin-catalyzed hydrolysis of Ac-Ala-Ala-Lys-(NO_2)Phe-Ala-Ala amide in cryosolvents. Conditions were the following: (a) 10 mM acetate, pH 5.5; S_0 = 0.16 mM; E_0 = 0.031 μM; ln (V_{obsd}/E_0) is plotted (●); (b) 60% MeOH–10 mM acetate, pH* 6.53; S_0 = 0.075 mM; E_0 = 0.178–8.14 μM; ln (V_{obsd}/E_0) is plotted (○); (c) 50% Me_2SO–0.01 M acetate, pH* 7.3; S_0 = 0.154 mM; E_0 = 0.5–10 μM; ln (k_{cat}/K_m) is plotted (△). The results in each set are from two or three separate experiments. The dashed line was used to calculate $E_{0,actn}$

Effect of Temperature on Penicillopepsin-Catalyzed Hydrolysis of Ac-Ala-Ala-Lys-(NO_2)Phe-Ala-Ala Amide. The Arrhenius plots shown in Figure 6 for an aqueous solvent above 0 °C and for 60% MeOH and 50% Me_2SO below 0 °C are nonlinear. In water, there is a linear part between 0 °C and about +9 °C. The slope of this gives a value of E_{actn} = 12.6 kcal mol^{-1}. Above +10 °C, there is a sharp falloff in the slope, the cause for which is at present unknown. The most likely cause is either a change in the rate-determining step or a change in molecular conformation. This aspect is at present under investigation. The plots for both cryosolvents show a pronounced downward curvature at lower temperatures. From the tangent to the slopes near 0 °C, activation energies of E_{actn} = 13 kcal mol^{-1} (60% MeOH) and E_{actn} = 15.3 kcal mol^{-1} (50% Me_2SO) can be calculated. Whereas in MeOH the activation energy appears to be the same initially as that in water near 0 °C, in Me_2SO the activation energy is probably significantly higher, and the more pronounced curvature of the plot is in accordance with this. Penicillopepsin activity was detectable in 67% MeOH at −76 °C (Table II), but no activity was observed in 50% Me_2SO at temperatures below about −45 °C, even after incubation for long periods (12 h) with enzyme concentrations up to 8.8 μM. When the temperature was raised to −40 °C, there was a slow reactivation. It appears that penicillopepsin is slowly and reversibly inactivated at low temperatures in Me_2SO.

The apparent smooth increase with decreasing temperature in the slope of the Arrhenius plots of the reactions in the cryosolvents is not due to the high viscosity of the solvents at lower temperature. All the measurements have been corrected for viscosity effects in the following manner. The activity of

Table II: Results of Low-Temperature Trapping Experiments with Penicillopepsin[a]

analysis	substrate	expt	residual marker (residues/molecule)	substrate hydrolyzed
p-nitrophenylalanine residue in acyl moiety	(I) Leu-Ser-(NO_2)Phe-Nle-Ala-Leu-OMe	test	0.24	++
		control	0.18	–
DNA group in acyl moiety	(II) DNS-Ala-Ala-Phe-Phe-OP4P$^+$-CH_3	test	<0.1[b]	?
		control	<0.1[b]	?
p-nitrophenylalanine residue in amino moiety	(III) Ac-Ala-Ala-Lys-(NO_2)Phe-Ala-Ala amide	test	<0.02[b]	++
		control	<0.02[b]	–
	(IV) Ac-Lys-(NO_2)Phe amide	test	<0.02[b]	(+)[c]
		control	<0.02[b]	–
	(V) Ala-Ala-Nle-(NO_2)Phe-Ala-Ala amide	test	<0.02[b]	++
		control	<0.02[b]	–
	(VI) Leu-Ser-Nle-(NO_2)Phe-Ala-Leu amide	test	<0.02[b]	++
		control	<0.02[b]	–

[a] Penicillopepsin (3 mg, 90 nmol) was incubated with substrate (3 μmol) at −76 °C as described under Methods. The isolated proteins were analyzed for their *p*-nitrophenylalanine content or for the presence of fluorescence from the DNS group. The supernatants were checked for hydrolysis of the substrates as described under Methods. The control values for substrates III–VI are lower than those for substrates I and II because the precipitates were more exhaustively washed. It was assumed that the putative amino intermediate (if covalent) would be stable and would withstand exhaustive washing, whereas a putative anhydride intermediate would be labile and might hydrolyze during exhaustive washing. The procedure adopted was used as a compromise. [b] Limit of detectability. [c] Parentheses indicate a low level of hydrolysis.

penicillopepsin was determined at 0 and −25 °C in aqueous solvents in the presence of glycerol up 75% (v/v). The rates obtained were plotted against log (η_c/η_w), where η_c is the viscosity of the glycerol–water mixtures [obtained from Wolf et al. (1982)] and η_w is that of water. This plot was then used to adjust the measured rates in the cryosolvents below 0 °C for the viscosity effect as follows. The relative viscosities η_t/η_0, where η_t is the viscosity at the experimental temperature and μ_0 that at 0 °C, were calculated from the values given for 60% MeOH (Tammann & Pillsbury, 1928) and 50% Me_2SO (Schichman & Amey, 1971), respectively. Correction factors for the initial rates of the enzyme reaction were then obtained from the plot obtained with glycerol solutions.

The curvature of the Arrhenius plot obtained in 60% MeOH is also not due to aggregation. UV absorption spectra taken at −50 °C showed no sign of light-scattering effects nor was the enzyme inactivated. The simplest explanation for the downward curvature is a decrease in motility, especially of the important Tyr-75 flap, as discussed above. As James et al. (1982) have pointed out, the flap is probably involved in hydrogen-bond interactions with the backbone of a good substrate bound productively in the active site, in the same way as it is involved with a pepstatin fragment. For this interaction to happen, the flap has to be in an "open" position while substrate binding takes place. If at the lower temperature the flap preferentially assumes a more closed position, access of substrate would be hindered. In fact, it is possible that under these circumstances the displacement of the flap before binding takes place may become the rate-limiting step.

The curvature of the Arrhenius plot obtained in 50% Me_2SO (Figure 6), on the other hand, is probably due to the conformational change that is responsible for the reversible inactivation observed at temperatures below −45 °C. A low temperature induced unfolding has been observed for β-lactoglobulin in 8 M urea by Pace & Tanford (1968). A similarly induced unfolding could explain, alternatively, the curvature of the Arrhenius plots in both solvents.

Search for Intermediates. Having established that MeOH and to a lesser degree Me_2SO provide suitable cryosolvent systems for a low-temperature study of penicillopepsin, we attempted to find evidence for intermediates in the reaction.

Burst Reactions. Although in principle it is possible to carry out kinetic experiments that might yield spectrophotometrically detectable bursts indicative of acyl intermediates with the substrate Ac-Ala-Ala-Lys-(NO_2)Phe-Ala-Ala amide, in practice it turned out that no clear-cut results could be obtained because the small absorbance changes following the release of the amino moiety occur in the wavelength range where the enzyme absorbance is comparatively large. Different substrates which can be studied at higher wavelengths and which are more sensitive are needed for studies designed to detect spectrophotometrically burst reactions indicative of the formation of intermediates at low temperatures.

Attempts at Trapping Intermediates. Because the possibility exists that reactions catalyzed by aspartyl proteinases proceed either via covalent amino or via covalent acyl intermediates, we chose for our trapping experiments substrates which carry a marker in either the acyl or the amino moiety. These are listed in Table II. The trapping procedure is described under Methods. The *p*-nitrophenylalanine marker could be detected after isolation of the protein and hydrolysis by amino acid analysis; the DNS marker was detected from the fluorescence spectrum of the isolated protein. The results obtained are shown in Table II. There is no evidence that either the acyl or the amino moiety has been trapped in the form of a covalent intermediate during the reaction. The evidence against the formation of an amino intermediate is particularly convincing if the assumption is correct that in an amino intermediate the amino moiety [(NO_2)Phe peptides from substrates III–VI, Table II] is linked through an amide bond to the β-carboxyl group of one of the active-site aspartic acids and that such a linkage would be stable in trichloroacetic acid and the washing solvents. After exhaustive washing, we were unable to detect any residual *p*-nitrophenylalanine. The same assumptions cannot be made about a covalent acyl intermediate. In this case, the acyl moiety [Leu-Ser-(NO_2)Phe from substrate I or DNS-Ala-Ala-Phe from substrate II, Table II] would presumably be linked through an anhydride bond to an active-site aspartic acid. Such a bond would be considerably more unstable during the same exhaustive washing that was used for the putative amino intermediates. With the milder washing procedure, not all the substrate or product was removed, as the control for substrate I (Table II) shows. (The residual *p*-nitrophenylalanine represents about 0.7% of the total present.) That the enzyme was active in 67% MeOH at −76 °C is shown by the fact that the hydrolysis products were detectable after electrophoretic separation of the supernatant in the test experiment, but not in the control, in which enzyme

was added after the trichloroacetic acid precipitation.

The results presented in this paper demonstrate the conformational stability of penicillopepsin in 60% MeOH at pH* values between 6 and 6.5 and in 50% Me_2SO from pH* 4 to 7.5. The enzyme is also active in these cryosolvents; in 67% MeOH, activity has been observed at −76 °C. The nonlinear Arrhenius plots suggest, however, that there is a shift in the rate-limiting step, which unfortunately leads in a direction away from that which would allow the accumulation of any possible covalent intermediates. The implications of these results and of those of the preceding paper on the cryoenzymology of pig pepsin (Dunn & Fink, 1984) on the mechanism of action of aspartyl proteinases are discussed in the Appendix.

Acknowledgments

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Registry No. I, 37589-79-0; II, 85353-23-7; III, 82867-31-0; IV, 88412-23-1; V, 92010-36-1; VI, 92010-37-2; aspartic proteinase, 78169-47-8; penicillopepsin, 9074-08-2.

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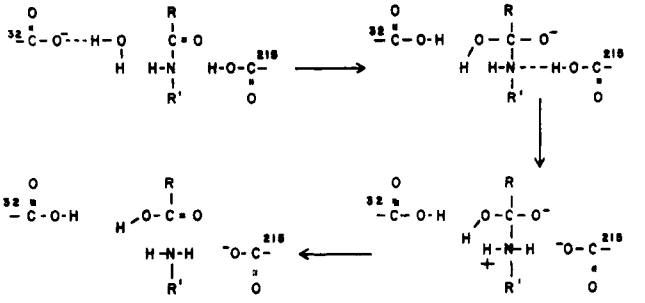
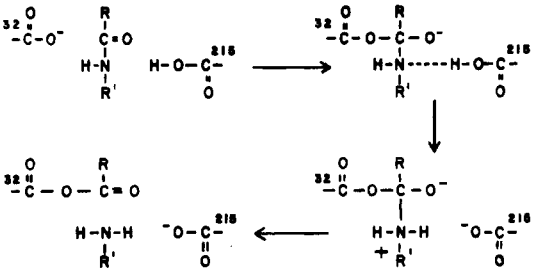
Appendix: Mechanism of Action of Aspartyl Proteinases

In the preceding two papers (Dunn & Fink, 1984; Hofmann & Fink, 1984), we presented evidence against the accumulation of either acyl or amino intermediates in the action of porcine pepsin (Dunn & Fink, 1984) and of penicillopepsin (Hofmann & Fink, 1984) with good substrates at low temperatures in methanol–water solvents. We discuss the implications of these findings on the mechanism of action of aspartyl proteinases and take the opportunity to present a critical evaluation of the present state of our knowledge in this field.

Scheme I: Peptide Bond Cleavage Involving General Base Catalyzed Attack by Water on the Carbonyl Group



Scheme II: Peptide Bond Cleavage by Nucleophilic Attack by Asp-32 on the Carbonyl Group To Give an Acyl Enzyme Intermediate

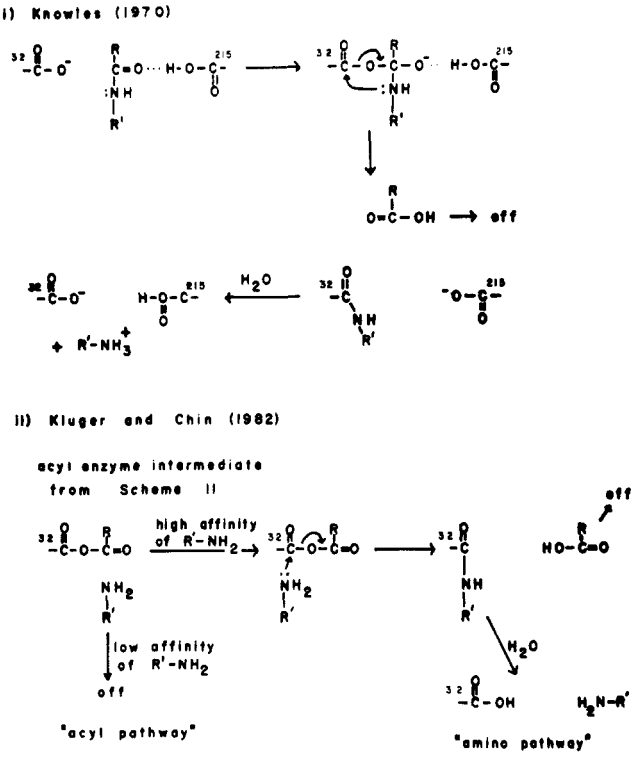


It is generally agreed that the mechanism of action of porcine pepsin and other members of the aspartyl proteinases involves the participation of two aspartyl residues at the active site [Asp-32 and Asp-215 in the numbering of the pepsin sequence according to Tang et al. (1973)]. These are located in the active-site groove and are oriented in such a way that they are linked through a hydrogen bond between one oxygen each of their side-chain carboxyl groups (James & Sielecki, 1983). However, there is at present no general agreement on the basis of the mechanism of action, let alone on its details.

There are two major alternatives for the peptide bond cleavage catalyzed by carboxyl groups. The first is a general base assisted attack of water upon the carbonyl group followed by prototrophic shifts and direct elimination to give the two products (Scheme I). In the second alternative, a nucleophilic attack would give a tetrahedral intermediate which would be followed by protonation of the nitrogen and expulsion of the amine component to give an anhydride intermediate (Scheme II). A covalent amino intermediate (Scheme III)—a possibility suggested by transpeptidation experiments discussed below—could form by transfer of the amino moiety to the catalytic carboxyl group during the collapse of the tetrahedral intermediate [Scheme III (i)], as suggested by Knowles (1970), or by an internal nucleophilic attack by the amine before release from the enzyme, as suggested by Kluger & Chin (1982) and Spector (1982).

The evidence for the second alternative involving one or two covalent intermediates comes entirely from transpeptidation experiments with relatively poor substrates. Transpeptidations involving a transfer of the amino moiety, and by implication an enzyme–amino intermediate, were first described for

Scheme III: Mechanisms Involving Formation of Amino Enzyme Intermediates



Cbz-Glu-Tyr by Neumann et al. (1959), who showed the formation of Tyr-Tyr during the hydrolysis of the substrate. Transpeptidations implying a covalent acyl intermediate were first observed by Takahashi et al. (1974), who showed that Leu-Leu and Leu-Leu-Leu are formed from Leu-Tyr amide by both pepsin and penicillopepsin. An extensive study of transpeptidation reactions (Wang & Hofmann, 1976) with a variety of substrates showed that under certain conditions the transpeptidation reactions by far exceeded the direct hydrolysis and that a leucyl residue was the preferred group in both acyl and amino transfers. The failure of free amino acids to exchange with the enzyme-bound amino acid intermediates, first observed by Sharon et al. (1962) for the amino transfer reaction and subsequently shown for both types of transfers (Takahashi & Hofmann, 1975; Wang & Hofmann, 1976), strongly suggested that the intermediates in the transfer reactions were covalent. Recent studies (M. Blum, P. Asselbergs, T. T. Wang, and T. Hofmann, unpublished results) strengthen this suggestion. They show that any productive complex that might be formed from the back-reaction between enzyme and free amino acid, when preincubated before a transpeptidation reaction, was not detectable and would account for less than 0.25% of a single turnover. Moreover, when the transpeptidation reaction was performed in the presence of excess free amino acid, no incorporation of the latter into the transpeptidation products was observed under conditions where incorporation of 0.01% of the free amino acid would have been detected.

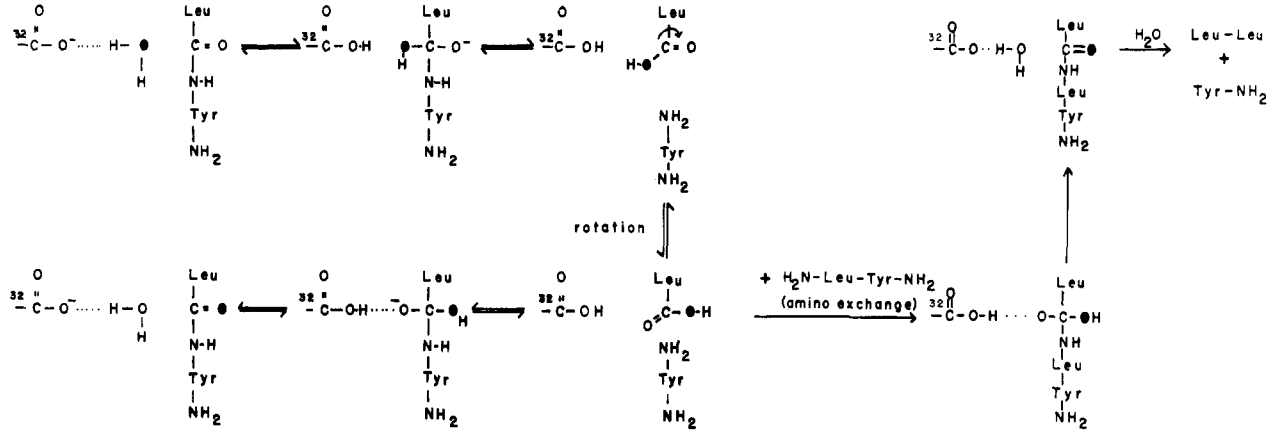
On the other hand, in spite of much effort in many laboratories, no supporting evidence for covalent intermediates has been obtained so far. Thus, Cornish-Bowden et al. (1969) failed to trap a putative acyl intermediate with methanol. Similarly, M. Blum, P. Asselbergs, T. T. Wang and T. Hofmann (unpublished results) using a variety of nucleophiles, including hydroxylamine, were unable to trap any acyl intermediates. However, these experiments do not exclude the possibility of covalent acyl intermediates for two reasons. First, the acceptor–substrate peptides used in the transpeptidation experiments are also nucleophiles and are clearly able to trap "intermediates" efficiently. Second, although β-lactamase has been shown to form a covalent acyl enzyme (Fisher et al., 1980; Cartwright & Fink, 1982), no transfer of the acyl group to nucleophiles has been observed.

Stopped-flow experiments with penicillopepsin and Ac-Ala-Ala-Lys-(NO_2)Phe-Ala-Ala amide (Hofmann & Hodges, 1982) showed no evidence for a burst release of (NO_2)Phe-Ala-Ala amide and by implication no evidence for a detectable amount of an Ac-Ala-Ala-Lys–enzyme complex (<5%).

Attempts to detect the formation of a covalent amino enzyme complex by rapid denaturation of a reaction mixture of pepsin or penicillopepsin with Cbz-Phe-[^{3}H]Leu also gave no evidence for the accumulation of a significant amount of intermediate (M. Blum, P. Asselbergs, T. T. Wang, and T. Hofmann, unpublished results); less than 0.3% of the enzyme had incorporated the labeled leucine residue. Stopped-flow experiments with pepsin acting on substrates with a fluorescent label on the N-terminal group also failed to provide evidence for the formation of an intermediate (Fruton, 1980).

Unfortunately, negative though these experiments are, they do not provide conclusive evidence against covalent intermediates. If the rate-limiting step occurs at or before the formation of a putative intermediate and if it is very much slower than any subsequent steps, intermediates would not accumulate to any significant extent and would not be detectable. Stronger evidence against a covalent acyl intermediate appears to be provided by the studies of Antonov et al. (1978, 1979, 1981). These authors observed the incorporation of ^{18}O from ^{18}O-enriched water during acyl transfer reactions with Leu-Tyr amide as substrate. The isotope was incorporated into the peptide carbonyls of both the product Leu-Leu and, to a less extent, the substrate. This was taken as support for a general base function of one of the catalytic aspartic acids, presumably Asp-32. However, one aspect of the data presented (Antonov et al., 1978) seems puzzling. If Asp-32 acts as a general base to assist the attack of water (as shown in Schemes I and IV), one would anticipate that by microscopic reversibility the ^{18}O would be lost in the reconversion of the tetrahedral intermediate into substrate. If, however, the reaction proceeds one step further to give the free carboxyl group and tryosinamide, then the rotation of the carboxyl (as indicated in Scheme IV) could move the ^{18}O into a position where reversibility could give starting material with the ^{18}O retained, as was indeed observed. Now, if this rotation is permitted to account for the incorporation of ^{18}O into substrate, it must also be present during exchange of the tyrosinamide product for a second molecule of Leu-Tyr amide to generate the transpeptidation product. This would mean, however, that the peptide bond of the Leu-Leu product could exchange a maximum of 50% of its oxygen and not over 80% as reported originally or 56–65% as reported subsequently (Antonov et al., 1981). In any case, these oxygen exchange experiments are not readily compatible with the observations made by M. Blum, P. Asselbergs, T. T. Wang, and T. Hofmann (unpublished results), as discussed above, which strongly suggest that the leucyl residue that is transferred is tightly bound to the enzyme. In that case, it is difficult to imagine that the carboxyl group of the bound leucine would be able to rotate freely and thus show complete oxygen exchange. Rather, the carbonyl group is likely to be held tightly, and thus the oxygen that the carboxyl would receive from the water would be displaced by the incoming second substrate molecule, and at most a partial isotope

Scheme IV: Pathway of Incorporation of ^{18}O (•) from $H_2{}^{18}O$ into Substrate and Transpeptidation Product during "Acyl-Transfer" Reaction



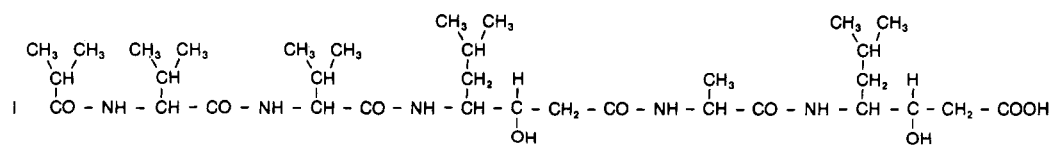
incorporation would be observed.

On the other hand, there is a route which can account for the incorporation of ^{18}O into both the original substrate and the product Leu-Leu, even if a covalent intermediate was involved. Pepsin and penicillopepsin catalyze condensation reactions, such as Leu-Leu and tyrosinamide to give Leu-Leu-Tyr amide (Wang & Hofmann, 1976) or Leu-Leu and Leu-Tyr amide to give Leu-Leu-Leu-Tyr amide (T. T. Wang and T. Hofmann, unpublished results). If these condensation reactions also occur during the longtime conversion of Leu-Tyr amide into products in the presence of ^{18}O-labeled water, then the condensation products will contain ^{18}O in the newly formed peptide bond. Leu-Leu-Tyr amide can be hydrolyzed to free leucine and Leu-Tyr amide (as well as Leu-Leu and tyrosinamide). This Leu-Tyr amide would now contain the oxygen isotope. Leu-Leu-Leu in turn can give rise to Leu-Leu and account for the incorporation of the isotope into this peptide. Although these reactions are slow, they could nevertheless occur to a very significant extent during the long incubation periods (72 h) and at the high enzyme concentrations used by Antonov et al. (1981).

Solvent deuterium isotope effects have been used to provide evidence both for and against a general base mechanism (Clement, 1973; Clement et al., 1968; Hollands & Fruton, 1969; Hunkapiller & Richard, 1972; Reid & Fahrney, 1967).

Major contributions toward our understanding of the mechanism are being provided by the three-dimensional structures which are available in detail for penicillopepsin (James et al., 1977, 1981, 1982; James & Sielecki, 1983) and *Rhizopus* pepsin (Subramanian et al., 1976, 1977; Bott et al., 1983). Both groups suggest that for steric reasons the formation of covalent acyl or amino intermediates is improbable and suggest a general base mechanism involving one of the two active-site carboxyl groups. Originally it was suggested that Asp-32 would be the group involved, because it appeared to be the group that would be unprotonated while Asp-215 would be the group with the higher pK_a. However, the refined structure of penicillopepsin at 1.8-Å resolution shows that the environment of the two aspartic acids is rather similar and a definite assignment of pK_a's is not possible (James & Sielecki, 1983). The assignment of the higher pK_a value to Asp-215 and the lower one to Asp-32 was originally based on their reaction with active site directed inhibitors (Chen & Tang, 1972), which showed that an epoxide inhibitor reacted specifically with Asp-32 whereas a diazoacetyl inhibitor reacted with Asp-215. Subsequent studies with similar inhibitors and different enzymes, however, showed that the epoxide inhibitor reacted apparently equally well with either of the two aspartic acids in penicillopepsin (Hsu et al., 1977), in *Rhizopus* pepsin (Nakamura & Takahashi, 1977), and in chymosin (Cheng & Takahashi, 1974), as did a diazo inhibitor with penicillopepsin (M. N. G. James and T. Hofmann, unpublished results). From this, it would appear that the location of the proton that is shared between the two carboxyl groups, and by implication the pK_a's of these groups, is determined by the nature of the ligand used to probe the ionization. Also, conformational changes that are associated with substrate binding (Fruton, 1980) make it likely that the fine structure of the active site in a productive enzyme–substrate complex is not exacetly the same as that seen in the X-ray analyses. Hence, the structural arguments against a covalent mechanism lose some of their persuasiveness.

The noncovalent mechanism is also supported indirectly by the fact that aspartyl proteinases are strongly inhibited by pepstatin (I in Figure 7) and many of its analogues. These inhibitors can be considered as transition-state analogues (Marciniszyn et al., 1976; Rich & Sun, 1980) of the noncovalent tetrahedral intermediate depicted in Scheme I.



Pepstatin

(isovaleryl - valyl - valyl - statyl - alanyl - statine = Iva - Val - Val - Sta - Ala - Sta)

II Iva - Val - Sta - OEt

III Iva - Val Val - Sta - OEt

FIGURE 7: Structure of pepstatin and analogues used in mechanistic studies of aspartyl proteases.

However, even in this case, recent work has made interpretations less straightforward. Thus, pepstatin analogue II (Figure 7) binds only weakly to penicillopepsin ($K_I > 10^{-4}$ M) compared to peptide III (Figure 7, $K_I = 2.4 \times 10^{-8}$ M) which has one valine residue more, although peptide II still has the statine residue with the feature of a transition-state analogue and consequently would be expected to bind tightly in accordance with the transition-state theory (Wolfenden, 1976). Clearly, the large difference in binding between peptides II and III is due to the presence of a valine in position P_3 and not to the statine residue.

As has been pointed out, the cryoexperiments do not support the covalent intermediate mechanism nor do they disprove it. These experiments were undertaken, among other reasons, in the hope that the energy of activation of the step controlled by k_2 in the following simplified scheme would be less than

$$E + S \leftrightarrow ES \xrightarrow{k_2} ES' \xrightarrow{k_3} E + P$$

that for the step controlled by k_3 so that at low temperature putative intermediates could accumulate. However, recent work by Dunn (1982) and by Rich & Bernatowicz (1982) on the interaction of inhibitors with pepsin as well as earlier work by Fruton (1980) with fluorescent substrates shows that binding to the enzyme involves slow conformational changes. One of these is probably the movement and proper orientation of the Tyr-75 flap (James & Sielecki, 1983). This raises the possibility that the rate-limiting step in the hydrolysis may be a conformational step in a minimal scheme of the following type:

$$E + S \leftrightarrow ES \overset{RLS}{\leftrightarrow} ES^* \leftrightarrow [EI^*] \leftrightarrow EP^* \leftrightarrow EP \rightarrow E + P$$

where ES* and EP* represent noncovalent complexes in which the enzyme is in a conformationally different state from ES and EP, respectively, and EI* is either a covalent or a noncovalent "intermediate". In this case, the energy of activation of the rate-limiting step is likely to be rather large, and the difference in the magnitude of the rate-limiting and subsequent steps would be increased with decreasing temperature and would tend to reduce the steady-state concentration of any possible intermediate.

From this discussion, is is apparent that there is at present no sound basis on which a decision about the mechanism of action of aspartyl proteinases can be made. If subsequent experiments also fail to support a covalent mechanism, then it becomes important to concentrate on studies of the nature of the intermediates that are involved in the transpeptidation reactions and that fail to exhibit the properties expected for a noncovalent complex. This point is relevant to the full understanding of the mechanism because there is no a priori reason to assume that the mechanism of hydrolysis of good substrates differes fundamentally from that of the transpeptidation reactions. It is even less likely that different active sites are involved in the hydrolysis and transpeptidation events.

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