A Ligand-induced, Temperature-dependent Conformational Change in Penicillopepsin

EVIDENCE FROM NONLINEAR ARRHENIUS PLOTS AND FROM CIRCULAR DICHROISM STUDIES

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The effect of temperature on the rate constants of hydrolysis of various substrates by penicillopepsin is dependent on the length of the substrate. For the series Ac-(Ala),-Lys-Nph-(Ala),-amide (where Ac- is acetyl- and Nph- is p-nitrophenylalanyl-), where m and n = 0–2, substrates lacking both P6 and P5 residues give linear Arrhenius plots with an energy of activation of about 55 kJ·mol⁻¹. The Arrhenius plots of substrates in which an alanine residue occupies P6 show a sharp break at an average transition temperature of 10.5 °C. The activation energies are ∼90 kJ·mol⁻¹ below and ∼54 kJ·mol⁻¹ above the transition temperature, respectively. For substrates in which P6 is occupied, the average transition temperature is 14.2 °C. In this case, the activation energies are 66 kJ·mol⁻¹ below and from 26 to 39 kJ·mol⁻¹ above the transition point. The most probable explanation of these phenomena is that substrate interaction at substates S6 and/or S5 of the enzyme induces a temperature-dependent conformational change. Physical evidence for this comes from the observation that the temperature dependence of a CD absorption band at 242 nm of a penicillopepsin-pepstatin complex shows a sharp break that corresponds to those observed in the Arrhenius plots of substrates with alanine at P6 and P5, whereas the same CD band in the free enzyme is linearly dependent on temperature.

Penicillopepsin is a well-characterized member of the family of aspartic proteinases. Its three-dimensional structure has been determined at high resolution (2) and is very similar to that of pig pepsin (3), rhizopuspepsin (4), and endothiapepsin (5, 6), which have been determined at high resolution (2) and is very similar to that of pig pepsin (3), rhizopuspepsin (4), and endothiapepsin (5, 6). The system of Schechter and Berger (1) for denaturing residues in peptide substrates as P6 to P5 and P1 to P10, and cuboiose, in the enzyme to which the side chains of these residues bind as S6 to S5 and S1 to S5, is used throughout. Amino acids in the sequence of penicillopepsin are numbered sequentially.

EXPERIMENTAL PROCEDURES

Materials—Highly purified penicillopepsin was prepared as described by Hofmann (18). The peptides of the series Ac-(Ala),-Lys-Nph-(Ala),-amide were prepared as described previously (11). Pepstatin was obtained from Bachem Bioscience (Bubendorf, Switzerland). Chemicals used for buffers were of the highest quality available.

Methods—All enzyme assays were carried out in 20 mM sodium acetate (pH 5.5) or in 10 mM sodium phosphate (pH 6.0). All buffers were adjusted to an ionic strength of 0.1 M with NaCl. A Uvikon 820 spectrophotometer (Kontron AG, Zurich, Switzerland) was used with cells of 2- or 10-mm light paths and at wavelengths of 296, 306, or 320 nm. The cells and wavelengths were chosen so that the initial absorbance of the substrate solution did not exceed 0.05. The enzyme concentrations ranged between 0.5 and 200 µg/ml (15 nM and 9 µM) depending on the rate of the reaction and were chosen to give initial rates in the first 2–10 min. The spectrophotometer cell holders, which were jacketed and insulated with foam rubber, were maintained at constant temperature (±0.1 °C) by circulating dilute ethanol from a Lauda R2R constant temperature water bath (Brinkmann Instruments). A stream of dry, filtered air over the faces of the quartz cuvettes was used to prevent condensation.

The abbreviations used are: Ac-, acetyl-; Nph-, p-nitrophenylalanyl-; Iva, isovaleryl; Sta, statine ((3S,4S)-4-amino-3-hydroxy-6-methylheptanoic acid). The system of Schechter and Berger (1) for denaturing residues in peptide substrates as P6 to P5 and P1 to P10, and cuboiose, in the enzyme to which the side chains of these residues bind as S6 to S5 and S1 to S5, is used throughout. Amino acids in the sequence of penicillopepsin are numbered sequentially.
CD spectra of the free enzyme and of its complex with pepstatin were recorded on a Jasco J-410 spectropolarimeter (Japan Spectroscopic Co. Ltd., Tokyo). Quartz cuvettes with light paths of 1 cm for measurement between 240 and 350 nm and of 0.1 cm for the range 200-250 nm were used in jacketed cuvette holders analogous to those described above. Temperatures were monitored inside the cells with a thermocouple connected to a digital monitor accurate to ±0.1 °C (Model DS-12, Bailey Instruments, Sudbury, N.J.). Spectra were recorded at chart speeds of 5 nm/min with a time constant set at 4 s. Because the signal in the region at 240-250 nm was very noisy under these conditions, spectra between 240 and 245 nm were run also at a chart speed of 0.2 nm/min with a time constant of 16 s to increase the accuracy of the measurement of the small temperature-dependent changes.

RESULTS

Effect of Temperature on Hydrolysis by Penicillopepsin of Substrates of Series Ac-(Ala),-Lys-Nph-(Ala),-amide—The effect of temperature on the rate constant of the hydrolysis by penicillopepsin of substrates of the series Ac-(Ala),-Lys-Nph-(Ala),-amide is shown in the form of Arrhenius plots for three examples in Fig. 1 (A-C). Activation energies calculated from these plots and from similar plots for the other substrates analyzed are given in Table I. The results show that all the peptides examined fall into one of three distinct groups. Peptides I (Fig. 1A) and II, in which there is no aminoacyl residue at either position P1 or P3, give linear Arrhenius plots and activation energies of 55 kJ mol⁻¹ (Table I), values that are well within the range of other enzyme-catalyzed hydrolysis reactions (19, 20). With substrates having an aminoacyl residue at P2, but not at P3 (peptides IV, V, and VII), the Arrhenius plots show a distinct break at 10.5 ± 2.0 °C. The two slopes give activation energies of 90 kJ mol⁻¹ below and 55 kJ mol⁻¹ above the transition point. The values for temperatures below the transition are significantly higher than those generally observed for enzyme-catalyzed hydrolysis reactions. The third group comprises peptides III, VI, and IX, substrates which have an alanine at P3 (peptide III) or at both P1 and P3. They also show a sharp break in the Arrhenius plots, but this is at significantly higher temperatures (average of 14.2 ± 1.56 °C). The activation energies are also significantly different, ranging from 65 to 69 kJ mol⁻¹ below and from 26 to 38 kJ mol⁻¹ above the transition point. The latter values are in the lower range of or below those observed generally for enzyme-catalyzed reactions (19, 20).

There are a number of relatively trivial factors that can be responsible for nonlinear Arrhenius plots, such as changes in the ionization state of the enzyme due to changes in the pH of the buffer, reversible inactivation of the enzyme (19), and temperature effects on Kₘ (21). The first two can be eliminated in this case because they affect all substrates studied equally. That the observed temperature effects are not due to effects on Kₘ was shown as follows. We have determined the Kₘ values of two representative substrates at the lower and upper ends of the temperature range studied (Table I, Footnote e). The Kₘ values were the same within experimental error at both temperatures for peptide VII, a peptide which lacks an alanine at P3, and for peptide IX, a peptide in which both P2 and P3 are occupied. We assume that this holds true for all peptides studied because the Kₘ values of all peptides are very similar (11). Although for practical reasons we were unable to carry out all the experiments with near-saturating substrate concentrations, Arrhenius plots obtained at substrate concentrations 17 to over 20 times the Kₘ values for peptides VII and IX (Table I, Footnote e) were the same as those obtained at the lower concentrations (between 3 and 6 times the Kₘ values). We conclude therefore that the observed discontinuities are due solely to effects on Kₘ and not to effects on Kₘ or other trivial factors.

Effect of Pepstatin Binding on Circular Dichroism Absorption Bands—Fig. 2 shows the CD spectra of penicillopepsin and its complex with pepstatin in the far-UV range (Fig. 2A) and the near-UV range (Fig. 2B). The binding of pepstatin causes several significant changes in the spectrum. There is a 20% increase in the negative band at 219 nm (after subtraction of a small negative signal for free pepstatin). This increase in apparent β structure is probably due to two factors. First, pepstatin is rigidly held in the active site of the enzyme and forms several backbone hydrogen bonds (12). Second, pepstatin binding renders the enzyme moiety more rigid, as shown by its increased temperature stability and by the decreased crystallographic isotropic B factors ("temperature factors") of at least two extended regions of the molecule (22). (The crystallographic study was carried out with Iva-Val-Val-Sta-ethyl ester, a short pepstatin homologue (22) which lacks the C-terminal Ala-Sta residues. We assume that intact pepstatin would give similar results.)

Several differences between the free enzyme and the pepstatin complex are also apparent in the near-UV spectrum. The largest increases occur in the positive peak at 291 nm and in the negative peak at 242 nm. Smaller increases are seen in the major peak at 280 nm and the accompanying bands at 254 and 264 nm. Small decreases in intensity are observable in the negative bands at 277.5, 287.5, and 294.5 nm.

Effect of Temperature on CD Bands of Penicillopepsin and

1 A. Cunningham and T. Hofmann, unpublished data.


**TABLE I**

Free energies of activation of hydrolysis of substrates of the series Ac-(Ala)$_n$-Lys-Nph-(Ala)$_m$-amide by penicillopepsin

The buffer used was 20 mM sodium acetate (pH 5.5); enzyme concentrations were varied between 15 nM and 9 μM for different substrates, depending on the $k_{cat}$ of the substrate.

| Peptide                  | No. | Subsite occupied | $k_{cat}$ (mM) | $t_0$ (°C) | $K_m$ (mM) |
|--------------------------|-----|------------------|----------------|------------|------------|
| Ac-Lys-Nph-amide         | I   | S1               | 55             | 55         | 0.64-0.85  |
| Ac-Ala-Lys-Nph-amide     | II  | S1, S2           | 56 ± 2.5       | 56 ± 2.5   | 0.31-0.56  |
| Ac-Lys-Nph-Ala-amide     | IV  | S1               | 82             | 56         | 0.08     |
| Ac-Ala-Lys-Nph-Ala-amide | V   | S1               | 91             | 55         | 8.7       |
| Ac-Lys-Nph-Ala-Ala-amide | VII | S1, S2           | 89             | 51         | 11.0      |
| Ac-Lys-Nph-Ala-Ala-amide'| VII | S1, S2           | 85             | 62         | 9.1       |
| Ac-Ala-Ala-Lys-Nph-amide | III | S1, S2           | 65             | 39         | 15.6      |
| Ac-Ala-Ala-Lys-Nph-Ala-amide | VI | S1, S2          | 69             | 38         | 13.5      |
| Ac-Ala-Ala-Lys-Nph-Ala-amide | VII | S1, S2        | 66 ± 4.6       | 26 ± 0.6   | 13.4      |
| Ac-Ala-Ala-Lys-Nph-Ala-amide | IX | S1, S2        | 55             | 32         | 13.5      |

$^a$ $E_a$, activation energy; average of two determinations, except where standard deviations are shown, when three to five determinations were made.

$^b$ $t_0$, transition temperature.

*At 25 °C, from Hofmann et al. (11).

$^d$ At 1.5 °C.

*Experiments at high substrate concentrations.

**Fig. 2.** CD spectra of penicillopepsin and its complex with pepstatin. The buffer used was 20 mM sodium acetate (pH 5.5) at 25 °C. - - , penicillopepsin; - - - - , penicillopepsin-pepstatin complex (1:1). A, 200-250 nm; B, 240-350 nm.

**Its Pepstatin Complex**—The effect of temperature on the intensity of the CD bands was studied between 1 and 28 °C. Fig. 3 (A-D) shows the changes for four selected bands: 294, 291, 242, and 214 nm. Linear changes were observed in all bands for both the free enzyme and the pepstatin complex except for the band at 242 nm. Its intensity changed linearly only for the free enzyme, but showed a marked sharp break with the inhibitor complex (Fig. 3D). The transition temperature for this change is at 15.6 °C, i.e. close to that observed for the Arrhenius plots of enzyme-substrate complexes in which S$_1$ is occupied (Fig. 1C). Linear changes were also seen for both the free enzyme and its complex for those bands not shown in Fig. 3.

**Attempts at Identifying Origin of 242 nm CD Band**—The nonlinear temperature dependence of the 242 nm CD band of the penicillopepsin-pepstatin complex provides clear evidence for a temperature-dependent conformational change. Since it is of interest to identify the region of the molecule that is involved in this change, we considered the possible origin of this band. There are two major sources that could give rise to CD bands in this region: disulfide bridges, and the other the $t_0$ transition of tyrosine and tryptophan rings (23-26). Constrained disulfide bridges give rise to a broad band in the 260-280 nm region with absolute intensities of the maxima in the range 6000-8000 degrees-cm$^{-2}$-dmol$^{-1}$ (23) and a narrower band of opposite sign but similar intensity in the 240-250 nm region. The sign of the bands is directly related...
to the diastereoisomerism of the disulfide bond, with left-handedness giving rise to a negative band in the 260–280 nm region and a positive band in the 240–250 nm region, whereas right-handedness leads to bands of the opposite sign (23). The single disulfide in penicillopepsin (residues 249–283) is right-handed and thus would give a positive band in the 260–280 nm region which would extend to 320 nm. The band in the 240–250 nm range would be negative, like the band we observed (Fig. 2B): but its expected "mean residue" ellipticity would be \(-20–30\) degrees \(\cdot\) cm\(^{-2}\) \cdot\) dmol\(^{-1}\), and not \(-80\) degrees \(\cdot\) cm\(^{-2}\) \cdot\) dmol\(^{-1}\), as found (Fig. 2B). It is therefore very likely that the right-handedness giving rise to a negative band in the 260–280 nm range originates from the 'L\(_{a}\) transition of aromatic rings, especially since the band is accompanied by an intense positive band at 230 nm (Fig. 2A). Although we attempted to modify chemically the single disulfide bridge of penicillopepsin at residues 249–283 and accessible tyrosine residues as likely candidates for CD bands in the 240 nm region (23–25), we were unsuccessful in obtaining any clear-cut results for the following reasons. Most chemical modifications that do not affect the native conformation of proteins require pH conditions near or above neutral either because of the stability of the reagents or because the reactive groups, such as amino groups, are needed in their unprotonated form. Penicillopepsin irreversibly denatures above pH 6–6.5 at low temperatures. Its complex with pepstatin, although somewhat more stable, denatures readily above pH 7. Because we were unable to reduce the disulfide with dithiothreitol below pH 6.5, even at a 1000-fold excess of reagent, we attempted to reduce the penicillopepsin-pepstatin complex at pH 6.8. Although we succeeded in partial reduction, as shown by the incorporation of radioactive iodoacetate into the protein after reduction, the reaction was accompanied by denaturation. The intensity of the 242 nm band decreased during the reduction, but we could not establish whether this decrease was due to reduction or denaturation.

Treatment of the penicillopepsin-pepstatin complex with acetylimidazole at pH 6.8 for 16 h with a 3000-fold excess over the tyrosine residues had no effect on the 242 nm CD band. This experiment only shows that the band does not originate from a tyrosine that is accessible to the reagent under the conditions of the experiments and does not exclude the possibility that the band originates from inaccessible tyrosines and/or tryptophans.

**Discussion**

There are a number of examples in the literature of nonlinear Arrhenius plots for enzyme-catalyzed reactions (27). They are attributable either to changes in the rate-limiting step of the overall reaction or to conformational changes in the enzyme, which may be intrinsic temperature effects or may be caused by changes in the environment. In the first case, the overall reaction would involve successive steps with different temperature coefficients without any changes in the conformation of the enzyme's active site. At higher temperatures, the step with the lower coefficient will tend to determine the overall reaction, whereas at lower temperature, the reaction with the higher coefficient will dominate. This leads to a discontinuity with downwards concavity. However, such a situation would result in gently curving Arrhenius plots, as exemplified in Fig. 1C (lower, theoretical curve), rather than in sharp breaks like those shown in Fig. 1 (B and C, upper curve). The lower, smooth curve of Fig. 1C was calculated as follows. It was assumed that over the temperature range examined, the rate-limiting step was controlled by two different rate constants, \(k_1\) and \(k_2\). It was further assumed that these rate constants had energies of activation which could be calculated from the slopes of the two straight lines shown in Fig. 1C which were assumed to represent the tangents at the highest and lowest temperatures, respectively, to a hypothetical curve. It was further assumed that the observed rate constant: \(k = k_1/k_2 = (k_1 + k_2)/k_2\). These assumptions allowed the calculation of a hypothetical \(k_2\) which would be observed if the temperature dependence of the overall reaction was only due to a change from one rate constant to the other. Clearly, such a curve does not fit the experimental points. The second possibility involves two conformations of the enzyme differing activity which are in equilibrium with each other. Massey et al. (27) have shown that this is the case for D-amino-oxidase. These authors calculated that sharp transitions are obtained if the enthalpy (\(\Delta H\)) of such an equilibrium is greater than 200 kJ.mol\(^{-1}\), i.e. of magnitudes that are in the range of those observed for conformational changes in proteins. They were able to demonstrate the existence of two forms of the enzyme by temperature dependent ultraviolet absorbance difference and fluorescence spectrophotometry.

The results presented in this paper strongly suggest that a temperature change causes conformational changes in penicillopepsin-substrate complexes when substrates S\(_{i}\) and/or S\(_{j}\) of the enzyme are occupied. They also suggest differences between the two substrates since the parameters of the nonlinear Arrhenius plots clearly fall into two distinct groups. For those substrates which occupy S\(_{i}\), the activation energies both above and below the transition temperature as well as the transition temperature itself differ significantly from those of the substrates which occupy S\(_{j}\) only. The effect induced by occupation of the enzyme substrate S\(_{i}\) overrules that of S\(_{j}\) because the parameters for peptide III (a substrate with a residue at P\(_{i}\), but not at P\(_{j}\)) are much closer to those of peptides VI and IX (which have residues at P\(_{i}\) and P\(_{j}\)) than to those in which occupation of P\(_{j}\) is lacking. Enzyme-substrate complexes can therefore exist in five different states. The first is the state which neither of the "activating substrates" is occupied and which is characterized by a free energy of activation \(-55\) kJ.mol\(^{-1}\) and by low catalytic efficiency (11). The second and third states are those of complexes in which only substrate S\(_{i}\) is occupied. The second state exists at temperatures below the transition temperature of \(-10\) °C and is characterized by unusually high free energies of activation, whereas the third state, which exists above the transition temperature, has an activation energy in the normal range of enzyme-catalyzed hydrolytic reactions. The catalytic efficiencies at 25 °C of these complexes are moderate (11). The fourth and fifth states are those of complexes in which substrate S\(_{j}\) is occupied. These complexes differ from the previous ones in catalytic efficiency and in the position of the transition temperature. The activation energies below the transition point are in the normal range, whereas those above are unusually low. The catalytic efficiency at 25 °C is high for peptides VI and IX and moderate for peptide III, which lacks an alanine at P\(_{j}\).

Direct evidence for a ligand-induced temperature-dependent conformational transition comes from the CD experiments. In these studies, pepstatin was chosen as a ligand because the residues -Val-Val- occupy the same positions as the residues P\(_{i}\) and P\(_{j}\), respectively, of a substrate, and the side chain of the statyl residue binds like the side chain of a leucyl residue in P\(_{i}\) (12, 13). We assumed therefore that pepstatin would cause similar temperature-dependent conformational changes as a substrate that has a residue at P\(_{j}\). Fig. 3D shows that whereas the increase in intensity of the negative 242 nm band is linear for the free enzyme, that for the
peptidylpeptidase A has a sharp break at ~15.6 °C. The changes in intensity of the other CD bands, three of which are shown in Fig. 3 (A–C), are all linear. The temperature of the break in the curve is close to that observed in the Arrhenius plots for substrates which have a residue at P1. Thus, inhibitor binding induces a phenomenon similar to that induced by substrates with residues at P1. We cannot study the effect of ligand binding at P2 because we have at present no suitable inhibitor with residue at P2, but not at P3.

Although there is no direct connection with the temperature-dependent transition, the question as to whether there are conformational changes in the enzyme when substrates and inhibitors bind deserves a brief comment. When the x-ray structures of penicillopepsin, rhizopuspepsin, and endothiappepsin are compared with those of their respective complexes with transition state analogue inhibitors, the only significant differences seen are those of the Tyr-75 "flap." This consists of 13 residues and moves from a flexible state in the free enzymes to tight interaction with the inhibitors (12–16). No other part of the enzyme molecules showed any changes, and temperature factors suggested that the structures around the active site were exceptionally rigid (22). However, kinetic experiments are difficult to explain without invoking some conformational changes other than the movement of the Tyr-75 flap. The fact that the catalytic efficiency of peptide bond hydrolysis (as expressed by $k_{cat}$) is controlled by ligand-induced conformational changes. A conformational change was also postulated by Fruton (28) as an essential component in the catalytic mechanism of peptide hydrolysis, especially as an explanation of the large increases in $k_{cat}$ upon binding of extended substrate chains observed with both pepstatin (7) and penicillopepsin (9). The first tentative evidence for a conformational change in the enzyme was obtained when we showed that Leu-Gly-Leu induced significant changes in the CD spectrum of penicillopepsin (29). Leu-Gly-Leu is not a substrate, but increases $k_{cat}$ of the hydrolysis of Leu-Tyramide 10-fold (29). The spectra in Fig. 2 (A and B) which show fairly large differences between free penicillopepsin and its pepstatin complex, tend to support, at least at first sight, the occurrence of structural changes upon ligand binding.

Whereas some of the changes are undoubtedly due to the stabilization of the Tyr-75 flap and the inhibitor in the active site, other changes probably originate from structural changes in the enzyme that do not appear in the crystal structure. Indeed, very recently, Sali et al. (17) found for the first time crystallographic evidence for a substantial structural change upon binding of an inhibitor to endothiappepsin. This change is described by the authors as a rigid body movement and consists of a change in the relative orientation of a 114-residue domain (residues 190–303) to the remainder of the molecule. This finding along with the experiments reported here therefore form the first direct demonstration of structural changes induced by ligand binding to aspartyl proteinases. The absence of significant structural differences, other than movement of the Tyr-75 flap, in the earlier x-ray analyses can probably be ascribed to the fact that the intermolecular forces responsible for stability of the crystal lattice counteract changes in the structure, such as that observed by Sali et al. (17).

Speculation on the nature of the conformational change(s) observed in the experiments reported here must await identification of the CD bands that undergo changes in intensity. The temperature-dependent transition is confined to the band at 242 nm. Although our attempts to elucidate its origin by chemical modification have failed, we have observed that this band is especially sensitive to conformational changes induced by temperature, pH, and urea. It increases markedly in the temperature range of 30–40 °C, long before the protein begins to denature and the other bands are affected; it is also the first band to change as the protein denatures at pH 7.0 and higher; and it is the first band to be affected by urea at concentrations above 5 M. These findings show that this band originates from a structural feature that is especially sensitive to environmental changes. Probably the only way by which the origins of the CD bands can be identified is by changing specific amino acids by site-specific mutagenesis of the penicillopepsin gene and by studying the properties of the mutant protein.

Finally, it is interesting to note that the temperature-dependent conformational changes due to pepstatin binding to penicillopepsin are not detectable by intrinsic fluorescence measurements or in UV difference spectra. The decrease in the intensity of intrinsic fluorescence with increasing temperature and the changes in the UV difference spectra with temperature are linear for both the free enzyme and its pepstatin complex. This suggests that any temperature-dependent changes do not affect the environment of the tryptophan residues, although 2 of them are located close to the catalytic site and adjacent to the "hinge" of the mobile Tyr-75 flap. The third lies between the two major domains of the molecule. The tryptophan residues are thus located at positions that would at first sight seem the most sensitive to conformational changes.

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