Effect of amino acid residues at the cleavable site of substrates on the remarkable activation of thermolysin by salts

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The activity of thermolysin in the hydrolysis of N-[3-(2-furyl)acryloyl]-glycyl-L-leucine amide and N-carboxbenzoxyl-L-aspartyl-L-phenylalanine methyl ester is remarkably enhanced in the presence of high concentrations (1–5 M) of neutral salts [Inouye (1992) J. Biochem. (Tokyo) 112, 335–340]. In this study, the effect of salts on such activity has been examined using a series of substrates, furylacryloyl dipeptide amides, which have various hydrophobic amino acids at the cleavable bond. Although the enzyme activity varies widely depending on the substrate employed, the degree of activation at a given concentration of NaCl is considerably similar. This indicates that the degree of activation is not dependent on the hydrophobicity of the amino acid side chains at the scissile bond of the substrates. The molecular activity, \( k_{cat} \) and Michaelis constant, \( K_m \), were evaluated separately for substrates N-[3-(2-furyl)acryloyl]-L-leucyl-L-alanine amide and N-[3-(2-furyl)acryloyl]-L-phenylalanine-L alanine amide, and the activation was found to be brought about only by an increase in \( k_{cat} \). The effectiveness of monovalent cations on the increase of \( k_{cat} \) was determined to follow the order of Na\(^+ > K^+ > Li^+\).

INTRODUCTION

Thermolysin (EC 3.4.24.4) is a thermostable neutral metalloproteinase produced in the culture broth of Bacillus thermoproteolyticus [1,2]. It requires essentially one zinc ion for enzyme activity and four calcium ions for structural stability [3–5], and catalyses specifically the hydrolysis of peptide bonds containing hydrophobic amino acid residues, especially at the P₁ site [6] (the nomenclature used for the amino acid residues (P) of the substrate and for naming the subites (S) of the active site being that of Schechter and Berger [7]). The amino acid sequence [8] and three-dimensional structure [9] are known, and the reaction mechanism has been proposed [10,11]. Holmquist and Vallee [12] first reported that some neutral salts, such as NaBr and NaCl, significantly activated the thermolysin-catalysed hydrolysis of N-[3-(2-furyl)acryloyl]- (FA)-glycyl-L-leucine amide (FAGLA) and its ester analogue FA-Gly-OLeu-NH₂. We have reported the activation of thermolysin by high concentrations of neutral salts in the hydrolysis and synthesis of N-carboxbenzoxyl-L-aspartyl-L-phenylalanine methyl ester (ZAPM), a precursor of a synthetic substrate employed, the degree of activation at a given concentration of NaBr and NaCl, significantly activated the thermolysin-catalysed hydrolysis of N-[3-(2-furyl)acryloyl]-glycyl-L-leucine amide and N-carboxbenzoxyl-L-aspartyl-L-phenylalanine methyl ester [6]. The molecular activity, \( k_{cat} \), and Michaelis constant, \( K_m \), can be evaluated separately in the case of ZAPM, and the activation has been demonstrated to be induced solely by increasing \( k_{cat} \). Recently, we have described a characteristic absorption difference spectrum that is observed on mixing thermolysin with NaCl and NaBr, suggesting a conformational change of the enzyme in the interaction with the salts [13,14]. The cause of the salt activation is currently not known, and there remains a possibility that the activation correlates with the difference spectrum.

For FAGLA and ZAPM, the activity of thermolysin increases progressively with increasing salt concentration, and does not show any saturating behaviour even at the saturated concentration of NaCl is remarkably enhanced in the presence of high concentrations (1–5 M) of neutral salts. This indicates that the degree of activation is not dependent on the hydrophobicity of the amino acid side chains at the scissile bond of the substrates. The molecular activity, \( k_{cat} \) and Michaelis constant, \( K_m \), were evaluated separately for substrates N-[3-(2-furyl)acryloyl]-L-leucyl-L-alanine amide and N-[3-(2-furyl)acryloyl]-L-phenylalanine-L alanine amide, and the activation was found to be brought about only by an increase in \( k_{cat} \). The effectiveness of monovalent cations on the increase of \( k_{cat} \) was determined to follow the order of Na\(^+ > K^+ > Li^+\).

EXPERIMENTAL

Materials

A three-times-crystallized-and-lyophilized preparation of thermolysin (Lot T8BA51; 8360 proteinase units/mg according to the supplier) was purchased from Daia Kasei, Osaka, Japan. This preparation was used without further purification. The thermolysin solution was prepared in 40 mM Tris/HCl buffer containing 10 mM CaCl₂, pH 7.5 (standard buffer), and filtered with a Millipore membrane filter, Type HA (pore size 0.45 mm), before use. The concentration was determined spectrophotometrically using the calculation that 1 mg/ml gave an absorbance value at 277 nm of 1.83 [8]. FA-dipeptide amides [FA-Gly-Y-NH₂; Y = Ala, (S)-2-amino-n-butryc acid (Abu), Val, (S)-2-amino-n-pentanoic acid (Nva) or Phe], dipeptide amides (Leu-Ala-NH₂ and Phe-Ala-NH₂) and FA-succinimide ester were purchased from Bachem (Bubendorf, Switzerland). FAGLA (Lot 370513) was obtained from the Peptide Institute (Osaka, Japan). All other reagents were of reagent grade and purchased from Nakalai Tesque (Kyoto, Japan). All amino acids were the l-forms.

Synthesis of FA-Leu-Ala-NH₂ and FA-Phe-Ala-NH₂

Furylacryloyl substrates (FA-X-Ala-NH₂; X = Leu or Phe) were synthesized by reacting FA-succinimide ester with appropriate

Abbreviations used: Dns, 5-(dimethylamino)-naphthalenyl-1-sulphonyl; FA, 3-(2-furyl)acryloyl; FAGLA, N-[3-(2-furyl)acryloyl]-glycyl-L-leucine amide; ZAPM, N-carboxbenzoxyl-L-aspartyl-L-phenylalanine methyl ester; Abu, (S)-2-amino-n-butryc acid; Nva, (S)-2-amino-n-pentanoic acid (L-norvaline).

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Hydrolysis of FA-dipeptide amide substrates

Hydrolysis of FA-dipeptide amides by thermolysin was measured by following the decrease in absorbance at 345 nm with the Shimadzu spectrophotometer UV-2200. The amount of FA-dipeptide amides hydrolysed was estimated by using the molar absorption coefficient $\varepsilon_{280}$ [13] in the standard buffer at 25°C. The $\Delta$A$_{280}$ value was confirmed not to be changed by the addition of NaCl, KCl and LiCl up to 5 M. Dependence of the specificity constant $k_{cat}/K_m$ for the hydrolysis of FA-X-Ala-NH$_2$ and FA-Gly-Y-NH$_2$ on hydrophobicity parameter $\pi$ of the side chains of the amino acids X and Y was examined according to the treatment of Fujita et al. [17]. The $\pi$ values for the side chains of Gly, Ala, Val, Leu and Phe of 0.00, 0.32, 1.27, 1.81 and 1.95 respectively, evaluated by Akamatsu et al. [18], were used. The $\pi$ values for the side chains of Abu and Nva were estimated to be 0.86 and 1.40 respectively, by subtracting the group branching factor (0.22) from the respective hydrophobic parameters, 1.08 and 1.62, estimated for ethyl and $n$-propyl substituents in aliphatic amines respectively [19]. Kinetic parameters ($k_{cat}$ and $K_m$) were determined by using the non-linear least-squares method with Taylor expansion [20].

RESULTS AND DISCUSSION

Effect of NaCl on the hydrolysis of FA-dipeptide amides

The effect of NaCl on the reaction rate of thermolysin-catalysed hydrolysis of eight kinds of FA-dipeptide amides with different amino acids at the P1 and P1’ sites of the scissile bond was examined. The reaction was carried out at substrate concentrations lower than the $K_m$ values (pseudo-first-order conditions). The first-order rate constant for substrate hydrolysis equals $(k_{cat}/K_m) [E]_o$, where $[E]_o$ is the total enzyme concentration, allowing us to estimate the specificity constant $k_{cat}/K_m$. Figure 1 shows dependence on the NaCl concentration of the relative activity for the hydrolysis of three representative substrates (FA-Gly-Ala-NH$_2$, FA-Gly-Leu-NH$_2$ and FA-Leu-Ala-NH$_2$). The activity increased apparently in an exponential fashion with increasing NaCl concentration, and the degree of the activation at 4 M NaCl was in the range of 11–17-fold, depending on the substrates. The activity seemed to increase further with [NaCl] higher than 4 M as long as its solubility was permitted. The log($k_{cat}/K_m$) values for eight substrates increased linearly with an increase in NaCl concentration, and the slopes of the lines were similar, whereas the log($k_{cat}/K_m$) value at 0 M NaCl varied considerably depending on the hydrophobicity of the amino acid side chains at the scissile bond of the substrates (Figure 2). However, it should be pointed out that there was a slight but distinct tendency for the slopes to increase in accordance with an increase in hydrophobicity of aliphatic side chains at the P1 and P1’ amino acids. As for the P1’ amino acid Y in FA-Gly-Y-NH$_2$, its hydrophobicity parameter ($\pi$) and the slope ($\alpha$) of the straight line obtained from Figure 2 (A) are as follows: Ala ($\pi$ 0.32, $\alpha$ 0.26 M$^{-1}$-s$^{-1}$); Abu ($\pi$ 0.86, $\alpha$ 0.27); Val ($\pi$ 1.27, $\alpha$ 0.27); Nva ($\pi$ 1.40, $\alpha$ 0.28); Leu ($\pi$ 1.81, $\alpha$ 0.28); and Phe ($\pi$ 1.95, $\alpha$ 0.26). Similarly, for the P1 amino acid X in FA-X-Ala-NH$_2$ the values are as follows: Gly ($\pi$ 0.00, $\alpha$ 0.26); Leu ($\pi$ 1.81, $\alpha$ 0.30); and Phe ($\pi$ 1.95, $\alpha$ 0.24). The degree of activation for substrates containing Phe, such as FA-Gly-Phe-NH$_2$ and FA-Phe-Ala-NH$_2$, is the lowest among eight substrates, although Phe is the most hydrophobic. Aromatic amino acids, including Phe, often behave differently from aliphatic amino acids in events as far as hydrophobicity is concerned [21], and such a phenomenon is also observed in Figure 3.

The $a$ values for eight different substrates are considerably similar and average 0.269 ± 0.016 M$^{-1}$, even though there is a large difference ranging 500-fold in the activity ($k_{cat}/K_m$) among them (Figure 2). In fact, the activity ($k_{cat}/K_m$) of thermolysin towards favourable substrates, FA-Gly-Leu-NH$_2$ and FA-Gly-Phe-NH$_2$, is 2.0 × 10$^5$ M$^{-1}$-s$^{-1}$, and that towards a poor substrate, FA-Gly-Ala-NH$_2$, is 40 M$^{-1}$-s$^{-1}$. Instead of this large difference in the activity, the degree of activation for these substrates at 4 M NaCl is in a considerably narrower range of 11–17-fold: 11-fold with FA-Gly-Leu-NH$_2$ and 17-fold with FA-Leu-Ala-NH$_2$ (Figure 1). Figure 2 shows that the activity ($k_{cat}/K_m$) at x M NaCl is expressed as
Activation of thermolysin by salts

Figure 2 Logarithmic relationship of the peptidase activity of thermolysin with NaCl concentration

The reaction was carried out in the standard buffer (pH 7.5) at 25 °C containing NaCl at the indicated concentrations with thermolysin of 0.1–0.85 µM and substrates of 14 µM–0.4 mM. The peptidase activity was expressed as $k_{cat}/K_m$ on the ordinate axis. (A) Substrates are in the form of FA-Gly-Y-NH$_2$, where Y is Ala ( ), Abu ( ), Val ( ), Nva ( ) or Phe ( ). (B) Substrates are in the form of FA-X-Ala-NH$_2$, where X is Gly ( ), Leu ( ) or Phe. [NaCl]$_0$ is the initial substrate concentration.

Hydrophobic effect on the NaCl-dependent activation of thermolysin

The log($k_{cat}/K_m$) values obtained at a constant NaCl concentration were plotted against the hydrophobicity parameter $\pi$ of the side chain of the P1' amino acid (Figure 3A). For linear aliphatic amino acids (Ala, Abu and Nva), a linear relationship was obtained at the respective NaCl concentrations, and the slopes are substantially the same (1.96 ± 0.01). The data points for branched aliphatic amino acids (Val and Leu) lie under the lines drawn for the linear aliphatic amino acids, and the lines connecting the points for Val and Leu are parallel to the lines for the linear aliphatic amino acids. The points for Phe are all under the lines for the branched aliphatic amino acids. An interesting feature is pointed out: all data points examined lie on six representative linear lines. Intervals between lines are all equal, as 0.25 unit in log($k_{cat}/K_m$). Points for Val and Leu at 0 M NaCl and Phe at 1 M NaCl lie on the first line (ordinate-axis intercept 0.75). On the second line (1.00), points for Ala, Abu and Nva lie at 0 M, Val and Leu lie at 1 M, and Phe lies at 2 M NaCl. On the third line (1.25), points for Ala, Abu and Nva lie at 1 M, Val and Leu lie at 2 M, and Phe lies at 3 M NaCl. On the fourth line (1.50), points for Ala, Abu and Nva lie at 2 M, Val and Leu lie at 3 M, and Phe lies at 4 M NaCl. On the fifth line (1.75), points for Ala, Abu and Nva lie at 3 M, Val and Leu lie at 4 M, and Phe lies at 5 M NaCl. On the sixth line (2.00), points for Ala, Abu and Nva lie at 4 M NaCl. The point for Phe at 0 M NaCl is expected on a line with a slope of 1.96 and an ordinate-axis intercept of 0.50. This result indicates that the enzyme activity $k_{cat}/K_m$ increases with an increase in the hydrophobicity at the P1' amino acid side chain in the presence of 0–4 M NaCl. If a linear aliphatic side...
Effect of NaCl on the kinetic parameters

The kinetic parameters, $k_{cat}$ and $K_m$, for the thermolysin-catalysed hydrolysis of FA-dipeptide amides employed in this study, except for FA-Leu-Ala-NH$_2$ and FA-Phe-Ala-NH$_2$, could not be determined separately because of the sparing solubility in comparison with the $K_m$ values, especially in the presence of NaCl. Therefore the enzyme reaction was inevitably conducted under the pseudo-first-order conditions ($[S] \ll K_m$), so that the initial rates are proportional to $k_{cat}/K_m$. The parameters for FA-Leu-Ala-NH$_2$ were barely evaluated in NaCl concentrations of only up to 2 M, $[S]_0$ being set to 0.1–1.5 $\times K_m$. By non-linear least-squares regression for the Michaelis-Menten (v versus [S]) plots, $k_{cat}$ and $K_m$ were determined to be 0.56±0.08 s$^{-1}$ and 0.80±0.04 mM, respectively, at 0 M NaCl; 1.20±0.08 s$^{-1}$ and 0.80±0.06 mM at 1 M NaCl; and 2.64±0.15 s$^{-1}$ and 0.82±0.10 mM at 2 M NaCl. With FA-Phe-Ala-NH$_2$, the parameters were evaluated safely in NaCl up to 1 M, and $k_{cat}$ and $K_m$ were 5.7±0.3 s$^{-1}$ and 83±5 µM, respectively, at 0 M NaCl, and 10.1±0.4 s$^{-1}$ and 84±8 µM at 1 M NaCl. Only $k_{cat}$ increased, whereas $K_m$ remained unaltered, with increasing NaCl concentration, which resulted in the increase in $k_{cat}/K_m$ shown in Figures 1 and 2. Being consistent with the results obtained with ZAPM [13], the activation of thermolysin in the hydrolysis of FA-Leu-Ala-NH$_2$ and FA-Phe-Ala-NH$_2$ has been demonstrated to be brought about only by the enhancement of $k_{cat}$, and $K_m$ is not altered in the presence of salts. Let us suppose that this could be adopted reasonably to FA-dipeptide amide substrates. Eqn. (3) is turned into eqn. (6), indicating the degree of increase in $k_{cat}$ in the presence of $x$ M NaCl:

$$\frac{(k_{cat})_x}{(k_{cat})_0} = 1.9^x$$

(6)

The parameters for six other substrates could not be determined definitely even in the absence of NaCl. In the case of FAGLA, $k_{cat}$ and $K_m$ were narrowly estimated to be $>60$ s$^{-1}$ and $>3$ mM, respectively, at 0 M NaCl, and $>120$ s$^{-1}$ and $>3$ mM at 1 M NaCl. In the presence of 5–20% organic solvents such as ethanol, dimethyl sulfoxide and ethylene glycol, the substrate solubility increased and the parameters could be evaluated. The organic solvents, however, inhibit the hydrolysis in a complicated manner (K. Inouye, unpublished work), suggesting that it is not easy to estimate the effect of NaCl on the kinetic parameters in the presence of the organic solvents. Substrates containing oligopeptides larger than FA-dipeptide amides may give smaller $K_m$ values, allowing us to determine $k_{cat}$ and $K_m$ values separately. With 5-(dimethylamino)-naphthalenyl-1-sulphonyl (Dns)-Ala-Phe-Ala and Dns-Gly-Phe-Ala, however, dependence of the thermolysin activity on NaCl concentration is different from that observed with FA-dipeptide amide substrates [27] (see below). This suggests that the effect of NaCl on the activity of thermolysin could be changed depending on substrate structure and interaction between substrates of thermolysin and substrates.

Effectiveness of monovalent cations on the salt activation of thermolysin

Thermolysin-catalysed hydrolysis of FA-Leu-Ala-NH$_2$ was examined at 1 M NaCl, KCl and LiCl. The $K_m$ and $k_{cat}$ values in
the absence of salt were $0.80 \pm 0.04 \text{ mM}$ and $0.56 \pm 0.08 \text{ s}^{-1}$ respectively. The $K_m$ values were $0.80 \pm 0.06$, $0.81 \pm 0.06$ and $0.78 \pm 0.08 \text{ mM}$ with 1 M NaCl, KCl and LiCl respectively, showing that the $K_m$ value is not changed on the addition of salts in the reaction medium. The $k_{cat}$ values were changed depending on the salts, and were $1.20 \pm 0.08$, $1.12 \pm 0.07$ and $0.91 \pm 0.08 \text{ s}^{-1}$ with 1 M NaCl, KCl and LiCl respectively.

It has been shown that the activation of thermolysin through increasing $k_{cat}$ in the hydrolysis of FA-Leu-Ala-NH$_2$ is dependent on the species of monovalent cations employed, and that the effectiveness follows the order of Na$^+$ > K$^+$ > Li$^+$, which is the same as that observed in the hydrolysis of FAGLA and ZAPM [13]. This order is different from that of the ionic radius of the monovalent cations, K$^+$ > Na$^+$ > Li$^+$, and that of the Hofmeister’s series, K$^+$ < Na$^+$ < Li$^+$ [28], suggesting a possibility that the activation of thermolysin is induced by specific interaction between the cations and the enzyme. As for anions, we have demonstrated that the difference between NaCl and NaBr or between KCl and KBr is not significant in the effects of activation of thermolysin [13], and that tryptophyl residue 115 interacts favourably with Cl$^-$ but not with Br$^-$ [14]. Further studies are needed to elucidate the relationship between binding of ions, especially cations, and the activation of the enzyme.

**Dependence of the salt activation of thermolysin on the substrate structure**

The variations in the thermolysin activity as a function of increasing NaCl concentration exhibit exponential behaviour for all FA-dipeptide amide substrates examined. The degree of activation is not much dependent on the structure of the substrates ($a = 0.269 \pm 0.016 \text{ M}^{-1}$), and ranges from 11- to 17-fold at 4 M NaCl. Rather different values have been reported, however. The value is 6–7 times for ZAPM at 3.8 M NaCl ($a = 0.22 \text{ M}^{-1}$) [13]. The thermolysin activity toward FAGLA and its ester analogue FA-Gly-OLeu-NH$_2$ increases progressively with increasing NaBr concentration until 5 M, and it becomes 25-fold that observed in the absence of salt; in contrast, the activity towards benzoyl-Gly-Phe-Ala and benzoyl-Gly-OPhe-Ala increases only 5-fold at 5 M NaBr [12]. It is evident that the degree of activation depends on the substrates, and the discrepancy in the degree of activation could be derived from the difference in the substrate structure at either P2 or P2’ residues, or both. Yang et al. [27] observed activation by 8- and 18-fold at 4 M NaCl with Dns-Ala-Phe-Ala and Dns-Gly-Phe-Ala respectively. Interestingly, they have reported that increasing [NaCl] increases $k_{cat}/K_m$ in a linear fashion but not progressively, and that it decreases $K_m$, but leaves $k_{cat}$ unaltered for both substrates. With ZAPM [13] and the FA-dipeptide amides used in this study, the activation of thermolysin by NaCl is brought about solely through an increase in $k_{cat}$, and $K_m$ is not affected at all by the addition of NaCl. It has been discussed that the different results between ZAPM and Dns-tripeptides were related to the fact that the Asp residue of nuclease H from some other difference in the structure of substrates. The P2’ residue (Ala) of the Dns-tripeptide substrates can contact directly the subsite S2’, while this contact might be less sufficient in the case of FA-dipeptide amides and ZAPM because of the smaller size of the amide and methyl moieties. The Dns group, being bulkier than the corresponding FA and Z groups, may interact with not only the S2 but also the S3 subsites. Because of the bulkiness, the salt effect on the thermolysin-catalysed hydrolysis of Dns-tripeptides might be different from that of FA-dipeptide amides and ZAPM.

Some reports on salt activation of enzymes other than thermolysin are found in the literature [29]. The activity of nuclease H of Micrococcus varians in the hydrolysis of thymidine-5’-monophospho-p-nitrophenyl ester increases with increasing [NaCl] in an exponential fashion, and at 5 M NaCl it is 50-fold higher than that in the absence of NaCl, whereas the activation profile toward DNA is bell-shaped with an optimal [NaCl] at 2.5 M [30]. The activity of a serine proteinase, halolysin, of an archaeabacterium in the hydrolysis of N-succinyl-Ala-Ala-Ala-p-nitroanilide is enhanced exponentially with an increase in [NaCl], while the activation of N-succinyl-Ala-Ala-Pro-Phe-7-amido-4-methyl-coumarin shows a bell-shaped effect [31]. The activation behaviour of these enzymes is dependent on substrates, and the effect of cations is in the order of K$^+$ > Na$^+$ > Li$^+$, which is different from that for thermolysin. Recently, it has been reported that aspartic acid proteases of avian myeloblastosis virus and human immunodeficiency virus type I and porcine pepsin are activated markedly in the presence of NaCl up to 5 M [32]. For these enzymes, $k_{cat}/K_m$ increases exponentially with an increase of NaCl, where $K_m$ is reduced progressively but $k_{cat}$ is not altered. Although they show an exponential activation behaviour like thermolysin, the cause of the activation is different. Varieties in the activation behaviour suggest that the activation may be attributable not only to a change in the characteristics of the medium by adding salts but also to specific interaction of salts with enzymes and substrates. Actually, the intensity of the absorption difference spectrum induced by adding NaCl or NaBr to thermolysin is 10-fold or greater than that expected from the solvent perturbation difference spectrum using model compounds [14]. This suggests that ions of salts interact preferably with thermolysin. The effects of the substrate properties on the activation of and interaction between salts and thermolysin are currently under examination.

**Influence of ionic strength**

Theoretical treatments of the influence of ionic strength ($\mu$) on the rates of reaction between a charged molecule and a neutral one gave the relationship:

$$\log(k/k_0) = b \cdot \mu \tag{7}$$

where $b$ is a constant, so that the logarithm of the rate constant becomes a linear function of the first power of the ionic strength [33]. It is noteworthy that the relationship shown in Figure 2 is similar to that expressed by eqn. (6). As has been discussed above, the relationship in Figure 2 is reasonably expressed by eqn. (6), so $1.9^\alpha = 10^{\mu b}$. If the ionic strength $\mu$ of the NaCl solution is expressed anyway with the NaCl molarity $x$, the b value is calculated to be 0.279, although the meaning of this value is unclear.

Generally, physicochemical treatments have been carried out so far in considerably dilute solutions, and avoided events in the concentrated salt solutions such as those studied in the present study. Concepts of ionic strength, activity coefficient, dielectric constant, hydration of ions or aqueous solution are all vague in such concentrated solutions. In order to get away from confusion brought about by the use of these physicochemical quantities, we use molarity in order to describe amounts of salts in the solution in the present paper. Studies on halophilic enzymes may be expected to expand the physicochemical limitations, so that
physical chemistry can interpret and predict the events in such concentrated salt solutions.

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