pH-dependent reversible inhibition of violaxanthin de-epoxidase by pepstatin related to protonation-induced structural change of the enzyme

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Abstract The redox enzyme violaxanthin de-epoxidase (VDE) was found to be sensitive to pepstatin, a specific inhibitor of aspartic protease. The inhibition was similar to that of aspartic protease in that it was reversible and accompanied by the protonation of the enzyme. Of the two peaks of VDE appearing on anion exchange chromatography, VDE-I predominated at pH 7.2. On lowering the pH of the chromatography, VDE-I decreased and VDE-II increased. Furthermore, re-chromatography of either peak yielded both peaks. These results suggest that VDE-I and VDE-II are interconvertible depending on pH, and thus, they represent the de-protonated and protonated forms of the enzyme, respectively. Presumably the protonation-induced structural change of the enzyme is responsible for the interaction with pepstatin, and also with substrate.

Key words: Pepstatin; Photoprotection; Protonation-induced conformational change; Violaxanthin de-epoxidase; Xanthophyll cycle

1. Introduction

The xanthophyll cycle plays an important role in protection of plants against excess light [1]. Only two enzymes are involved in the cycle, violaxanthin de-epoxidase (VDE) and zeaxanthin epoxidase [2]. The former catalyzes the de-epoxidation of violaxanthin to zeaxanthin through the intermediate antheraxanthin, and the latter catalyzes the reverse reaction. VDE is located in the thylakoid lumen and is activated by the acidification of the lumen by photosynthetic electron transport. In contrast, zeaxanthin epoxidase is located on the stromal surface of thylakoid membranes and functions irrespective of the lumenal pH. Thus, the reactions can proceed separately in opposite directions. Since violaxanthin is a light-harvesting pigment, while antheraxanthin and zeaxanthin function as energy-dissipating ones [3], this system serves to regulate the energy supply to photosystem II.

In spite of extensive studies on the enzymatic properties and sequence analysis [4–8], the reaction center of VDE has not been identified to date. Generally, inhibitor study can help reveal the reaction center type, but no active site-specific inhibitors have been found for this enzyme; although dithiothreitol is known to inhibit VDE, its action is not site-specific and the inhibition derives from alteration of the conformation of the enzyme by cleavage of the multiple disulfide bonds [7,9]. In a previous study [9], we purified VDE from spinach and showed that it was inhibited by pepstatin, an acetylated pentapeptide that is known to be a specific inhibitor of aspartic protease [10]. This finding suggested that the reaction centers of VDE and aspartic protease have a common structural feature, but the inhibition of VDE has not been characterized in detail. In the present study, we show that it is pH-dependent and reversible, and related to the protonation of VDE, which are characteristics also found in the inhibition of aspartic protease.

2. Materials and methods

2.1. Materials

Spinach was purchased at a local market. Monogalactosyldiacylglycerol (MGDG) was the product of Serdary Research Laboratories (London, Ont., Canada). Violaxanthin was a gift from Dr. S. Takaichi of Nippon Medical School. Pepstatin A was obtained from Sigma (St. Louis, MO, USA). Other chemicals were from Wako Pure Chemical Industries (Osaka, Japan).

2.2. VDE reaction

VDE was extracted by sonicating spinach thylakoids in 50 mM Tris–HCl (pH 7.5) and 1 mM $MgCl_2$ as described previously [11]. VDE activity was assayed spectrophotometrically by monitoring A_{505} minus A_{540} with a dual-wavelength spectrophotometer (UV-2200; Shimadzu, Kyoto, Japan) at room temperature as follows. 5 μl of 13.2 mM MGDG (in ethanol) was placed in a sample tube, with which 5 μl of 0.66 mM violaxanthin (in ethanol) was mixed. To the mixture, 1 ml of 50 mM CH_3COONa–HCl (pH 4.8) and 50 μl of VDE sample were added in that order. The absorbance difference gradually rose during the course of the stabilization of the mixed micelles of MGDG and violaxanthin in the aqueous solution. It took about 10 min for the absorbance difference to reach a plateau. The reaction was started by adding 20 μl of 0.55 M ascorbic acid. The activity was calculated from the initial rate of increase of the absorbance difference with the difference absorption coefficient of 63 mM^{-1} cm^{-1} [6]. One unit (U) of activity is defined as 1 μmol violaxanthin de-epoxidized per minute.

2.3. Inhibition of VDE by pepstatin and restoration by pH 7.5 treatment

VDE extract (50 μl) was mixed with the same volume of 12.5 mM CH_3COONa–HCl (pH 4.8), which resulted in a solution with a final pH of 5.8. To the mixture, 2.7 μl of pepstatin solution (usually, the concentration was 11.4 mM to give a final concentration of 0.3 mM) in dimethyl sulfoxide (DMSO) was added. This solution was well mixed by repeated sucking and ejection with a micropipette (Pipetman; Gilson). After incubation of the mixture at 15°C for 10 min, the pepstatin-treated VDE was subjected to the assay. DMSO somewhat decreased the activity by itself, but this effect was negligible when photosystem II membranes were used as substrate [9]. Thus, the effect of DMSO should be related to possible alteration of the structure of

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Abbreviations: DMSO, dimethyl sulfoxide; MGDG, monogalactosyldiacylglycerol; VDE, violaxanthin de-epoxidase

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mixed micelles; violaxanthin without lipid cannot be the substrate of VDE and the micellar structure of the substrate is essential for the reaction [4,11]. In this study, the DMSO-treated sample was regarded as the control to evaluate the inhibition by pepstatin.

For restoration of VDE, 20 μl of 0.1 M K_2HPO_4 was added to the pepstatin-treated sample to raise the pH to 7.5. The sample was well mixed by repeated sucking and ejection with a micropipette. After incubation at room temperature for 10 min, the sample was assayed.

2.4. Chromatography

To remove polyphenol oxidase whose degradation products partially overlap with the 43-kDa polypeptide of VDE on SDS–PAGE [12], VDE extract from spinach thylakoids equivalent to 130 mg chlorophyll was subjected to chromatography on an affinity column retaining antibodies against dithiothreitol-sensitive tetrameric protease (DSTP, the same as polyphenol oxidase) [13]. The flow-through fraction from the anti-DSTP column, which contained VDE, was subjected to HiTrap SP chromatography in the presence of 0.1% (w/v) Tween 20, as described previously [9]. The preparation from the HiTrap SP column was divided into three portions and subjected to anion exchange chromatography as described previously [9], but at three different pHs. Briefly, the sample was dialyzed against 20 mM sodium phosphate (pH 7.2, 6.6 or 6.0) and 0.05% Tween 20; the dialysate was then loaded on a Mono Q column (1 ml; Pharmacia, Uppsala, Sweden) that had been equilibrated with the same medium. After washing with the equilibration medium, the column was eluted with 50 ml of a linear gradient of 0–0.3 M NaCl in 20 mM sodium phosphate (at the respective pHs) and 0.05% Tween 20; since the detergent interferes with the activity assay with mixed micelles [11], the concentration was decreased from 0.1% in the previous study [9] to 0.05%.

3. Results

3.1. VDE is inhibited by treatment with pepstatin

When VDE extract was treated with 0.3 mM pepstatin under standard conditions followed by 10-fold dilution with the assay medium, activity was inhibited by 95%, compared to the control that had been treated with DMSO alone. In contrast, no inhibition was observed when 0.03 mM pepstatin was provided with VDE that had been diluted with the assay medium. Thus, we adopted the pretreatment system to observe the inhibition. In the pretreatment, at least 4 min passed before the maximal inhibition was observed (Fig. 1A), suggesting that pepstatin may not immediately bind to the enzyme. Based on this result, we chose a period of 10 min for the pepstatin treatment. VDE was maximally inhibited by the treatment at 0.3 mM pepstatin or above with half inhibition at 0.12 mM pepstatin (Fig. 1B).

3.2. The inhibition by pepstatin is pH-dependent and reversible

Effects of pH were studied by treating VDE with 0.3 mM pepstatin at different pHs, which was followed by the assay at pH 4.8. The extent of the inhibition was almost the same in the range of pH 3.7–5.8, while the inhibition dramatically diminished when the treatment was performed at pH 7 or above (Fig. 2A). This observation suggests that pepstatin did not effectively bind to de-protonated VDE; the enzyme has been suggested to be de-protonated at neutral pH [5,14,15]. To examine the reversibility of the inhibition, the pepstatin-treated VDE was brought to pH 7.5 by adding a small volume of 0.1 M K_2HPO_4 and incubated at room temperature for 10 min. We expected that if pepstatin bound at pH 5.8 was released at pH 7.5, the inhibitor could not effectively rebind to the enzyme even when assay medium of pH 4.8 was added thereafter. In fact, the activity was 70% restored by the temporary pH rise (Fig. 2B), suggesting that

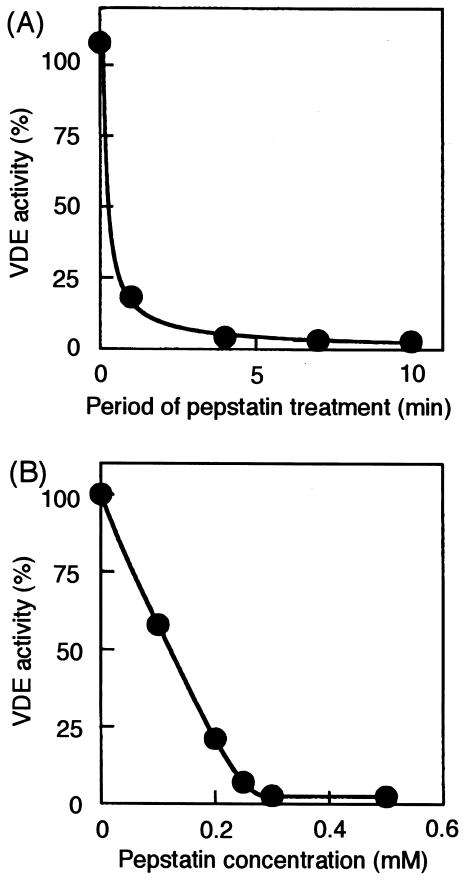

Fig. 1. Characterization of the inhibition of VDE by pepstatin. A: Time course of pepstatin treatment. VDE extract was treated with 0.3 mM pepstatin at pH 5.8 at 15°C for the designated periods. The sample was 10-fold diluted with the assay medium for the reaction at pH 4.8. The activity of the sample treated with DMSO for 10 min (10.0 mU/ml) was taken as 100%. B: Effect of pepstatin concentration. VDE extract was treated with the designated concentrations of pepstatin under the standard conditions prior to the assay. The activity of the sample treated with DMSO (8.6 mU/ml) was taken as 100%.

the inhibition is reversible. Restoration at pH 7.5 took more than 5 min to reach the maximal level (Fig. 2B). This result suggests that pepstatin was not immediately released from the enzyme upon the pH 7.5 treatment.

3.3. Protonation-induced structural change of VDE

We showed in a previous study [9] that VDE separates into two peaks, VDE-I and VDE-II, when subjected to anion exchange chromatography on Mono Q at pH 6.5 in the presence of Tween 20. However, such heterogeneity of the VDE molecule has not been reported by other authors [6,12,16] who performed similar chromatography at pH 7.2 in the absence of detergent. From this apparent discrepancy, we hypothesized that the molecular heterogeneity of VDE would derive from the protonation of the enzyme. To examine this hypothesis, we subjected VDE to Mono Q chromatography at various pHs. In this experiment, the sample had been partially purified so that no polypeptides overlapped on SDS–PAGE with the 43-kDa VDE polypeptide (see Section 2). VDE-I predominated when the chromatography was performed at pH 7.2, but as the pH of the chromatography was lowered, VDE-I decreased and VDE-II increased (Fig. 3). Further-

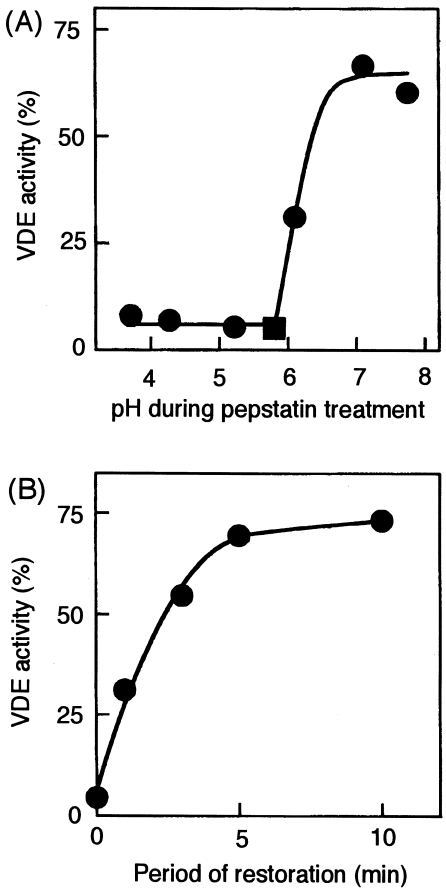

Fig. 2. pH-dependent reversible inhibition of VDE by pepstatin. A: Effects of pH upon pepstatin treatment. VDE extract was treated with 0.3 mM pepstatin at the designated pHs at 15°C for 10 min, and then subjected to the assay at pH 4.8. To obtain the pH values indicated in the figure, 12.5 mM CH_3COONa–HCl (pH 4.8) used in the standard conditions (square) was replaced by 50 mM buffer (circle), either CH_3COONa–HCl (pH 3.0), CH_3COONa–HCl (pH 4.0), CH_3COONa–HCl (pH 5.0), MES–NaOH (pH 6.0), HEPES–HCl (pH 7.0), or TAPS–NaOH (pH 8.0). The activity of the sample treated with DMSO at pH 5.8 (5.2 mU/ml) was taken as 100%. B: Time course of restoration of pepstatin-inhibited VDE at pH 7.5. VDE extract, which had been treated with 0.3 mM pepstatin under the standard conditions, was incubated at pH 7.5 for the designated periods prior to the assay. The activity of the sample treated with DMSO under the standard conditions but not incubated at pH 7.5 (9.1 mU/ml) was taken as 100%.

more, when VDE-I was subjected to re-chromatography at pH 6.0, not only VDE-I appeared but also VDE-II (Fig. 4). Similarly, both peaks appeared when VDE-II was subjected to re-chromatography at pH 7.2 (Fig. 4). These findings indicate that VDE-I and VDE-II are interconvertible by pH change, and thus represent the de-protonated and protonated forms of VDE, respectively. It is likely that VDE undergoes a structural change along with the protonation, which allowed the separation of the two forms by the chromatography (see Section 4). The VDE-I and VDE-II preparations showed the same enzymatic properties [9] and it was also the case with respect to the inhibition by pepstatin (data not shown). This fact suggests that VDE-I and VDE-II reflect the respective forms upon binding to the column, but after elution, the two peaks become enzymatically equivalent by equilibration between the two forms at any given pH.

4. Discussion

In the present study, we characterized the inhibition of VDE by pepstatin. To observe the inhibition, treatment of VDE with a relatively high (sub-millimolar) concentration of pepstatin was necessary. This finding suggests that the affinity of the inhibitor to the enzyme is not so high upon binding. However, the observation of inhibition after 10-fold dilution of the treated enzyme suggests that once the binding is accomplished, the inhibitor is hardly released by dilution. Thus, the interaction of VDE and pepstatin is not simple. We interpret the result taking account of the molecular structure of VDE proposed by Bugos et al. [8]. They indicated referring to the deduced amino acid sequences of VDE that this enzyme has

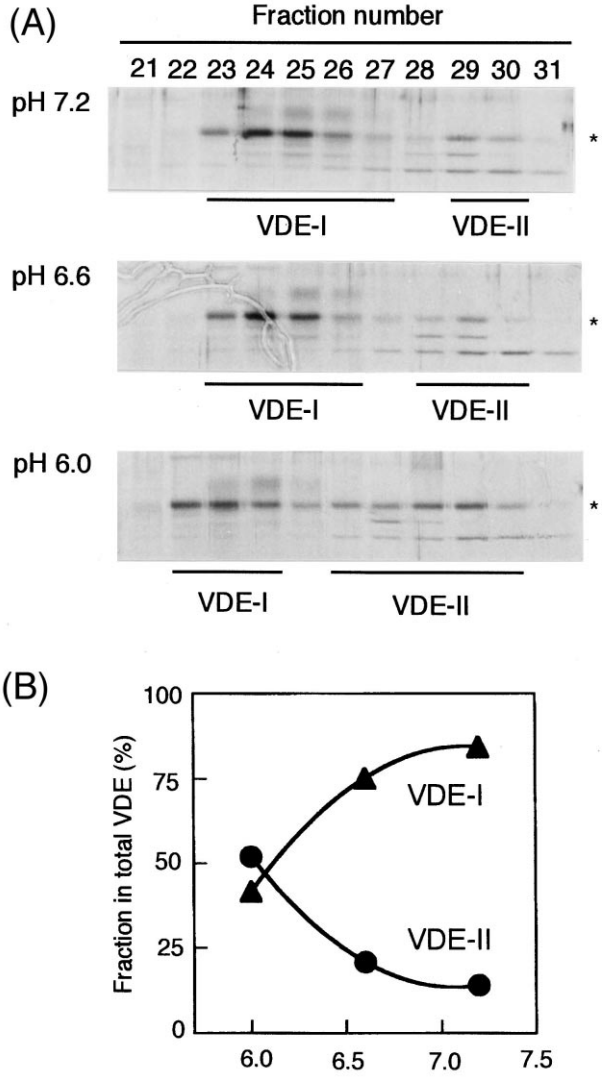

Fig. 3. Separation of VDE-I and VDE-II on anion exchange chromatography at different pHs. A: The flow-through fraction from the anti-DSTP column (see Section 2) was subjected to anion exchange chromatography on Mono Q at pH 7.2, 6.6 and 6.0. Fractions with the activity were subjected to SDS–PAGE, and the gels were stained with silver. The asterisk indicates the 43-kDa polypeptide of VDE. The first peak of VDE was designated VDE-I and the second VDE-II [9]. B: Relative amounts of VDE-I and VDE-II at different pHs. The relative amounts of VDE-I and VDE-II in the total VDE were estimated from the results of A by image analysis [9].

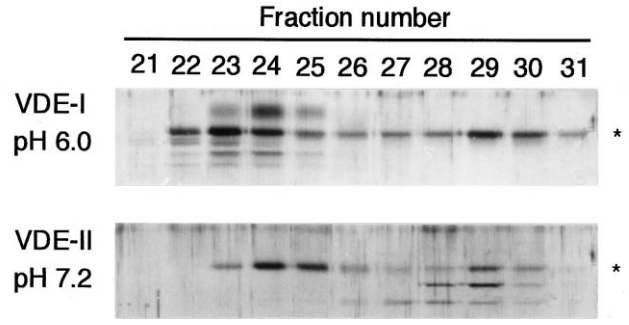

Fig. 4. Re-chromatography of VDE-I and VDE-II obtained by Mono Q chromatography. VDE-I and VDE-II preparations were pooled and subjected to re-chromatography on Mono Q at pH 6.0 and pH 7.2, respectively. The asterisk indicates the 43-kDa polypeptide of VDE. Note that the re-chromatography is effective in the purification of VDE, as revealed by the polypeptide profile.

an anti-parallel β-barrel structure, in which a lipocalin signature, which is indicative of binding of a small hydrophobic molecule, is situated. This structure is consistent with their previously proposed model, in which the VDE molecule has a cavity formed by the protein folding, into which the substrate enters [4]. If it is assumed that pepstatin enters the cavity, the above discrepancy can be explained; it is hard to enter and hard to exit. The finding that neither the inhibition by pepstatin nor the restoration at pH 7.5 occurred immediately upon the treatments is consistent with our interpretation.

The inhibition of VDE by pepstatin suggests that the reaction centers of VDE and aspartic protease may have a common structural characteristic. However, this does not necessarily imply that the active site of VDE is an aspartic acid residue(s), considering the mechanism of inhibition proposed above; pepstatin might interfere with the substrate entering the cavity by binding to a site different from the active site. Experiments to examine possible competition between the substrate and pepstatin were performed, but no clear results were obtained (Kawano and Kuwabara, unpublished). Further study is needed to clarify how pepstatin inhibits VDE.

The pH dependence of the inhibition of VDE by pepstatin appears to be similar to that of the rate constant of the initial part of the activity in isolated pea chloroplasts [14] in that it drastically changes in a narrow pH range around 6.0. Hill plot analysis of the rate constant showed a Hill coefficient of 5.3, indicating a strong cooperativity with respect to protons [14]. The similarity suggests that the pH-dependent structural change of VDE, which was inferred from the interconversion between VDE-I and VDE-II (Fig. 4), affects the interaction with pepstatin as well as with substrate. Bratt et al. [5] suggested that the protonation of the enzyme is related to the cooperativity observed in the rate constant [14], as revealed by the pH dependence of the binding of the enzyme to thylakoid membranes, although the binding showed a slightly weak cooperativity (Hill coefficient) of 4 with an inflection point at pH 6.6 instead of 6.0. Taking together the results in this study, it is likely that the protonation of VDE causes not only the increase in the bulk hydrophobicity of the enzyme but also the structural change to more readily accept the substrate.

Note that pepstatin induces protonation of aspartic protease; the pK_a of one of the two active site aspartic acid residues in plasmepsin II increases from 4.7 to 6.5 through interaction with pepstatin [17]. Thus, the affinity of pepstatin to the protease is extraordinarily enhanced by acidification of the surrounding environment [18]. Whether pepstatin induces the protonation of VDE should be examined in the future; it could be an indication of the presence of a reactive amino acid residue.

From the present study, we suggest that VDE-I and VDE-II are related to the de-protonated and protonated forms of VDE, respectively, based on the pH-dependent changes in their relative amounts (Fig. 3) and on their interconvertibility (Fig. 4). This assignment is apparently inconsistent with the order of elution of the two peaks from the anion exchange column [9]: VDE-I (de-protonated) was eluted earlier than VDE-II (protonated) in a gradient of increasing NaCl concentration. One might suspect that a hydrophobic interaction between the protonated form and the column resin might retard the elution. However, this situation seems unlikely since the elution medium contained a neutral detergent Tween 20 at 0.1% to eliminate such hydrophobic interaction [9]. Thus, we speculate that the protonation of VDE may not simply decrease the surface charge of the protein but also cause a structural change that enhances the binding to the anion exchange column resin. This hypothesized conformational change of VDE remains to be investigated in the future.

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