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# Protein splicing in the yeast Vma1 protozyme: evidence for an intramolecular reaction

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**Abstract Protein splicing is an autocatalytic reaction of a single polypeptide in which a spliced intervening sequence is excised out and the two external regions are ligated with the peptide bond to yield two mature proteins. We examined the reaction mechanism using a folding-dependent in vitro protein splicing system. Protein splicing proceeds at an optimal pH of 7 and is an intramolecular reaction. The reaction is not inhibited by potential protease inhibitors, suggesting that its mechanism is different from those catalyzed by known proteases.**



*Key words:* Intramolecular reaction; Protease inhibitor; Protein splicing; Refolding; Vma1 protozyme

## 1. Introduction

Protein splicing is a chemical reaction in which an in-frame intervening polypeptide is excised from a precursor protein and the flanking N- and C-terminal regions are ligated with the peptide bond to produce two mature proteins. In the yeast *Saccharomyces cerevisiae*, the 120 kDa nascent translation product of the *VMA1* gene, termed Vma1 protozyme [1], autocatalytically excises out the 50 kDa DNA endonuclease (VDE; *VMA1*-derived endonuclease, see [2]) and splices the two external polypeptides to form the 70 kDa catalytic subunit of the vacuolar $H^+$-ATPase [3,4]

Substitution of amino acids around the splice junctions has shown that the structure around the cleavage sites of the substrate is important for the splicing reaction [5–10]. Mutational analysis has demonstrated the crucial requirement of the thiol- or hydroxyl-containing residues at the two splice junctions ($Cys^{284}$ and $Cys^{738}$ as for the Vma1 protozyme) and the asparagine residue immediately preceding the C-terminal junction ($Asn^{737}$). The C-terminal cleavage step is found to be accomplished by cyclization of this asparagine residue [9,11]. The invariant histidine residue ($His^{736}$) preceding $Asn^{737}$ seems to assist the reaction at the C-terminal cleavage step [9,10]. Moreover, we have recently demonstrated that another conserved His residue ($His^{362}$), which is 78 amino acid apart from the N-terminal $Cys^{284}$, participates in the reaction at the N-terminal cleavage step [12].

For more extensive biochemical studies of the splicing reaction, we have previously established an in vitro protein splicing system with various recombinant VDE derivatives as substrates, and demonstrated that upon refolding of the VDE domain under critical conditions, the excision-coupled splicing reaction takes place autocatalytically [13]. In this report, we describe that the splicing reaction proceeds intramolecularly at neutral pH and its splicing mechanism differs from those of known protease reactions.

## 2. Materials and methods

XC-VDE [13]and MIIYVG-VDE-VMA1C (see Fig. 2A) polypeptides were expressed as described previously [13]. To make an expression vector for the VMA1N-VDE-CGER polypeptide (see Fig. 2A), a *Nco*I site was introduced at the initiation codon of the *VMA1* gene by PCR using two oligonucleotide primers (5′-GCGCCATGGCTGGTGCAATTGAAAAC-3′ and 5′-AAGGCCTGCAGTTTCTTCGTA-3′) and pMVMA1 [6] as a template. The *Nco*I–*Pst*I fragment of the PCR product and the *Pst*I–*Kpn*I fragment of the *VMA1* gene were introduced into the *Nco*I and *Kpn*I gaps of pET-Linker [13]. Then the *Kpn*I–*Bam*HI gap of the resultant plasmid was filled with the *Kpn*I–*Bam*HI fragment of pET-XC-VDE [13]. The MIIYVG-VDE-VMA1C and VMA1N-VDE-CGER polypeptides were partially purified as described previously [13].

In our standard refolding protocol [13], recombinant VDE derivatives accumulated in an *E. coli* insoluble fraction were collected and solubilized in the unfolding buffer (6 M guanidine-HCl, 50 mM Tris-HCl, 1 mM EDTA, 10 mM DTT, pH 8.0) at a protein concentration of 0.1–1 mg/ml. After 1 h incubation at room temperature, the mixture was clarified by centrifugation, and dialyzed against the refolding buffer (50 mM Tris-HCl, 1 mM EDTA, pH 8.0) for over 2 h at 4°C. Then the reaction mixture was analyzed by SDS-PAGE. The excised VDE migrates faster than the recombinant VDE substrates. The rate of protein splicing was determined by measuring the amount of VDE formed under conditions indicated. Protease inhibitors were purchased from Sigma.

## 3. Results

### 3.1. Refolding conditions for protein splicing

We examined refolding conditions which allow efficient splicing of XC-VDE, which is a recombinant VDE bracketed by 6 proximal and 4 distal amino acids [13], by monitoring the rate of VDE production under conditions indicated. The excised VDE was observed within 30 min of dialysis against the refolding buffer, but did not appear at all by dilution of the denaturing agent (data not shown). The excision occurred when the concentration of guanidine-HCl was reduced less than 0.8 M by dialysis, and the reaction completed within 2 h. XC-VDE was not excised when 8 M urea was used as an unfolding agent (data not shown). The unfolding step with 6 M guanidine-HCl at pH 8.0 required reducing agents such as 10 mM DTT for the following successful refolding. The effect of pH on the splicing reaction was examined with Britton-Robinson buffers at pH ranging from 4 to 10 at room temperature (Fig. 1A). The reaction proceeded most efficiently

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*Abbreviations:* DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; PAGE, polyacrylamide gel electrophoresis; VDE, *VMA1*-derived endonuclease; Vma1p, the 70 kDa catalytic subunit of vacuolar $H^+$-ATPase; Vma1 protozyme, the 120 kDa *VMA1* gene translation product


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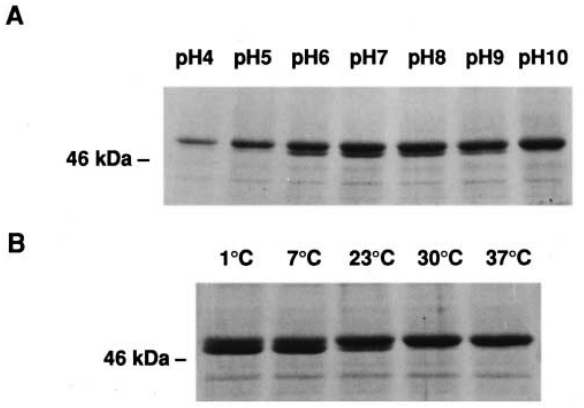


Fig. 1. The effect of pH and temperature on in vitro peptide cleavage of XC-VDE. A: The insoluble pellet of *E. coli* cell lysate which expressed XC-VDE was solubilized with ~50 vol. of an unfolding buffer of the indicated pH conditions (6 M guanidine-HCl, 12.5 mM Britton-Robinson buffer of the indicated pH, 1 mM EDTA and 10 mM DTT), and after 1 h incubation at room temperature, the suspension was centrifuged and the supernatant was dialyzed against 100 vol. of a refolding buffer with the same pH conditions (25 mM Britton-Robinson buffer of the indicated pH, 1 mM EDTA) for 2 h at room temperature. B: The unfolding buffer contained 6 M guanidine-HCl, 50 mM sodium-phosphate buffer, 1 mM EDTA and 10 mM DTT, pH 7.0. After 1 h incubation at room temperature, the supernatant was dialyzed against 50 mM sodium phosphate, 1 mM EDTA and 10 mM DTT, pH 7.0 buffer which had been equilibrated at the indicated temperature.

at pH around 7.0. Interestingly, amounts of VDE formed under these conditions decreased as the temperature of the refolding step increased (Fig. 1B), suggesting that the refolding of XC-VDE competent for protein splicing depends on temperature.

### 3.2. *Effect of protease inhibitors upon protein splicing*

The protein splicing reaction initiates the first cleavage of the peptide bond between $Gly^{283}$ and $Cys^{284}$ in the Vma1 protozyme, followed by formation of the branched intermediate [9]. Here we attempted to characterize an intrinsic endopeptidase activity of XC-VDE. Proteases are classified into four classes: serine-, cysteine-, aspartic-type proteases and metalloproteases [14]. According to the simple protocol for characterizing the catalytic type of proteases by use of the specific inhibitors (3,4-DCI, E64, pepstatin and 1,10-phenanthroline) [14], we tested whether the excision reaction was classified into one of the four catalytic types. After incubation at 1 h in the unfolding buffer, each protease inhibitor was added to the buffer solution at an excess molar ratio to the XC-VDE, then dialyzed against the refolding buffer containing the same inhibitor. We found that the inhibitors had no effect on the peptide cleavage of XC-VDE (data not shown). This suggests that the peptide cleavage process of protein splicing occurs in a manner different from known peptidase reactions.

### 3.3. *Evidence for intramolecular splicing*

To test whether protein splicing is an intramolecular or intermolecular event, two kinds of precursors, which were different in the length of external regions, were prepared for the use as substrates. One precursor VMA1N-VDE-CGER is a VDE derivative combined with the entire 30 kDa N-terminal region of Vma1p and with 4 amino acids at the C-terminus (Fig. 2A). The other precursor MIIYVG-VDE-VMA1C is a VDE combined with 6 amino acids at the N-terminus and with the entire 40 kDa C-terminal region of Vma1p (Fig. 2A). The purified precursors in the unfolding buffer were mixed, and the mixture was placed in a dialysis tube and subjected to the refolding cycles (Fig. 2B). If protein splicing occurs between the two different molecules, the 70 kDa chimeric ligation product would be produced by swapping the external regions. However, even after the renature steps repeated (Fig. 2B, lanes 5–8), no detectable amount of the 70 kDa spliced product was observed as judged by Western blotting analysis using monoclonal antibodies against the N-terminal Vma1 protein region (R70) and C-terminal Vma1 protein region (5M39) as in Fig. 2C, lane 8. This result suggests that the protein splicing is likely to occur in an intramolecular reaction of the single precursor molecule under our experimental conditions.

## 4. Discussion

Protein splicing is an autocatalytic process, which does not require any extrinsic protease to assist the excision-coupled ligation reaction, because the purified precursors undergo protein splicing in vitro [13,15]. In the present study, we investigated this unique chemical reaction using an in vitro system.

We assessed whether the peptide cleavage process of protein splicing is similar to those of known proteases, such as cysteine proteases. Cysteine proteases are a class of enzymes requiring the thiol group of a cysteine residue for their catalytic activity. Their activity depends on a catalytic dyad of cysteine and histidine [16]. In the papain family, highly conserved Asn is essential for catalysis, forming the Cys–His–Asn catalytic triad. The residues Cys, His, and Asn are also important for protein splicing: mutations of $Cys^{284}$ at the N-terminal splice site, $His^{736}$, $Asn^{737}$ and $Cys^{738}$ at the C-terminal splice site result in defects of protein splicing [5–10]. The $His^{362}$ residue apart from the N-terminal splice site is also crucial for protein splicing [12]. Therefore we first expected that protein splicing might utilize similar mechanism to cysteine proteases, thus might be inhibited by cysteine protease inhibitors. However, E64, an inhibitor of many cysteine proteases did not inhibit the cleavage reaction of XC-VDE. Furthermore, cleavage was not prevented by typical inhibitors of the other three types of proteases: serine-, aspartic- and metalloproteases (3,4-DCI, pepstatin and 1,10-phenanthroline, respectively). Although there remains a possibility that the inhibitor may not enter the active site efficiently to act, the cleavage reaction mechanism of XC-VDE is likely to be different from the known protease reactions.

According to the model of Xu and Perler [10], protein splicing proceeds through a branched intermediate, which is formed by nucleophilic attack of the thiol or hydroxyl group of the side chain at the C-terminal splice junction onto the ester or thioester linkage which had been formed at the N-terminal splice junction. Although all the current models postulate an single-molecular splicing reaction, an intermolecular head-to-tail interaction mechanism is also possible, being consistent with the previous experimental data. Intermolecular splicing could take place if the C-terminal Cys residue attacks onto the N-terminal thioester bond of the 'another' molecule. In order to justify the intramolecular reaction model, two distinct precursors were mixed and allowed to splice in vitro.

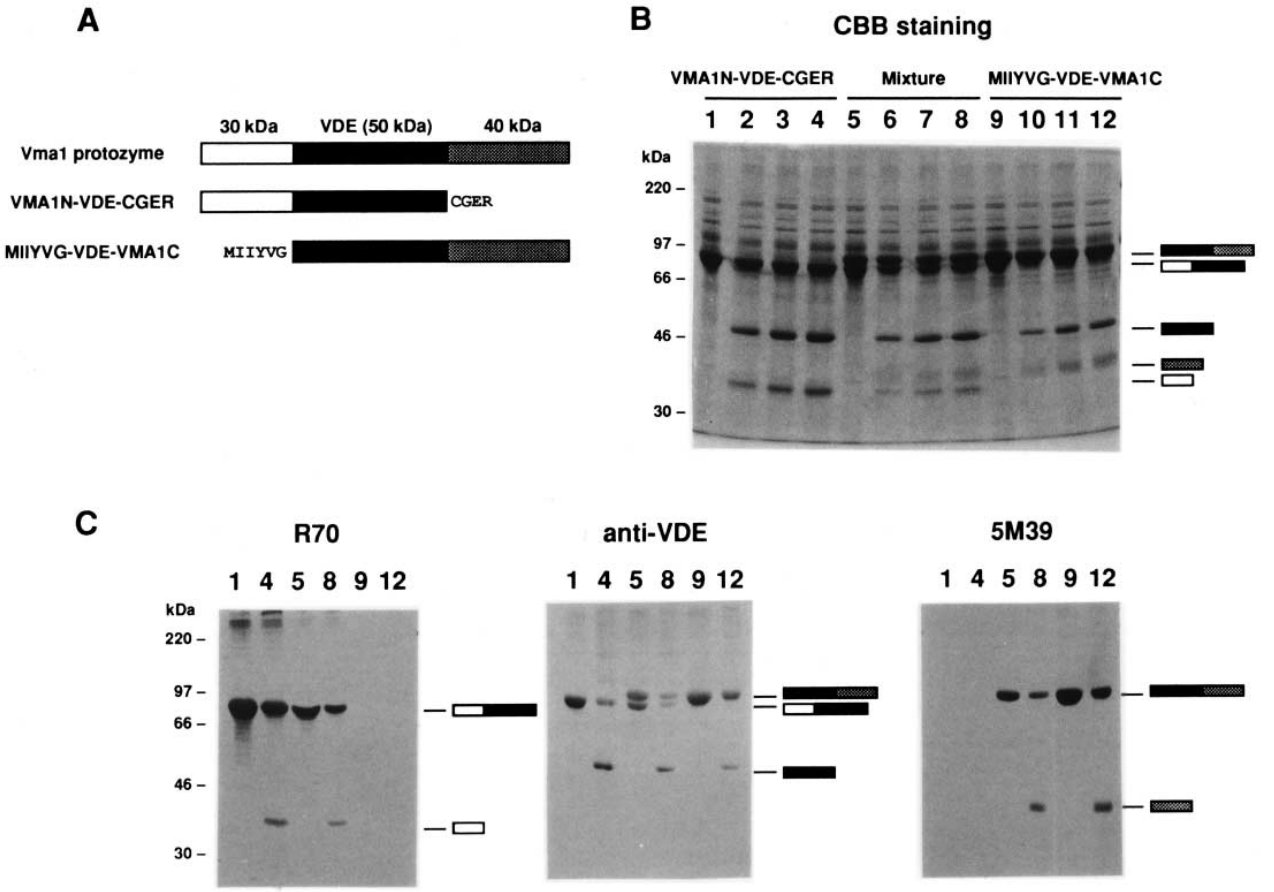


Fig. 2. Protein splicing of mixed two precursors. A: The two kinds of spliced polypeptides, VMA1N-VDE-CGER and MIIYVG-VDE-VMA1C, were used in this experiment. B: Unfolded polypeptides were partially purified by Sephacryl S-300 superfine gel filtration column chlomatography (lanes 1 and 9, respectively; protein concentration was 1 mg/ml), and dialyzed against the folding buffer at 4°C. Cycles of denature–renature were repeated to accumulate the splicing products (lanes 1–4: VMA1N-VDE-CGER, lanes 9–12: MIIYVG-VDE-VMA1C) To detect the intermolecular splicing product, the same volumes of the two precursors (1 mg/ml each) were mixed and subjected to refolding cycles (lanes 5–8). C: The 70 kDa spliced product (VMA1N-VMA1C), which was predicted to be produced by intermolecular protein splicing of the two different precursors, was not detected (lane 8).

No detectable amount of the intermolecular splicing product was formed when analyzed by SDS-PAGE and Western blotting, supporting that the splicing reaction is in fact carried out within one precursor molecule.

We described here unique biochemical features of the catalytic mechanism of protein splicing. The results of the present study may lead to a better understanding of the chemical basis of protein splicing, though the three-dimensional structure of the splicing precursor would be required for a whole insight of the structure-function relationship of this compelling chemical reaction.

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