The open reading frame III product of cauliflower mosaic virus forms a tetramer through a N-terminal coiled-coil

(Received for publication, July 1, 1998, and in revised form, August 20, 1998)

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The open reading frame III product of cauliflower mosaic virus is a protein of 15 kDa (p15) that is essential for the virus life cycle. It was shown that the 34 N-terminal amino acids are sufficient to support protein-protein interaction with the full-length p15 in the yeast two-hybrid system. A corresponding peptide was synthesized and a recombinant p15 was expressed in Escherichia coli and purified. Circular dichroism spectroscopy showed that the peptide and the full-length protein can assume an α-helical conformation. Analytical centrifugation allowed to determine that p15 assembles as a rod-shaped tetramer. Oxidative cross-linking of N-terminal cysteines of the peptide generated specific covalent oligomers, indicating that the N terminus of p15 is a coiled-coil that assembles as a parallel tetramer. Mutation of Lys22 into Asp destabilized the tetramer and put forward the presence of a salt bridge between Lys22 and Asp24 in a model building of the stalk. These results suggest a model in which the stalk segment of p15 is located at its N terminus, followed by a hinge that provides the space for presenting the C terminus for interactions with nucleic acids and/or proteins.

Cauliflower mosaic virus (CaMV) is the type member of the caulimovirus group, a family of plant pararetroviruses (for review, see Ref. 1). The virion is made of 420 subunits of the RNA genome. The Open Reading Frame III Product of Cauliflower Mosaic Virus contains a C-terminal proline-rich nonspecific DNA-binding domain that is conserved in the caulimovirus group and similar to the C terminus of the histone-like proteins (4). It was suggested that this domain could be involved in compaction of the viral genomic DNA (4). p15 is essential for the virus life cycle (5–7), although its exact function remains unknown.

The analysis of the amino acid sequence of the N terminus of p15 predicted a coiled-coil structure. A coiled-coil is a protein structure that allows the oligomerization of a protein via association of two or more α-helices. Coiled-coils are found in a large class of fibrous proteins like keratin, myosin, and fibrinogen, and more recently, they have been recognized as a dimerization element of transcription factors (8). Sequences that are capable of forming coiled-coils are characterized by a heptad repeat of seven residues denoted “a” to “g” (9–11) in which the “a” and “d” positions are occupied predominantly by hydrophobic amino acids, whereas polar residues are found elsewhere. A hydrophobic interface is formed between the helices and represents the major driving force for the stabilization of the oligomer (12). Positions “e” and “g” are frequently charged residues which might contribute to stability, the specificity of helix association, and their relative order of oligomerization (13–17). There can be two, three, four, or five helices in the bundle, in parallel or in opposite orientation (for review, see Ref. 8).

We have characterized the coiled-coil domain of p15, located at its N terminus, and have shown that the protein assembles into a parallel tetramer through this domain to adopt a rodlike structure.

EXPERIMENTAL PROCEDURES

Plasmids—The CaMV ORF III sequence and its derivatives were amplified by PCR from the Strasbourg strain cloned in pBR322 (pCa37; Ref. 18). The following sense oligonucleotides, 5′-AAACCCGGGGAT-TCACCTAGTCTTTATCAACATCAGATCCAAAG3′ (11711), 5′-AAAGCCGGGAAATCCACCAGGTTTTGCAAAATCGATT3′ (11715), 5′-AAAGATCTTAC- CAGCTTAAAGCTTTAGGGTTTC-3′ (11753), 5′-AAAGATCTTATT-TGTTCTATTGTTCTTTAGG3′ (11756), 5′-AAAGATCTGTCAT- CCTAAAAATGTACCTGCCATCTCCGAGAC-3′ (11710) were used for the amplification. The combinations of oligonucleotides for the PCR reaction were: 11711 and 11710 for the full-length p15, 11711 and 11715 for p(33–129). All PCR reactions were performed as follows: 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min for 30 cycles. An EcoRI and an NcoI site at the N terminus and a BglII site at the C terminus were generated by PCR for cloning in pGAD424, pGBT9 (CLONTECH), and pET-3D (Novagen). The entire sequence of each construct was verified by DNA sequencing.

The clone pA34–107 was generated by a BamHI digest of p15wt cloned into pGAD424 that allowed the deletion of an p15 internal BamHI fragment. The linearized plasmid was ligated and used in the two-hybrid assay. The fusion of the N terminus of p15 was then generated without adding or changing any amino acids of the protein. The construct p1–34 was generated by elution of the EcoRI-BamHI fragment of p15wt in pGAD424 and cloning in the pGAD424 vector linearized with the same enzymes. This cloning generated a fusion of 5 amino acids at the C terminus of (MNRRY) of the first 34 amino acids of ORF II of CaMV that are not present in the wt sequence. The PCR mutagenesis was done according to Vogel and Das (19) with primers 11710 and 11711. The PCR products were cloned in pGAD424 as described previously.
The RTBV genome served as template for the PCR amplification of the ORF II. PCR was performed using the forward oligonucleotide 5'-ACGTAAATGCCCATATGAGCCCGTA TATCAAATCATTTCAAG-3' and the reverse oligonucleotide 5'-GGATATTTTCTTTTAATTCC-3'. The PCR fragment was digested with NdeI and BamHI and cloned into the procaroytic expression vector pET-3A (Novagen). The resulting plasmid allowed the expression of the complete ORF II product.

Purification of the CaMV p15—The bacterial pellet from 3 liters of culture was resuspended in 50 ml of TP buffer (20 mM Tris, pH 7.0, 20 mM NaCl, 1 mM EDTA, 1 mM EGTA, 5% glycerol, and mixture of inhibitors of proteases (Boehringer Mannheim)), and the cell suspension was lysed by sonication or with a French press. The lysate was heated at 65 °C for 15 min and centrifuged at 30,000 × g for 60 min at 4 °C. The supernatant was loaded on a CM-Sepharose column that was previously pre-equilibrated in TP buffer. p15 was eluted with TP buffer + 100 mM sodium chloride. The eluted protein was diluted 10-fold with 0.5× TP buffer and passed through the column once more. The eluted protein was then diluted 5-fold in TP buffer, pH 8.8, and loaded on a DEAE-Sepharose column. The protein was eluted with TP buffer, pH 8.8, plus 250 mM NaCl and concentrated with Centriprep P50 (Amicon). This procedure yielded around 10 mg of pure protein/liter of culture medium.

Peptides—The peptide sequences were as follows: pep(wt) is GSCECKQNLQIKVEKSVLSDQKSMADIAKIE, pep(K->N) is GSCECKQNLQIKVEKSVLSDQKSMNADIAKIE, and pep(K->D) is GSCECKQNLQIKVEKSVLSDQKSDADIAKIE. The peptides were synthesized, freeze-dried, dissolved in water to a concentration of 1 mg/ml, and stored as stock solution at –80 °C.

SDS-PAGE and Electroblotting—The proteins were mixed with 1/3 of the final volume of loading buffer containing 5% SDS, 30% glycerol, and 0.01% bromphenol blue. The SDS-PAGE was performed as described elsewhere (20).

Analytical Ultracentrifugation—Analytical ultracentrifugation was performed according to Ref. 21. The recombiant protein was analyzed at 20 °C in 12.5 mM Tris, pH 8.3, and 90 mM glycine. The protein concentration was adjusted to 0.2 mg/ml, loaded on a DEAE-Sepharose column. The protein was eluted with TP buffer, pH 8.8, plus 250 mM NaCl and concentrated with Centriprep P50 (Amicon). This procedure yielded around 10 mg of pure protein/liter of culture medium.

RESULTS

Mapping the Interaction of CaMV p15 with Itself in the Yeast Two-hybrid System—The yeast two-hybrid system (28) was used to study the interaction of p15 with itself. The ORF III sequence of CaMV was used in-frame with the C terminus of the GAL4 activation domain. B, PCR mutants of p15 that do not interact with wt p15. The mutations in pm1, pm2, pm3, and pm5 are indicated. All the mutants have a stop codon introduced interrupting the open reading frame at amino acid 28 or before. The shaded box in pm3 represent amino acids that are not found in the native sequence of p15, resulting from a frameshift at amino acid 18. Results of the β-galactosidase filter assay are given in the last column. (+) indicates the appearance of blue color and denote a positive protein-protein interaction. (−) indicates the absence of color and interaction between the two proteins. C, amino acid sequence of p15. The hydrophobic residues found in position a and d of the amphiphatic α-helix are in bold. The C-terminal DNA binding domain of p15 is shown in italics (4).
The amino acids that are not native of the p15 are in italics. The hydrophobic residues that are found in position a or d of the α-helix are in bold. The Lys at position 22 is labeled and is involved in a salt bridge with amino acid Arg5. B, reduced pep(wt) was incubated for 1, 10, and 60 min at 4 °C at a protein concentration of 0.5 mg/ml with 10 mM oxidized glutathione and 1 mM reduced glutathione. Samples were then alkylated, analyzed by SDS-PAGE, and stained with Coomassie Blue. Positions of the monomeric (1), dimeric (2), trimeric (3), and tetrameric (4) cross-linked products are marked to the left and the position of molecular mass marker proteins on the right. The C lane was not incubated with oxidized glutathione prior to alklylation and denaturation with the SDS-PAGE loading dye. C, chemical cross-linking of pep(wt). Cross-linking was performed with 5 and 10 mM sulfo-GMBS for 2 h at 4 °C according to the manufacturer’s protocol (Pierce). As a control, peptide cross-linked through oxidation of the 2 N-terminal cysteines for 10 min was done as described in B.

Random mutagenesis was performed on the p15 gene using a modified PCR reaction with Taq polymerase. The fidelity of DNA synthesis of Taq polymerase can be altered by modification of the buffer composition. The presence of metal ions as well as altered nucleotide ratios have been shown to decrease the fidelity of DNA synthesis (19, 29). Following PCR mutagenesis, a bank of mutated DNA fragments encoding the p15 gene was cloned into the pGAD vector and cotransformed in yeast with the wt p15 sequence in pGBT III. Five colonies that did not show β-galactosidase activity were selected. Their plasmids were isolated and sequenced. One of the five colonies was an empty pGBT vector. The other four plasmids revealed mutations, a frameshift ending at a new stop codon (pM3) (Fig. 1A). These mutants could probably not interact with p15 because they are too short. This result reinforces the importance of the N terminus of p15 in the protein-protein interaction. Since only mutations found close to the N terminus of p15 could influence the interaction, it is tempting to suggest that only this region is involved in the oligomerization of the protein.

**Determination of the Oligomeric Structure of p15**—When expressed in *Escherichia coli* BL21(DE3) cells, p15 represented more than 20% of the total protein. The purified protein was analyzed by analytical ultracentrifugation to determine the quaternary structure. The calculation yielded a molecular mass of 57 kDa in 12.5 mM Tris pH 8.3 and 90 mM glycine. The sedimentation coefficient of p15 was of 2.8 s$^\text{20,w}$. The combined analytical ultracentrifugation data show that the protein is a tetramer and has a rodlike shape.

The p15 N-terminal Peptide Forms an α-Helical Coiled-coil—The analysis of the amino acid sequence of the N terminus of p15 revealed a heptad repeat that is characteristic of a coiled-coil (Fig. 1C). To test the ability of this region to form a coiled-coil structure, a peptide corresponding to residues 3–32 of p15 with a molecular mass of 4149 Da was synthesized. The presumptive coiled-coil sequence was preceded by the sequence GSCCKGQ and called pep(wt) (Fig. 2A). The two cysteines were used to allow disulfide cross-linking of associated peptides. The position of the cysteines at the N terminus of pep(wt) is important to assess the orientation of the α-helices to each other in the coiled-coil. Based on the results obtained in the two-hybrid system, it was concluded that the N-terminal 34 amino acids of p15 are involved in the oligomerization of the protein. Furthermore, the analytical centrifugation showed us that p15 is a tetramer.

To investigate the formation of an oligomeric structure, pep(wt) was reduced at 37 °C, slowly cooled to 4 °C and oxidized with glutathione for different times. After alkylation, the cross-linked peptide complexes were separated by electrophoresis on non-reducing SDS-polyacrylamide gels. One minute of oxidation was sufficient to cross-link the peptide into a parallel tetramer, which is the major cross-linked form observed on the gel (Fig. 2B). Oxidation for longer periods gave similar results (Fig. 2B). We also observed the formation of a tetramer as the highest multimer when pep(wt) was chemically cross-link with sulfo-GMBS (Fig. 2C). The mass of the tetramer is overestimated on the SDS gel when compared with the protein markers. This could be due to the dense packing of the coiled-coil making it resistant to complete denaturation by SDS, thus causing retardation of the protein in the gel because less SDS is bound to it.

To assess the formation of a coiled-coil, the secondary structure of the peptide was examined by CD spectroscopy. The spectrum, recorded at 20 °C in 200 mM Tris, pH 7.5, 200 mM NaCl, and 1 mM EDTA at a concentration of 0.5 mg/ml, displayed minima around 208 and 222 nm, characteristic for an

![Fig. 2](image_url) **Disulfide cross-linking of pep(wt).** A, amino acid sequence of pep(wt). The amino acids that are not native of the p15 are in italics. The hydrophobic residues that are found in position a or d of the α-helix are in bold. B, reduced pep(wt) was incubated for 1, 10, and 60 min at 4 °C at a protein concentration of 0.5 mg/ml with 10 mM oxidized glutathione and 1 mM reduced glutathione. Samples were then alkylated, analyzed by SDS-PAGE, and stained with Coomassie Blue. Positions of the monomeric (1), dimeric (2), trimeric (3), and tetrameric (4) cross-linked products are marked to the left and the position of molecular mass marker proteins on the right. The C lane was not incubated with oxidized glutathione prior to alklylation and denaturation with the SDS-PAGE loading dye. C, chemical cross-linking of pep(wt). Cross-linking was performed with 5 and 10 mM sulfo-GMBS for 2 h at 4 °C according to the manufacturer’s protocol (Pierce). As a control, peptide cross-linked through oxidation of the 2 N-terminal cysteines for 10 min was done as described in B.

![Fig. 3](image_url) **Circular dichroism spectra of purified p15 and pep(wt).** A, purified pep(wt) was dissolved in 10 mM Tris, pH 7, and 50 mM NaCl. B, the reduced pep(wt) was dissolved in 200 mM Tris, pH 7.5, 200 mM NaCl, and 1 mM EDTA. All spectra were recorded at 20 °C.
a-helical structure (Fig. 3A).

The purified tetramer of p15 was also analyzed by CD spectroscopy. The protein showed a typical a-helical spectrum (Fig. 3B) with two minima, at 208 and 222 nm. The calculation predicts an a-helix content of 65%, 27% random coil, and 8% &-turn.

Because of the discrepancy in apparent molecular weight on the gel, we verified the oligomerization state by mass spectrometry. The cross-linked pep(wt) was applied to a reverse phase high performance liquid chromatography interfaced with an electrospray ionization mass spectrometer. The spectrum of peak A (Fig. 4A) showed a mass of 16,585 Da corresponding to the tetramer form with 8 oxidized Cys. Peak B, with a mass of 4759 Da, corresponded to the monomer, taking into account the two attached glutathione on the Cys, and peak C with a mass of 8293 Da corresponds to the dimer with 4 oxidized cysteines.

**Fig. 4.** A, mass spectra of pep(wt). Reverse phase chromatography and mass spectra of the cross-linked pep(wt). The various cross-linked forms of pep(wt) were separated on FPLC by reverse phase chromatography. The mass spectra of peak A shows a mass of 16,585 Da corresponding to the tetramer, peak B a mass of 4759 Da corresponding to the monomer, and peak C a mass of 8293 Da corresponding to the dimer. B, schematic "helical wheel" representation of the tetrameric a-helical coiled-coil of p15 as seen from the N terminus. The upper part shows the hydrophobic interactions within the tetramer. The dashed line in the bottom part shows the interchain ionic interaction between Lys and Asp found in the "e" and "g" positions, respectively. The solid line shows a putative stabilizing interchain hydrophobic interaction. Dashed arrows also represent the e-g interaction.
The trimer form was previously found as the least abundant form of oligomers on the SDS-polyacrylamide gel (Fig. 4A). The trimer was not detected with the liquid chromatography-electrospray ionization mass spectrometer.

A helical wheel representation of the coiled-coil of p15 (Fig. 4B) highlights the importance of the hydrophobic residues in the formation of the stalk and the contribution of the electrostatic interaction between Lys22 and Asp24 in position “e” and “g,” respectively, for the stability of the tetrameric coiled-coil. The hydrophobic residues Leu29 and Leu31 could also contribute to the stability of the stalk.

**Gel Filtration of Pep(wt)—**To discard the possibility of an artifact caused by the oxidative cross-linking of pep(wt), the homogeneity of the oligomer of the reduced pep(wt) was verified in native condition by gel filtration on a Sephadex G-50 medium (Pharmacia). Two globular protein markers were chymotrypsinogen A (25 kDa) and cytochrome C (12.4 kDa), as shown on the right side of the gel. Fractions 8, 9, and 10 contain the reduced pep(wt). Fraction 9 was oxidized 10 min to cross-link, denatured with SDS loading dye, and loaded on the gel as described before. Lane 9C represents the oxidized cross-linked form of pep(wt) as shown in Fig. 3. The gel was stained with Coomassie Blue.

**Fig. 5. Gel filtration of pep(wt).** Separation of the reduced pep(wt) on Sephadex G-50 (Pharmacia). SDS-gel electrophoresis was performed with fractions 6–18. The two globular protein markers were chymotrypsinogen A (25 kDa) and cytochrome C (12.4 kDa), as shown on the right side of the gel. Fractions 8, 9, and 10 contain the reduced pep(wt). Fraction 9 was oxidized 10 min to cross-link, denatured with SDS loading dye, and loaded on the gel as described before. Lane 9C represents the oxidized cross-linked form of pep(wt) as shown in Fig. 3. The gel was stained with Coomassie Blue.

**DISCUSSION**

The formation of α-helical coiled-coil structures is a common mechanism of protein subunit assembly. Well-characterized examples are myosin, intermediate filaments, laminin, and transcription factors (30–32). The ORF III product of CaMV (p15) contains a heptad repeat at its N terminus, typical of a coiled-coil domain. Using the yeast two-hybrid system, we showed that the first 33 amino acids of p15 are sufficient to allow oligomerization of the full-length p15. Oxidative and chemical cross-linking of the stalk peptides (pep(wt)), comprising amino acids 3–32 of p15, could form a parallel tetramer as shown by SDS-PAGE and mass spectroscopy. Finally, the CD spectrum of pep(wt) confirmed the α-helical structure of the tetramer. The homogeneity of the oligomer of the reduced pep(wt) was verified by gel filtration, suggesting that the peptide is found uniquely as a tetramer in solution.

p15 is a thermostable protein as evidenced by the purification protocol (see “Experimental Procedures”). Electrostatic interactions have been shown to improve thermal stability in coiled-coils (17). The mutation K22N decreased thermal stability. Furthermore, mutation K22D introduced a negative charge that destabilized the tetramer and favored the formation of a dimer. In a tetrameric and pentameric coiled-coil, positions “a” and “d” are substantially buried in the stalk and are close to each other; residues at the “e” and “g” positions are partially buried; residues “b,” “c,” and “f” remain completely exposed (27). Only dimers were observed when a negative charge was introduced at position e22 probably because the tetramer stalk cannot accommodate two negative charges in position e22 and g24.

Sequence alignment of related proteins found in other caulimoviruses suggests also the presence of N-terminal coiled-coils. Thus, oligomerization of this class of proteins would seem to be important for its function (Fig. 7A). This is supported by the observation that deletion of 20 amino acids in the coiled-coil of CaMV p15 leads to a non-infectious virus (33). As shown in studies of GCN4 leucine zipper mutants, the “a” and “d” positions exhibit preferences for leucine and isoleucine, respectively, in a tetrameric coiled-coil (12). The coiled-coil domain of p15 shows no strong bias for leucine at the “a” position, while the “d” position, as in the case of the GCN4-tetramer, is occupied by apolar Cγ-branched residues (mostly Ile). This provides additional support for the idea that the isoleucine at “d” position can favor coiled-coil tetramerization. To visualize the spatial arrangement of the ORF III product tetramer, a model of the three-dimensional structure was developed (Fig. 8A). The data and the modeling suggest that Asp24 can interact simultaneously with both Lys22 and Lys26. The modeling also suggests that the p15 tetramer has two interhelical salt bridges between charged side chains at the “g” and “b” positions (Asp17-Lys19, Asp24-Lys26) (Fig. 8A). This type of ion pair was also found in the crystal structure of the four-stranded coiled-coil of GCN4 (12) and can provide an additional interaction favoring the tetramerization of p15. A hydrophobic interaction be...
## Badnaviruses

### Alignment of coiled-coils from different plant pararetroviruses.

**A** alignment of the ORF III N-terminal domain of different caulimoviruses. GCN4 mut sequence corresponds to the yeast GCN4 leucine zipper mutant that forms a tetramer. **B** alignment of the putative coiled-coils of the ORF II of the known plant badnaviruses. The residues found in position “a” and “d” that are part of the core of the tetramer coiled-coil are shown in black cartridges and bold. The residues in italics outside the black cartridge are charged residues that could be involved in an interchain ionic interaction. The hydrophobic residues outside the black cartridge with a black point below the letter could be involved in stabilizing a hydrophobic bond.

Badnaviruses have a genomic organization related to the one of caulimoviruses. ORF II of badnaviruses has been suggested to be the homologue of the ORF III of caulimoviruses based on sequence homology and function of their C-terminal nucleic acid binding domain (7). We could also find a putative coiled-coil domain in the ORF II of the badnavirus RTBV in the middle of the protein (Fig. 7B). The purification and chemical cross-linking of the ORF II product showed that also this protein oligomerizes as a tetramer (data not shown). It is tempting to extrapolate this feature to the other badnavirus (Fig. 7B). The putative coiled-coil of CoYMV shows some residues of the same charge in positions b\(^{57}\) and e\(^{58}\), and b\(^{64}\) and e\(^{65}\), respectively. This arrangement would destabilize the tetrameric coiled-coil, however, the virus might have compensated this deficiency by an increase in length of the coiled coil structure. We can also find putative salt bridges between the couples e\(^{56}\), g\(^{59}\) and e\(^{60}\), g\(^{63}\) in the sugarcane bacilliform virus, b\(^{57}\), e\(^{58}\), c\(^{59}\) in cacao swollen shoot virus, e\(^{60}\), e\(^{62}\) in the RTBV and a\(^{67}\), g\(^{69}\) in CoYMV coiled-coil. Potential hydrophobic stabilizing interactions are found between positions e\(^{66}\) and g\(^{69}\) in the CoYMV, and between positions e\(^{67}\) and g\(^{69}\) in the sugarcane bacilliform virus coiled-coils.

All the coiled-coils presented in Fig. 7 (A and B), with the exception of RTBV, which has the smallest coiled-coil, are interrupted once with a Gln or Thr in position “a” or “d” in the stalk of the multimer. This seems to be a common feature of other coiled-coils, and the nature of this phenomenon is not understood. The assembly domain of cartilage oligomeric matrix protein forms a homo-pentamer coiled-coil with a conserved Gln in the interior “d” position. The substitution of this residue for Leu improved the thermal stability of the coiled-coil and did not lead to any difference in the assembly of the multimer (34). It is possible that nature favors the presence of a Gln or Thr in this position to allow protein turnover in the cell (34). The tetramer structure has a continuous central channel with a radius that varies from 1.0 to 1.3 Å. The radius of the pore excludes a 1.4-Å radius of a water molecule probe or an anion, for example chloride, which was found in a pentameric coiled-coil structure (35). However, the oxygen atoms of Gln\(^{18}\), in the CaMV p15 coiled-coil, could form hydrogen bonds in the stalk and possibly provide a higher specificity for a parallel coiled-coil structure (Fig. 8B) (34).

The coefficient of sedimentation and the evidences brought
by the oxidative and chemical cross-linking as well as the gel filtration of pep(wt) suggest that the protein adopts a rodlike structure presenting the C-terminal functional domain. The C terminus is likely to be important for the function since a deletion of the last 5 amino acids of the protein completely abolishes the viability of the virus (33). The rod-shaped structure of the coiled-coil probably extends to the two central Cys residues (Cys64 and Cys66) because of an extension of the amphipathic α-helix (Fig. 1C). The amino acid sequence after those two Cys is rich in the α-helix breakers Gly and Pro. This region of the protein probably serves as a hinge, providing the space necessary for the C-terminal domain to bind nucleic acids (4). The tetramerization of p15 through a coiled-coil domain can modify the structure of the viral genomic DNA prior to assembly (4). It is not excluded that the tetramerization of p15 could also play a role in the specificity of recognition of nucleic acids.

Acknowledgments—We especially thank Dr. J. Hofsteenge and H. Rothnie for helpful discussions and editorial assistance. We are grateful to F. Fischer for peptide synthesis and K. Kirstner for the supply of the CD spectra spectrophotometer and to Renate Mattis for technical assistance.

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