cavity (Fig. 3) shows that the density does not entirely fill the cavity as defined by the x-ray model. Although only a monomer is shown in Fig. 3, the area of the cavity region close to the dimer interface has very little density associated with it. This is the part of the cavity that is formed mainly by two opposing α helices from different subunits. It is marked in the x-ray model of Fig. 3 by the single α helix forming a backdrop to the cavity region. The neutron maps at 10% D_2O and 93% D_2O (not shown) also indicate a similar lack of density, which suggests that this region is either full of solvent or more probably that it is the location of highly hydrated lipid headgroups, which have very little contrast.

The two phospholipid molecules placed in the negative density in Fig. 3 show a hypothetical packing arrangement within the lipoprotein complex. In the scattering length density of the 93% D_2O map, where the highest contrast arises from close-packed acyl chains, the density in the cavity merges smoothly into the β sheet of the surrounding protein. This is strong evidence for the major interaction between lipid and protein to be through hydrophobic contacts. If the interaction were through hydrated phospholipid headgroups, one would expect to see a very low density region in contact with the protein. Two molecules of phospholipid are shown to indicate that the size of the neutron cavity is sufficient to accommodate a microdomain of phospholipid bilayer. Based on ^{31}P nuclear magnetic resonance studies, the possibility that the phospholipid in lipovitellin is in such an anisotropic environment has been suggested (6).

It was hoped that the neutron diffraction studies would also indicate the location of the phosphorylated segment of phosvitin. As noted above, the x-ray model is not complete because no electron density was found to suggest the position of phosphorylated side chains. The prediction is that a component containing mainly phosphoserine would appear as positive contrast in a 40% D_2O map as can be seen in Fig. 1.

The regions of positive contrast in the 40% D_2O map are shown in Fig. 4, again along with the x-ray model. There are only three larger regions of density that have a higher contrast than normal protein. One of these regions appears to be an extension of the α helix that forms the dimer interface and may therefore mediate closer contacts between the lipovitellin monomers. There is little to be said about the other two positive regions except that they are located in solvent channels within the crystal lattice. They occupy volumes outside of the existing x-ray model. In addition to these three larger positive peaks, numerous small positive peaks, again all outside of the x-ray model, are also visible in Fig. 4. Without the indication of a single aggregate site in this positive density, speculation about the location of the missing phosphoprotein is difficult. The simplest explanation in agreement with the appearance of the positive neutron scattering peaks at 40% D_2O suggests either that it is located in one of the three larger positive peaks or that the missing phosphoprotein is probably highly disordered and hydrated.

The contrast variation technique, when used in conjunction with single-crystal diffraction, predicts the location of the phospholipid within the molecular model of lipovitellin derived from x-ray studies. Although the resolution is only at 12 Å, the neutron-defined lipid cavity is located in a region that was unoccupied in the x-ray model. We have therefore demonstrated that in lipovitellin the stored phospholipid is located in a cavity in the protein formed primarily by β-sheet structure. We suggest that the lipid is stabilized through hydrophobic interaction with the presumed apolar face of the juxtaposed β sheets.

REFERENCES AND NOTES

1. L. Banaszak, W. Sharrock, P. A. Timmins, *Annu. Rev. Biophys. Biophys. Chem.* **20**, 221 (1991).
2. T. Meininger, R. Raag, S. Roderick, L. J. Banaszak, *J. Mol. Biol.* **179**, 759 (1984).
3. R. Raag, K. Appelt, N.-H. Xuong, L. J. Banaszak, *ibid.* **200**, 553 (1988).
4. B. Poliks and L. Banaszak, unpublished results.
5. P. A. Timmins and G. Zaccai, *Eur. Biophys. J.* **15**, 257 (1988).
6. L. J. Banaszak and J. Seelig, *Biochemistry* **21**, 2436 (1982).
7. C. T. Roche, M. G. Strauss, R. Brenner, *IEEE Trans. Nucl. Sci.* **NS-32**, 373 (1985).
8. M. Roth, A. Lewit-Bentley, G. A. Bentley, *J. Appl. Crystallogr.* **17**, 77 (1984).
9. M. Roth, *Acta. Crystallogr.* **A43**, 780 (1987).
10. D. L. Worcester and N. P. Franks, *J. Mol. Biol.* **100**, 359 (1976).
11. M. Roth, in *Crystallographic Computing 5*, D. M. Moras, A. D. Podjarny, J. C. Thierry, Eds. (Oxford Univ. Press, Oxford, in press).

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Vacuolar Chitinases of Tobacco: A New Class of Hydroxyproline-Containing Proteins

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The fungicidal type I chitinases contribute to the defense response of plants against pathogens. Two tobacco chitinases represent a different class of hydroxyproline-containing proteins. Hydroxyproline-rich proteins are predominantly extracellular, structural glycoproteins that lack enzymatic activity and contain many hydroxyproline residues. In contrast, type I chitinases are vacuolar enzymes. They are not glycosylated and contain a small number of hydroxyproline residues restricted to a single, short peptide sequence.

Hydroxylation of prolyl residues is an important posttranslational modification of some secreted structural proteins. In animals, the major hydroxyproline-containing protein (HCP) is collagen, in which hydroxyproline residues are essential for stabilizing the triple-helix structure of the molecule (*1*). In plants, several secreted structural glycoproteins contain hydroxyproline (*2*): the hydroxyproline-rich glycoproteins (HRGPs) or extensins, which are components of cell walls with chains of one to four arabinoses O-linked to hydroxyproline; the arabinogalactan proteins (AGPs), which are soluble proteins and have polymers of galactose and arabinose O-linked to hydroxyproline; and certain lectins of solanaceous plants, with O-glycosylated hydroxyproline in domains structurally similar to HRGPs. The 4-*trans*-hydroxyproline (4Hyp) and the less common 3-*trans*-isomer found in HCPs result from the posttranslational hydroxylation of peptidyl prolines present in repeated peptide motifs such as GPPG (*3*) in collagens (*4*), SPPPP in HRGPs, and APA in AGPs (Table 1).

Here, we show that the type I tobacco chitinases CHN-A and CHN-B are HCPs. Type I chitinases are vacuolar proteins (*5*) implicated in the defense of plants against pathogens (*6*). The mature tobacco isoenzymes consist of a conserved lectin domain linked to the catalytic domain by a short spacer rich in T, P, and G residues (*7*, *8*). The primary structures of the isoforms differ by only three conservative substitutions and a deletion of five amino acids in the spacer (Fig. 1).

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The difference in apparent relative molecular mass (M_r) between CHN-A and CHN-B, as measured by SDS–polyacrylamide gel electrophoresis, is about 1.5 kD greater than that predicted from the deduced primary structure (*8*). Because similar anomalies have been reported for HCPs, we measured the 4Hyp content of the purified tobacco enzymes (Fig. 2). 4Hyp was detected in both CHN-A (3.5 moles of 4Hyp per mole of CHN-A) and CHN-B (1.2 moles per mole). No 4Hyp (<0.1 moles per mole) was detected in a truncated chitinase (Fig. 1) that contained only the catalytic domain, which indicates that none of the 16 prolines in this domain is hydroxylated.

The exact position of the 4Hyp residues in the amino acid sequence was established by peptide analysis (Fig. 2). Amino acid analysis of all peptides identified one major, 4Hyp-containing peptide, which was isolated and sequenced from purified CHN-B (Fig. 2B). It encompassed residues 40 to 61, which contained the spacer region. Only the central Pro residues of the spacer were hydroxylated (Fig. 2C). CHN-A yielded one major (CHN-A_d) and three minor 4Hyp-containing peptides (CHN-A_a through CHN-A_c), all spanning residues 40 to 66 (Fig. 2A). In CHN-A_d, the four central Pro residues were fully hydroxylated, but a small amount of phenylthiohydantoin-4Hyp (PTH-O) was found at position 44, in addition to PTH-Pro, indicating partial hydroxylation. This was confirmed by analysis of peptide CHN-A_c, in which an excess of PTH-O was found at position 44 (Fig. 2C). Likewise, analysis of peptides CHN-A_a and CHN-A_b showed Pro^{52} to be hydroxylated. Thus, the 4Hyp content of CHN-A and CHN-B results exclusively from hydroxylation of Pro residues in the spacer region. CHN-B shows a constant pattern of hydroxylation, whereas hydroxylation of Pro^{44} and Pro^{52} of CHN-A is variable (Fig. 2C).

Table 1. Representative 4Hyp-containing amino acid sequences of plant proteins (*2, 3*).

Species	Protein	Sequence
Tobacco	CHN-A	CPGGOTOTOOTOOGGG
	CHN-B	CPGGOTOPGGG
Tomato and melon	HRGP	SOOOOK
Tomato		SOOOOVKPYHPTOVYK
		SOOOOSOK
		SOOOOTOVYK
		SOOOOVYKYK
Sugar beet	HRGP	SOOVHEYPOOTOVYK
Carrot	AGP	ADAOAOSOAOO
		DEAOAOAOSO
		GOAOAOAOAO
Lolium	AGP	AEAOAOAOAS

Plant HRGPs and AGPs are highly glycosylated, with a large fraction of their total carbohydrates present as arabinose-containing oligosaccharides attached to 4Hyp residues (*2*). There is evidence that neither 4Hyp nor other residues of CHN-A and CHN-B is glycosylated. (i) PTH-O was recovered at normal yields in sequencing reactions. (ii) Less than 0.1 mole of sugar per mole of protein was detected (*9*). (iii) Hydrolysis with trifluoromethanesulfonic acid (*10*) did not reduce the apparent M_r of the proteins. (iv) The molecular masses determined by mass spectrometry of the native proteins agreed with molecular masses deduced from cDNA clones (*11*).

Secretion as well as transport of plant proteins to the vacuole is through the Golgi compartment, which contains enzymes that catalyze O-glycosylation of 4Hyps in dicotyledonous plants (*12*). The finding that no 4Hyps are O-glycosylated in chitinase and not all 4Hyps are O-glycosylated in HRGPs (*13*) suggests that the glycosyl transferases recognize 4Hyp only in a particular structural context, that O-glycosyl groups can be selectively removed later in protein processing, or that chitinases never reach the compartment that contains the transferases.

The sequence Thr/Ser-4Hyp present in chitinases is found in all plant HCPs (Table 1). The proposal (*2*) that Pro residues of HRGPs preceded by an hydroxyl–amino acid are recognized as a substrate by the hydroxylase does not extend to chitinases. Such Thr/Ser-Pro sequences occur five times in the catalytic domain of CHN-A, which does not contain 4Hyp. Individual plant HCPs contain different 4Hyp-con-

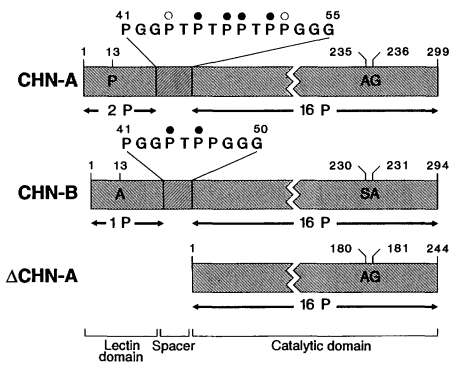
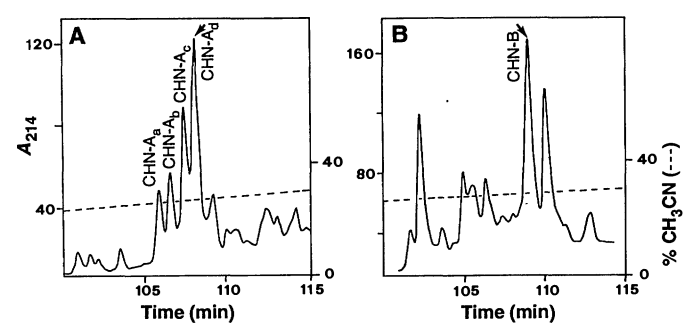


Fig. 1. Primary structures of tobacco chitinases CHN-A, CHN-B, and ΔCHN-A, which encompasses residues 56 to 299 of CHN-A (*3*). Sequences of CHN-A and CHN-B were deduced from cDNA clones (*7, 8*) and are numbered from the NH_2-terminus of the mature proteins. Amino acid substitutions and numbers of prolines are indicated within and below the bars, respectively. Non–4-hydroxylated, partially 4-hydroxylated (○), and fully 4-hydroxylated (●) Pro residues in the spacer were determined as described (Fig. 2). ΔCHN-A was extracted with 50 mM sodium acetate (pH 4) from leaves of *Nicotiana sylvestris* plants transformed with a truncated tobacco gene CHN48 (*5*). The protein was purified as described (*7*) except that elution was from CM-Trisacryl (Pharmacia) in 130 mM NaCl and 10 mM sodium phosphate (pH 7). The NH_2-terminus of the protein was verified by sequencing.



C

Peptide	41	42	43	44	45	46	47	48	49	50	51	52	53	54
	Sequence													
CHN-A$_a$	P	G	G	O	T	O	T	O	O	T	O	O	G	G
CHN-A$_b$	P	G	G	P>O	T	O	T	O	O	T	O	O	G	G
CHN-A$_c$	P	G	G	O>P	T	O	T	O	O	T	O	P	G	G
CHN-A$_d$	P	G	G	P>O	T	O	T	O	O	T	O	P	G	G
CHN-B	P	G	G	O	T	O	P	G	G					

Fig. 2. Isolation and sequencing of 4Hyp-containing peptides from CHN-A (**A**) and CHN-B (**B**) (*3*). CHN-A and CHN-B (*7*) were oxidized with performic acid and digested with endoproteinase Asp-N (Boehringer Mannheim) as described (*19*). The peptides were fractionated by high-performance liquid chromatography with a C_{18} (Vydac) column (*19*). Amino acid compositions were determined with the use of an acetonitrile gradient (0.17% per minute) to separate the 4Hyp derivative from excess reagent (*20*). Peptides were sequenced by Edman degradation (*21*). The PTH amino acid obtained in the first cycle with the dipeptide 4Hyp-Gly (Bachem) was used as a standard for PTH-O. PTH-O yielded two peaks eluting closely before and after PTH-Ala; 3-hydroxyproline does not give a signal during Edman degradation (*22*). Absorbance (*A*) at 214 nm is measured in milliabsorbance units. (**C**) The relevant sequences of the 4Hyp-containing peptides numbered from the NH_2-terminus of the mature proteins (*3*). Symbols: P > O, more P than O detected; O > P, more O than P detected.

taining sequences (Table 1). One possible explanation is that plants have several prolyl hydroxylases that differ in specificity. Although plant prolyl-4-hydroxylases differ from the vertebrate enzyme in specificity (*14*), enzymes that recognize different substrate sequences in a single plant species have not been found.

Alternatively, prolyl hydroxylases from plants might recognize the conformation of the substrate. A common feature of the sequences in Table 1 is the clustering of the 4Hyp residues. Studies of the structure of HRGPs and AGPs performed by spectroscopic methods (*15*) and studies of model peptides performed by x-ray methods (*16*) have shown that sequences that contain clusters of imino acids form poly(L-proline) type II helices. It is this conformation that is recognized by the prolyl-4-hydroxylase of *Vinca rosea* (*17*).

We conclude that CHN-A and CHN-B are examples of a class of HCPs that differ from those described previously in specific ways. (i) CHN-A and CHN-B are vacuolar enzymes (*5*), whereas other HCPs are predominantly secreted, structural proteins (*2, 4*). (ii) The 4Hyp content of chitinases is much lower than in other HCPs, and hydroxylation takes place exclusively at a few unique Pro residues in a short spacer joining two Pro-containing domains. (iii) Unlike other plant HCPs, the 4Hyps in chitinase are not O-glycosylated.

4Hyps in repeated sequences stabilize the polyproline II conformation of collagen, HRGP, and AGP (*1, 15*). The function of the 4Hyps in chitinase is not known. One possibility is suggested by the structural homology of chitinase and bacterial β-1,4-glucanases, which have a lectin domain connected to a catalytic domain by a spacer that contains repeats of the dipeptide Thr-Pro (*18*). Deletion of the lectin domain or of the spacer changes the specificity of this enzyme for different physical forms of cellulose. Modification of the spacer in chitinase by prolyl hydroxylation at specific sites might alter the relative positions of the lectin and catalytic domains and, hence, modulate enzyme activity or specificity. The tobacco chitinases are abundant regulated proteins particularly well suited for the study of the function and specificity of prolyl hydroxylation. The fact that these are intracellular enzymes also raises the possibility that limited prolyl hydroxylation of proteins is a more general phenomenon than previously recognized.

REFERENCES AND NOTES

1. R. A. Berg and D. J. Prockop, *Biochem. Biophys. Res. Commun.* **52**, 115 (1973); S. Sakakibara *et al.*, *Biochim. Biophys. Acta* **303**, 198 (1973).
2. A. M. Showalter and J. E. Varner, *Biochem. Plants* **15**, 485 (1989); A. M. Showalter and D. Rumeau, in *Organization and Assembly of Plant and Animal Extracellular Matrix*, W. S. Adair and R. P. Mecham, Eds. (Academic Press, New York, 1990), pp. 247–281.
3. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; G, Gly; H, His; O, 4Hyp; K, Lys; P, Pro; S, Ser; T, Thr; V, Val; and Y, Tyr.
4. J. Gross, *Harvey Lect.* **68**, 351 (1973).
5. J.-M. Neuhaus, L. Sticher, F. Meins, Jr., T. Boller, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 10362 (1991).
6. T. Boller, *Ox. Surv. Plant Mol. Cell Biol.* **5**, 145 (1988); H. J. M. Linthorst, *Crit. Rev. Plant Sci.* **10**, 123 (1991); F. Meins, Jr., J.-M. Neuhaus, C. Sperisen, J. Ryals, in *Genes Involved in Plant Defense*, T. Boller and F. Meins, Jr., Eds. (Springer-Verlag, Vienna, in press).
7. H. Shinshi, D. Mohnen, F. Meins, Jr., *Proc. Natl. Acad. Sci. U.S.A.* **84**, 89 (1987).
8. H. Shinshi, J.-M. Neuhaus, J. Ryals, F. Meins, Jr., *Plant Mol. Biol.* **14**, 357 (1990); M. van Buuren, J.-M. Neuhaus, H. Shinshi, J. Ryals, F. Meins, Jr., *Mol. Gen. Genet.* **232**, 460 (1992).
9. A mixture of CHN-A and CHN-B was acid-hydrolyzed, and the neutral-sugar fraction was analyzed by thin-layer chromatography [R. G. Spiro, *Methods Enzymol.* **8**, 3 (1966)]. Samples of CHN-A and CHN-B with transferrin as an internal standard were assayed with a DIG glycan detection kit (Boehringer Mannheim).
10. A. S. B. Edge, C. R. Faltynek, L. Hof, L. E. Reichert, Jr., P. Weber, *Anal. Biochem.* **118**, 131 (1990).
11. Means of >21 scans in positive and negative ionization modes [M. Schär, K. O. Börnsen, E. Gassmann, *Rap. Comm. Mass Spec.* **5**, 319 (1991)] yielded 32,168 ± 73 (32,022) daltons for CHN-A and 31,597 ± 47 (31,500) daltons for CHN-B. Values in parentheses were deduced from cDNA clones (*8*).
12. R. L. Jones and D. G. Robinson, *New Phytol.* **111**, 567 (1989).
13. D. T. A. Lamport, *Biochemistry* **8**, 1155 (1969).
14. D. G. Robinson, M. Andreae, P. Blankenstein, in *Organization and Assembly of Plant and Animal Extracellular Matrix*, W. S. Adair and R. P. Mecham, Eds. (Academic Press, New York, 1990), pp. 283–299.
15. R. B. Homer and K. Roberts, *Planta* **46**, 217 (1979); G.-J. van Holst and J. E. Varner, *Plant Physiol.* **74**, 247 (1984); G.-J. van Holst and G. B. Fincher, *ibid.* **75**, 1163 (1984).
16. T. Matsuzaki, *Acta Crystallogr. Sect. B Struct. Sci.* **30**, 1029 (1974).
17. M. Tanaka, K. Sato, T. Uchida, *J. Biol. Chem.* **256**, 11397 (1981).
18. H. Shen *et al.*, *ibid.* **266**, 11335 (1991).
19. J. Hofsteenge, S. R. Stone, A. Donella-Deana, L. A. Pinna, *Eur. J. Biochem.* **188**, 55 (1990).
20. R. Knecht and J.-Y. Chang, *Anal. Chem.* **58**, 2375 (1986).
21. R. M. Hewick, M. W. Hunkapiller, L. E. Hood, W. J. Dreyer, *J. Biol. Chem.* **256**, 7990 (1981).
22. J. H. Waite, *ibid.* **258**, 2911 (1983).

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Calcium-Dependent Transmitter Secretion Reconstituted in *Xenopus* Oocytes: Requirement for Synaptophysin

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Calcium-dependent glutamate secretion was reconstituted in *Xenopus* oocytes by injecting the oocyte with total rat cerebellar messenger RNA (mRNA). Co-injection of total mRNA with antisense oligonucleotides to synaptophysin message decreased the expression of synaptophysin in the oocyte and reduced the calcium-dependent secretion. A similar effect on secretion was observed for oocytes injected with total mRNA together with an antibody to rat synaptophysin. These results indicate that synaptophysin is necessary for transmitter secretion and that the oocyte expression system may be useful for dissecting the molecular events associated with the secretory process.

Synaptic transmission between nerve cells depends on impulse-triggered, Ca^{2+}-dependent transmitter secretion from the presynaptic nerve terminal (*1*), a process poorly understood at the molecular level. Physiological studies in a variety of systems have provided important clues to the process of synaptic vesicle exocytosis and its regulation. The application of protein purification as well as molecular cloning techniques to the study of synaptic vesicle proteins has led to the identification and characterization of the major components of these organelles. However, attempts to determine the precise function of the synaptic vesicle proteins have been hampered by the inaccessibility of the small nerve terminal to experimental manipulations. We have now examined the role of synaptophysin, a major integral membrane protein of synaptic vesicles (*2*), in transmitter secretion by reconstituting Ca^{2+}-dependent transmitter release in *Xenopus* oocytes. The use of *Xenopus* oocytes for expressing neuronal properties in a millimeter-size cell (*3*) offers an opportunity for studying secretion mechanisms in vitro. Recently, Ca^{2+}-dependent

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