Cloning, expression, and spectroscopic characterization of *Cucumis sativus* stellacyanin in its nonglycosylated form

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Abstract

The cDNA encoding the 182 amino acid long precursor stellacyanin from *Cucumis sativus* was isolated and characterized. The protein precursor consists of four sequence domains: I, a 23 amino acid hydrophobic N-terminal signal peptide with features characteristic of secretory proteins; II, a 109 amino acid copper-binding domain; III, a 26 amino acid hydroxyproline- and serine-rich peptide characteristic of motifs found in the extensin family, extracellular structural glycoproteins found in plant cell walls; and IV, a 22 amino acid hydrophobic extension. Maturation of the protein involves posttranslational processing of domains I and IV. The copper-binding domain (domain II), which shares high sequence identity with other stellacyanins, has been expressed without its carbohydrate attachment sites, refolded from the *Escherichia coli* inclusion bodies, purified, and characterized by electronic absorption, EPR, ESEEM, and RR spectroscopy. Its spectroscopic properties are nearly identical to those of stellacyanin from the Japanese lacquer tree *Rhus vernicifera*, the most extensively studied and best characterized stellacyanin, indicating that this domain folds correctly, even in the absence of its carbohydrate moiety. The presence of a hydroxyproline- and serine-rich domain III suggests that stellacyanin may have a function other than that of a diffusible electron transfer protein, conceivably participating in redox reactions localized at the plant cell wall, which are known to occur in response to wounding or infection of the plant.

Keywords: blue copper protein; cDNA; *Cucumis sativus*; expression; inclusion bodies; refolding; spectroscopy; stellacyanin

Stellacyanin is a copper-containing glycoprotein of unknown function isolated originally from *Rhus vernicifera* (Keilin & Mann, 1940; Omura, 1961; Blumberg et al., 1964; Peisach et al., 1967). Its spectroscopic properties and electron transfer reactivity place it in the class of mononuclear blue or type 1 copper proteins, such as plastocyanin, azurin, pseudoazurin, plantacyanin, rusticyanin, amicyanin, auracyanin, and halocyanin, proteins that are collectively termed cupredoxins (Adman, 1985, 1991). Blue copper centers

also occur in multicopper blue oxidases (ceruloplasmin, ascorbic acid oxidase, laccase) and in copper-containing nitrite reductases. These proteins are distributed widely and, in those cases where function has been established, are known to play essential roles in the rapid, long-range electron transfer occurring in a variety of biological processes, including photosynthesis and bacterial energy conversion (Adman, 1991; Canters & Van de Kamp, 1992; Solomon et al., 1992; Solomon & Lowery, 1993; Wuttke & Gray, 1993; Malmström, 1994; Guckert et al., 1995).

Stellacyanin was described initially in 1940 by Keilin and Mann as a blue pigment co-product observed during the isolation of laccase from the extracellular secretion of the Japanese lacquer tree *R. vernicifera* (Keilin & Mann, 1940). Omura (1961) reported its purification and identification as a copper-containing protein. In 1964–67, Peisach, Blumberg, and coworkers further characterized this protein, described its unusual visible absorption and EPR spec-

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Abbreviations: EPR, electron paramagnetic resonance; ESEEM, electron spin echo envelope modulation; RR, resonance Raman; BCB, blue copper binding protein; RT-PCR, reverse transcriptase-mediated PCR; IPTG, isopropyl- β -D-thiogalactopyranoside.

troscopic properties relative to that of other cupredoxins known at that time, and designated it stellacyanin (Blumberg et al., 1964; Peisach et al., 1967).

High-resolution X-ray crystallographic studies of plastocyanin, azurin, pseudoazurin, and amicyanin have revealed that the typical copper binding sites of cupredoxins consist of four liganding amino acid residues, two His, one Cys, and one Met. In the case of azurin, an additional fifth ligand, the carbonyl oxygen of a glycine residue, is also coordinated to copper. These ligands are conserved in all currently available sequences of blue copper proteins other than stellacyanins. But early in the characterization of *R. vernicifera* stellacyanin, it was noted that this protein contains no Met at all (Peisach et al., 1967; Bergman et al., 1977). Stellacyanin also differs from other cupredoxins in the unusually fast rates with which it undergoes redox reactions either with small inorganic complexes or electrodes, observations suggesting that its copper center is considerably more solvent-exposed (Mauk et al., 1980; Tollin et al., 1986). ESEEM studies also indicated a greater accessibility to solvent for stellacyanin relative to azurin (Mims et al., 1984).

Significant advances in the molecular biology of mononuclear copper proteins have taken place in recent years, particularly for plastocyanin and azurin, both of which were structurally characterized by X-ray crystallography in 1978 (Adman et al., 1978; Colman et al., 1978). The first nucleotide sequences of genes encoding mononuclear blue copper proteins were determined for plastocyanins from *Spinacia oleracea* and *Silene pratensis* (Smeekens et al., 1985; Rother et al., 1986). Subsequently, the nucleotide and deduced amino acid sequences of the genes for various mononuclear blue copper proteins from different microorganisms became available, notably for azurin, pseudoazurin, amicyanin, and more recently for halocyanin (Canter, 1987; Yamamoto et al., 1987; Arvidsson et al., 1989; Chistoserdov et al., 1991; Ubbink et al., 1991; Mattar et al., 1994). In addition, an artificial gene for rusticyanin has been constructed and amplified using PCR based on the amino acid sequence (Casimiro et al., 1995). The availability of these genes has stimulated research in this area significantly, particularly in the case of plastocyanin and azurin, where various mutant proteins have been engineered.

The unusual sequence, spectroscopic, and electrochemical properties of *R. vernicifera* stellacyanin have long made it an intriguing candidate for high-resolution X-ray structural analysis, but efforts to crystallize the protein in many different laboratories have been in vain, probably because of the high carbohydrate content (almost 40% of the molecular weight) (Peisach et al., 1967) and heterogeneity caused by polyploidy (Obokata et al., 1993) and/or post-translational modifications (in addition to glycosylation), both of which are observed frequently in higher plants. Here we report the isolation and characterization of the cDNA encoding *Cucumis sativus* stellacyanin and expression, purification, and spectroscopic properties of its nonglycosylated form with the aim of further detailed studies of this protein in both structural and functional levels.

Results

Isolation of cDNAs encoding *C. sativus* stellacyanin and expression of its copper binding domain

For isolating a full-length cDNA encoding stellacyanin from cucumber, a 269-bp partial clone was amplified using RT-PCR (see

Materials and methods). A λ gt11 cDNA library was synthesized from poly(A)⁺ RNA isolated from the cucumber peelings, and approximately 500,000 primary recombinant phages were screened with the 269-bp RT-PCR product. More than 500 positive signals were obtained. Twenty recombinant phages were selected randomly and sequenced. They contain identical in-sequence inserts ranging from 600 to 700 bp. Further analysis with restricted nuclear DNA indicated a single gene per haploid genome (data not shown), which is consistent with the identity of the sequences from 20 independent recombinant phages checked. Sequencing of the longest cDNA showed that this clone was 702 nucleotides long and contained a start codon at nucleotides 22–24 that initiates an open reading frame of 182 amino acids (Fig. 1). Comparison of the amino acid sequences of the mature protein and the one deduced from the nucleotide sequence of the cDNA combined with hydrophathy analysis suggests a complex mechanism for the posttranslational modification of the precursor protein (Fig. 2A,B). Precursor *C. sativus* stellacyanin consists of four distinct domains. Domain I is a 23 amino acid hydrophobic N-terminal signal peptide. Domain II consists of a 109 amino acid copper-binding domain. Domain III is a 26 amino acid peptide with hydroxyproline- and serine-rich repetitive sequence motifs characteristic of the hydroxyproline-rich proteins of the extensin family, extracellular structural glycoproteins found in plant cell walls (Showalter, 1993; Kieliszewski & Lamport, 1994). The C-terminal residue of the mature protein, Ser¹³⁷, is followed by domain IV, an additional

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adaptor
GAATTCGCGGCGGC TTTTTCACACCTCTCATCCATG GCC GCC GGA TCA GTT GCC TTT 45
                                     M A A G S V A F
                                     -20
GTT TTG GGT TTG ATT GCA GTG GTT TTT CTT CAC CCT GCC ACC GCT CAG TCG 96
V L G L I A V V F L H F C C T A Q S
-10
ACG GTT CAT ATC GTC GGC GAC AAC ACC GGT TGG AGT GTT CCA TCG AGC CCC 147
T V H I V G D N T G W S V P S S P
10
AAT TTC TAT TCT CAA TGG GCT GCT GGA AAG ACC TTC CGC GTT GGC GAT TCT 198
N F Y S Q W A A G K T F R V G D S
20
CTC CAA TTT AAC TTT CCA GGC AAT GCC CAC AAC GTA CAT GAA ATG GAA ACA 249
L Q F N F P A N A H N V H E M E T
40
AAA CAG TCG TTT GAT GCA TGT AAT TTT GTC AAT TCT GAT AAC GAT GTC GAG 300
K Q S F D A C N F V N S D N D V E
60
AGG ACA TCT CCA GTG ATA GAG AGA CTC GAC GAA CTC GGC ATG CAT TAC TTT 351
R T S P V I E R L D E L G M H Y F
80
GTT TGC ACC GTT GGA ACA CAT TGT TCA AAT GGT CAA AAG TTA TCC ATC AAT 402
V C T V G T H C S N G Q K L S I N
90
GTC GTT GCT GCC AAT GCC ACT GTT TCG ATG CCA CCA CCT TCA TCA TCG CCT 453
V A A N A T V S M P P P P S S S P
110
CCT TCA TCA TCG CCT CCT TCA TCG GTG ATG CCA CCA CCG TCT CCT TCA TCC 504
P S S S P P S S V M P P P S P S S
130
TCC AAC GCA CTT ATG GCA ACC CTT TAT CTT ACT TTC TCC GCC CTT CTT GTT 555
S N A L M A T L Y L T F S A L L V
140
ATG GCC TTC TTT TAG ATATATATGTATGAGTTTCTTAATTAAATTCATGTGAGAGTTTG 617
M A F F Stop
150
AGAGATATTAGTACTTAGGTGAGATTGGATTTCATTGTCTCTCTCCATTTTTCATCAAT 684
TCTAGTTTTCCTCCAA GCGGCGCGAATTC
adaptor

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Fig. 1. Nucleotide sequence of the cDNA encoding *C. sativus* stellacyanin. The sequence is numbered from the first nucleotide of the cDNA insert. The deduced amino acid sequence (single-letter code) is shown below the nucleotide sequence and numbered beginning with the first Gln residue of the mature protein. The inverted solid triangle and vertical arrow indicate the posttranslational cleavage sites of the precursor protein from amino-terminal and carboxyl-terminal ends, respectively.

Fig. 2. **A:** Hydropathy profile of cucumber stellacyanin precursor protein. Numbering (+1) refers to the amino terminus of the mature protein. Hydropobicity values were calculated using window size of seven residues according to Kyte and Doolittle (1982). The inverted solid triangle and arrow indicate maturation sites. **B:** Schematic representation of precursor stellacyanin. Protein domains are indicated by Roman numerals. One-letter codes are used for the amino acid sequence of the copper binding domain.

22-amino acid hydrophobic polypeptide extension. A C-terminal extension has also been earlier found in precursors of two allelic forms of multicopper enzyme laccase from *Neurospora crassa* (Germann et al., 1988).

The glycosylation sites (a single N-linked site via Asn¹⁰⁹ and multiple O-linked sites via several hydroxyproline residues of domain III) as well as the signal peptide for *C. sativus* stellacyanin were removed using PCR, and the copper-binding domain of the nonglycosylated protein was expressed and metallated (see Materials and methods). Unlike the glycoprotein isolated from cucumber peelings, the nonglycosylated copper binding domain of stellacyanin crystallized readily, supporting our hypothesis that it is the carbohydrate moiety that hinders crystallization of the mature protein. The 1.6-Å resolution crystal structure is reported in the companion paper (Hart et al., 1996).

Spectroscopy

The electronic absorption spectra in the visible region, EPR, ES-EEM, and RR spectra of cucumber stellacyanin were recorded and are displayed in Figures 3, 4, and 5. It is interesting to note that the characteristic spectroscopic properties of stellacyanin are retained even when the carbohydrate moiety is removed. This result suggests that the carbohydrate-free copper binding domain folds as it does in the mature glycoprotein.

Fig. 3. **(A)** Electronic absorption and **(B)** EPR spectra of nonglycosylated cucumber stellacyanin at two different pH values. Spectra 1: 10 mM acetate buffer, pH 5.5. Spectra 2: same protein solution as in spectra 1 at pH 11 adjusted with 1 M NaOH. The protein concentration for all spectral measurements was 0.5 mM. EPR spectra were measured at 77 K. Instrument settings: microwave frequency, 9.645 GHz; microwave power, 10.4 mW; modulation amplitude, 8 gauss.

Discussion

Comparative analysis of stellacyanins from four different sources

Three other cupredoxins that are similar to *R. vernicifera* stellacyanin are now known. These are: (1) the subject of this paper, i.e., stellacyanin isolated from cucumber peelings (Mann et al., 1992); (2) umecyanin from horseradish roots (*Armoracia laphatifolia*) (Van Driessche et al., 1995); and (3) a gene product (BCB) predicted from the nucleotide sequence of a negatively light-regulated gene isolated from *Arabidopsis thaliana* (Van Gysel et al., 1993). Although umecyanin and cucumber stellacyanin were isolated originally in 1970 and 1979, respectively, their amino acid sequences became available only recently (Paul & Stigbrand, 1970; Aikazyan & Nalbandyan, 1979). The copper binding domains of these three proteins share more than 40% amino acid identity with *R. vernicifera* stellacyanin and only 10% and 15% with azurin and plastocyanin, respectively.

Although these proteins, unlike *R. vernicifera* stellacyanin, contain Met, protein sequence alignments (see Fig. 6) suggest that they all contain Gln in place of the Met normally found as a copper ligand in other cupredoxins and in type 1 sites in multicopper oxidases and nitrite reductases. In addition, like *R. vernicifera*

Fig. 4. ESEEM spectra of nonglycosylated cucumber stellacyanin at two different pH values. Spectrum 1: 10 mM acetate buffer pH 5.5. Spectrum 2: ESEEM spectra of *R. vernicifera* stellacyanin at pH 5.5. Spectrum 3: same protein solution as in spectrum 1 at pH 11 adjusted with 1 M NaOH. The protein concentration was 0.5 mM. ESEEM spectra were measured at 4.2 K. All samples contain 50% ethylene glycol. Microwave frequency, 8.5 GHz; $g = 2.05$; τ was the second harmonic of the proton Larmor frequency (for suppression of proton lines).

stellacyanin, they are glycoproteins (in the case of BCB, a putative N-linked glycosylation site at Asn⁷⁶ residue is analogous to the same residue in umecyanin, which had been identified to be glycosylated) and they contain three conserved cysteine residues. The gene sequences for both umecyanin and *R. vernicifera* stellacyanin are not available currently. Umecyanin has a heterogeneous carboxyl terminus, probably due to a nonspecific proteolysis during the purification of the protein. In addition, umecyanin and BCB have almost 90% identity in amino acid sequence. On this basis, we predict that the umecyanin precursor and more likely also the precursor of *R. vernicifera* stellacyanin will ultimately be found to carry a carboxy-terminal extension as well, analogous to the domains III and IV in cucumber and *Arabidopsis* proteins. Based on the structural similarity inferred from sequence alignments in the region of the copper binding site, we consider all of these proteins to be stellacyanins, a special subclass of type 1 copper centers with Gln rather than Met coordination to copper.

Two discrepancies between the amino acid sequences determined previously and those deduced subsequently were found in inserts from all 20 recombinant phages analyzed. First, sequencing of the mature protein was found to be blocked, and it was proposed that this was due to the presence of pyroglutamate at the N terminal position (Mann et al., 1992). However, according to the nucleotide sequence, glutamine rather than glutamic acid is the first

Fig. 5. A: Frozen solution (77 K) RR spectra of ~4 mM cucumber stellacyanin in 100 mM sodium acetate buffer at pH 5.5 (top) and 11.2 (bottom) with 568.2-nm excitation (160 mW) and 3-cm⁻¹ slit widths. Three spectra were added together, each recorded at 0.2 cm⁻¹ increments with photon counting for 1 s. B: Expanded RR spectra of the 275-cm⁻¹ region for cucumber stellacyanin at pH 5.5 (bottom) and 11.2 (top) with solution and excitation conditions as in A. Six spectra were added together, each recorded at 0.2 cm⁻¹ increments with photon counting for 1 s.

amino acid in the mature protein. The N terminus is therefore probably blocked by another mechanism, possibly acetylation rather than cyclization. Second, five amino acids following Ser¹²⁰, PPSSS in the deduced sequence, are missing in the sequence obtained by protein sequencing, and five amino acids following Pro¹³⁴, VMPPP found in the protein sequence, are missing in the sequence derived from the cDNA. The amino acid sequence of cucumber stellacyanin determined by protein sequencing (lower line, only C-terminal region) is compared below with that deduced from nucleotide sequence (upper line, only domains III and IV):

¹¹³SMPPPPSSPPSSSPSSVMPPP SPSSSNALMATLYLTFSALLVMAFF
¹¹³SMPPPPSS PPSSVMPPPPVMPPSPS

We conclude that the protein sequence of cucumber stellacyanin must now be revised. The revised sequence based on the cDNA sequence reveals the presence of a palindromic site generated by a glycopeptide PPSSPPSSSP (starting from Pro¹¹⁶). Such a site is thought to be a prerequisite for ordered self-assembly of a number of different extracellular matrix proteins prior to their covalent crosslinking (Kieliszewski & Lampion, 1994).

Fig. 6. Alignment of the amino acid sequences of closely related mononuclear blue copper proteins, stellacyanins from four different sources. UMC, umecyanin from *A. laphatfolia* (residues 1–105) (Van Driessche et al., 1995). BCB, copper binding domain (23–125) of the 21.5-kDa product of the negatively light-regulated gene from *A. thaliana* (Van Gysel et al., 1993). CST, copper binding domain (1–109) of stellacyanin from *C. sativus* (this study). STC, stellacyanin from *R. vernicifera* (Bergman et al., 1977). Residues that are identical in three or more proteins are boxed. Dashes indicate gaps introduced to allow optimal alignment. Symbols used to indicate the side chain character are: (), aromatic residue; (Cu), the known or postulated copper ligands; -S-S-, disulfide bridge between the residues Cys⁶⁰ and Cys⁹⁵. Numbering refers to the amino acid positions in cucumber stellacyanin.

Spectroscopic properties

The spectroscopic properties of the protein were found to be virtually identical to those of *R. vernicifera* stellacyanin (Peisach et al., 1967). The visible absorption spectrum (Fig. 3A) contains two peaks assigned as thiolate sulfur-to-copper charge transfer transitions. As has been described previously, the ratio of the extinction coefficient of the band at 450 nm to that at 600 nm is indicative as a rhombic rather than an axial type 1 copper geometry (Lu et al., 1993). The EPR spectrum (Fig. 3B) is also characteristic of a rhombic site (Lu et al., 1993). The shift of the visible absorption spectrum at pH 11 to higher energy is virtually identical to that of *R. vernicifera* stellacyanin and indicates that a structural change has slightly modified the interaction between the sulfur and Cu(II).

The ESEEM spectrum (Fig. 4) at neutral pH is characteristic of ¹⁴N superhyperfine interactions from the remote nitrogen atom of a coordinated imidazole (Mims & Peisach, 1978; Jiang et al., 1990). In the protein spectra, a small splitting in the feature assigned to the double quantum transition is observed, indicative of a dissimilarity in coupling between the two ligand imidazoles and the electron spin of Cu(II). These features contrast with data obtained for azurin (Van de Kamp et al., 1993) and copper-imidazole complexes, for which the double quantum transition is sharper, and therefore suggest that the imidazole moieties are equivalent.

At elevated pH, there is a marked alteration of the combination lines, which appear in the 2–3-MHz region. These lines merge and sharpen, and a detailed multifrequency study plus simulations indicate that these changes are indeed the result of changes in the combination lines only and may be attributed to a reordering of the imidazole rings relative to the equatorial plane of the metal com-

plex. At neutral pH, the quadrupole tensors of the two imidazole remote nitrogen atoms have different orientations relative to the molecular axis; structural reorientation at higher pH brings these tensors into a more symmetric and equivalent orientation (C.J. Bender, unpubl. results).

These same multifrequency ESEEM studies and their simulations also preclude any assignment of peaks to a new form of copper ligand interaction, as has been suggested by Thomann et al. (1991). Spectra for the *R. vernicifera* stellacyanin recorded over the entire X-band frequency range (8–12 MHz) indicate that the peaks do not shift in a manner commensurate with the ¹⁴N Larmor frequency, which would be the case if an amide nitrogen were responsible for any of the observed peaks. A similar argument holds against the assignment of any peak to protons. We are therefore confident that the lineshape changes in the 2–4-MHz region of the spectrum are attributable to changes in the combination lines, and our simulations suggest that these changes can be explained by the reorientation of the imidazole rings.

Our conclusions from the ESEEM data are in strong agreement with the fact that the identical, pH-dependent, reversible changes in optical and EPR spectra have been documented earlier for plantacyanins as well (Nersissian & Nalbandyan, 1988). Plantacyanins, also known as cucumber basic protein, CBP, are strongly basic mononuclear blue copper proteins from plants (Markossian et al., 1974). According to the X-ray structural data, they have a methionine as an axial ligand rather than glutamine as in stellacyanin (Guss et al., 1988). The similarity in the pH-dependent behavior of stellacyanins and plantacyanins argues strongly that this change is not attributable to the glutamine ligand in stellacyanin. In addition, we found that a mutant form of cucumber stella-

cyanin, in which the Gln⁹⁹ residue was substituted by methionine, possesses the same pH-dependent reversible spectroscopic transformations (A.M. Nersissian, G. Williams, J.S. Valentine, unpubl. results) as the wild-type protein, demonstrating that these pH-dependent changes occur even in the absence of the Gln⁹⁹ ligand.

Laser excitation of cucumber stellacyanin within the 600-nm absorption band produced the RR spectra that are characteristic of a tetrahedrally distorted type I Cu site, in agreement with its rhombic EPR signal and increased absorbance at ~450 nm. The frequencies and intensities of the enhanced RR bands were found to be pH dependent as well. Figure 5 shows the effect of pH on the low-temperature frozen solution RR spectra of the protein at low (5.5, top) and high pH (11.2, bottom) obtained with 568.2 nm Kr⁺ laser excitation, in close resonance with the protein blue absorption bands. The spectra of both pH forms are characterized by four intense bands in the 340–430 cm⁻¹ Cu-S(Cys) stretching region (Fig. 5A), a doublet of moderate intensity at ~270/280 cm⁻¹ (Fig. 5B), and a broad band at ~125 cm⁻¹ (not shown), which are typical of the type I Cu centers (Woodruff et al., 1988; Sanders-Loehr, 1993). Clusters of moderately strong, pH-sensitive bands were also observed between 700 and 800 cm⁻¹, which contained combinations of the dominant 350–410-cm⁻¹ fundamentals and, in addition, a distinct band at 751 (pH 5.5) and 753 cm⁻¹ (pH 11.2) due to C-S stretching of the cysteine ligand (Ferris et al., 1979).

The most intense peak in the cucumber stellacyanin spectrum occurs at 385 cm⁻¹, which is analogous to the similar peak in *R. vernicifera* stellacyanin (Nestor et al., 1984). Because the strongest peak in the RR spectra of blue copper proteins results from a predominantly Cu-S(Cys) stretching vibrational mode (Dave et al., 1993; Andrew et al., 1994; Qiu et al., 1995), the Cu-S bond strength in the two proteins should be very similar. The positions of the other three major peaks differ, however, and are shifted ~5–6 cm⁻¹ toward the higher frequencies relative to those of *R. vernicifera* stellacyanin. In addition, the 411-cm⁻¹ band is much more enhanced than its counterpart in *R. vernicifera* stellacyanin at 405 cm⁻¹. Thus, the normal-mode compositions are quite different for cucumber and *R. vernicifera* stellacyanins, most likely reflecting different conformations of the cysteine ligand and its close environment. Raising the pH from 5.5 to 11.2 preserves the overall vibrational pattern but lowers the frequency of the most intense RR band in the spectrum from 385 to 378 cm⁻¹. The 352- and 411-cm⁻¹ bands also shift downward (1–2 cm⁻¹) at high pH, whereas the shoulder at ~380 cm⁻¹ is apparently shifted up (~2 cm⁻¹). Relative band intensities also change at pH 11.2; the 351-cm⁻¹ peak gains intensity, whereas those at 378 and 409 cm⁻¹ lose intensity. These observations indicate that the high-pH form of stellacyanin possessed weaker and, consequently, longer Cu-S bond than the low-pH form.

Additional changes were observed in the low-frequency region, 100–300 cm⁻¹, where vibrational modes associated with His ligands are expected to occur. One of the bands in this region at 281 cm⁻¹ gained intensity and upshifted to 283 cm⁻¹ when the pH changed from 5.5 to 11.2, whereas the other shifted from 125 to 128 cm⁻¹ (not shown). Because these two bands in the RR spectra of blue copper proteins result from the predominantly Cu-N(His) stretching and Cys(S)-Cu-N(His) bending vibrational modes, respectively (Fraczkiewicz, 1996), the shifts of the 281- and 125-cm⁻¹ bands in the pH 11.2 spectrum of cucumber stellacyanin to higher frequencies suggests slightly shorter Cu-His distances and/or different conformations of the His ligands at the high pH copper site (see below).

What is a stellacyanin and what is its function?

The identification of this cucumber protein as a stellacyanin is based on (1) its high overall sequence identity with *R. vernicifera* stellacyanin, (2) the presence of a Gln residue in the position of the conserved Met residue found in blue copper proteins other than stellacyanins, (3) its spectroscopic properties, which are virtually identical to those of *R. vernicifera* stellacyanin, (4) the pH-dependent reversible changes in the EPR and visible absorption spectra, which are similar to those observed in *R. vernicifera* stellacyanin, (5) the presence of three invariant cysteine residues of which one (Cys⁸⁹) serves as a copper ligand, whereas the two others (Cys⁶⁰ and Cys⁹⁵) form a disulfide-bridge (Engeseth et al., 1984; Hart et al., 1996), and (6) the fact that like *R. vernicifera* stellacyanin it is also glycosylated.

The physiologic role of cucumber stellacyanin (and of the other stellacyanins) remains unknown. Two structurally and functionally distinct protein domains, a type I copper electron transfer center (domain II) and an extensin-like cell wall structural element (domain III), have been fused into a chimeric protein. Precursor cucumber stellacyanin carries two strongly hydrophobic terminal domains (domain I and domain IV), which are absent in the sequence of the mature protein isolated from cucumber peelings, suggesting that it undergoes posttranslational processing from both N terminus and C terminus. Domain I is a N-terminal 23 amino acid classic signal peptide that can act as an endoplasmic reticulum targeting sequence with a subsequent translocation of the protein to the cell wall through Golgi apparatus. Its cleavage site (Ala-Thr-Ala) matches with -3, -1 rule proposed by von Heijne (1986), which required a small and neutral residue in positions -1 (the C-terminal residue of the signal peptide) and -3. It appears very likely that domain IV, a C-terminal, 22 amino acid, hydrophobic extension acts as an enhancer for integration or immobilization of the protein into the cell wall. This domain is apparently cleaved subsequently. Despite the fact that all biochemically characterized stellacyanins, i.e., stellacyanins from cucumber and *R. vernicifera* as well as horseradish umecyanin recovered as a soluble proteins, it has been proposed by Van Gysel et al. (1993), that domain IV in BCB may be replaced with a phosphatidylinositol glycan moiety, which could serve as a membrane anchor, similar to several cell-surface attached proteins (Ferguson & Williams, 1988; Van Gysel et al., 1993). Furthermore, it is interesting to note, that carboxyl-terminal processing occurs also during the vacuolar targeting of different vacuolar proteins, such as lectins and chitinase (Bednarek & Raikhel, 1991).

It is currently well-documented that mechanical wounding or infection of the plant by pathogens causes a rapid accumulation of hydrogen peroxide, which in turn mediates an accelerated oxidative crosslinking of cell wall structural proteins. This process is believed to be a one of the most rapid inducible defense responses in plants, being initiated within 2 min (Bradley et al., 1992; Levine et al., 1994). The factors participating in this primary defense response are unknown.

We suggest that stellacyanin, because it contains both a redox active copper and an extensin-like domain, is highly likely to be localized at the plant cell wall and to participate in redox reactions at that site. Stellacyanin is thus a plausible candidate to participate in the reactions leading to generation of hydrogen peroxide at the site of wounding or infection of plants.

The sequence reported in this paper has been deposited in the GenBank database with accession number U65511.

Materials and methods

Cloning of cDNAs

A partial cDNA clone was amplified by RT-PCR (Chelly et al., 1988; Lee et al., 1988). The oligonucleotide primers for PCR were designed from the known amino acid sequence of the mature protein isolated from cucumber peelings (Mann et al., 1992). Primer 1, a sense 27-mer, with a 5' overhang *Sma* I restriction site (underlined) CATATCCCGGGNGAYAAAYACNGGNTGG, corresponds to the DNA sequence complementary to all possible coding sequences for the N-terminal region Gly⁸-Asp-Asn-Thr-Gly-Trp¹³, whereas primer 2, an antisense 32-mer with a *Kpn* I restriction site (underlined) TGGGTACCNACNGTRCANACRAARTARTGCAT, is complementary to the coding sequences for the C-terminal region Met⁸⁴-His-Tyr-Phe-Val-Cys-Thr-Val-Gly-Thr⁹³. To introduce a *Kpn* I restriction site the GGT and ACC genetic codes were used for Gly⁹² and Thr⁹³, respectively. In the above sequences, N represents any nucleotide A, T, C or G; Y represents both C or T; and R represents G or A.

Poly(A)⁺ RNA was purified on oligo-d(T) cellulose columns (Pharmacia) from total RNA extracted from cucumber peelings. The quality of the poly(A)⁺ RNA preparation for library construction and the reverse transcription reaction was examined by cell-free translation in vitro using wheat germ extract (Stratagene). One microgram of poly(A)⁺ RNA in 20 μ L of water was heated at 65 °C for 5 min and transferred directly into the tube containing reverse transcription buffer (50 mM Tris/HCl, pH 8.2, 6 mM MgCl₂, 40 mM KCl, and 1 mM dithiothreitol), 10 units of RNasin (Boehringer), 1 μ L of oligo d(T) (1.5 μ g/ μ L), and 1 mM of dNTPs in a final volume of 30 μ L. The first-strand cDNA pool was extended using 15 units of avian myeloblastosis virus reverse transcriptase (Boehringer) for 2 h at 41 °C. After removing excess oligo d(T) and salts using spin columns (Pharmacia) equilibrated with 1 $\times$ PCR buffer (Promega), 10 μ L of the first-strand cDNA pool was used as a template for the PCR.

PCR was conducted in the presence of 1 μ M final concentration of the sense and antisense primers, 3 mM MgCl₂, and 0.2 mM dNTPs. The reaction mixture was heated for 5 min at 94 °C and cooled to 72 °C. Then 2.5 units of *Taq* DNA polymerase (Promega) were added and overlaid immediately with mineral oil. The reaction mixture was cooled to 50 °C and annealing was allowed for 2 min followed by an extension step for 30 min at 72 °C. PCR was conducted for 30 cycles under the following conditions: 1 min denaturation at 94 °C, 1 min annealing at 50 °C, and 2 min extension at 72 °C with a final extension for 10 min at 72 °C.

After purification by electrophoresis on a 2% low melting agarose (Sigma) gel, the single PCR product of the expected size was phenol extracted, digested by *Sma* I and *Kpn* I and inserted into the corresponding sites of pBluescript KS⁺ (Stratagene). A riboprobe of the sequence-correct amplified partial clone was used for re-screening a λ gt11-based cDNA library using the double-strand cDNA pool derived from the same preparation of poly(A)⁺ RNA that had been applied for RT-PCR. The amplified partial DNA fragment was labeled with [α ³²P]dCTP by the random primer technique. Approximately 500,000 primary (unamplified) recombinant phages were screened by in situ plaque hybridization at high stringency. More than 500 positive signals were obtained on both original and duplicate lifts. Twenty were selected randomly and purified by two to three replating cycles. The phage DNA inserts were excised with *Eco*R I, subcloned into pBluescript KS⁺, and

sequenced by dideoxynucleotide chain termination method (Sanger et al., 1977) using α -³⁵S-dATP.

For library construction, a cDNA synthesis kit and *Eco*R I-digested λ gt11 phage DNA from Pharmacia were used. The cDNA was ligated into λ gt11 with *Eco*R I linkers containing an internal *Not* I site and packaged in vitro using Gigapack Gold (Stratagene) packaging extract.

Expression, refolding, and purification of the nonglycosylated copper binding domain

The cDNA encoding the complete precursor of cucumber stellacyanin was modified by PCR to remove the signal peptide and the carboxyl-terminal hydroxyproline-rich domain that is responsible for the posttranslational O-linked glycosylation of the mature protein. The two primers required were designed in such a way that primer 1, CCTGCCCATATGACAGTCGACGGTTCATATCGTCGGC, created a *Nde* I restriction site (underlined) at the 5' end, primer 2, GTAGTTACAGCAACGACGGTTAATT, replaced the Ala¹¹⁰ GCC codon, creating a stop codon (UAA) that alters the N-linked glycosylation site (Asn-Ala-Thr). The resulting 342-bp fragment encodes a truncated 110-amino acid long copper binding domain with a Met residue at position 0 and an Asn residue (109) at the carboxyl terminus. The amplified fragment was ligated into the *Sma* I site of the pBluescript KS⁺ and checked for the integrity of the entire fragment, including the desired modifications.

The insert was subcloned as a *Nde* I (newly generated by PCR)/*Bam*H I (from the multicloning site of pBluescript) fragment into the corresponding sites of pET3a expression plasmid, operating with the T7 gene 10 promoter. The protein was expressed in *Escherichia coli* BL21(DE3), which houses the gene for T7 RNA polymerase under the control of the IPTG-inducible lac UV5 promoter (Studier et al., 1990). Cells were grown at 37 °C in LB media containing 50 μ g/mL ampicillin. IPTG was added to a final concentration of 1 mM at an OD₆₀₀ of 0.8 and expression was performed growing cells at 37 °C for 4 h. Cells were harvested at 4,000 $\times$ g for 15 min at 4 °C.

Stellacyanin expression leads to the aggregation of overproduced protein and formation of insoluble inclusion bodies, which accounts for almost 30% of the total cellular protein. After disruption of the cells by sonication, the inclusion bodies were pelleted by centrifugation at 10,000 $\times$ g for 10 min and washed twice with 5 mM phosphate buffer, pH 7.2. Routinely, an aliquot of the inclusion bodies isolated from 3–5 L of bacterial culture was solubilized in 10–20 mL of a buffer containing 10 mM phosphate, pH 7.2, 6 M urea, 10 mM 2-mercaptoethanol, and 0.5 mM EDTA. Insoluble material was removed by centrifugation at 12,000 $\times$ g for 20 min. Refolding of the protein was performed by 10-fold gradual dilution (2 mL/h) of the clarified solution with 5 mM phosphate buffer, pH 7.2, containing 0.5 mM EDTA. The refolded protein was subjected to anion-exchange chromatography on a DEAE-cellulose column and reconstituted with copper by dialyzing twice against 10 mM acetate buffer, pH 5.5, containing 1 mM CuSO₄. This procedure has enabled us to recover more than 95% of the solubilized protein in its correctly folded form. Purification of the protein was completed by gel-filtration chromatography on Sephadex G-75 sf. The yield of purified protein, which gives a single band of 12 kDa on 15% SDS-PAGE, was almost 100 mg per 1 L of bacterial culture.

Concentration of the refolded apoprotein was calculated using the molar extinction coefficient, $\epsilon = 14,000 \text{ M}^{-1} \text{ cm}^{-1}$, for the

aromatic chromophores (two Trp and two Tyr residues) at 280 nm. This value was estimated from the $\epsilon_{280} = 5,500 \text{ M}^{-1} \text{ cm}^{-1}$ and $1,490 \text{ M}^{-1} \text{ cm}^{-1}$ for a Trp and a Tyr residues, respectively, according to (Pace et al., 1995). The value of the $\epsilon = 4,100 \text{ M}^{-1} \text{ cm}^{-1}$ at 600nm for the copper chromophore was estimated from the ratio $A_{280}/A_{600} = 3.4$ for completely reconstructed and fully oxidized holoprotein.

Spectroscopic measurements

Electronic absorption spectra were recorded on a Cary 3 spectrophotometer at room temperature. EPR spectra were measured using a Bruker ER200d-SRC spectrometer operating with an ER043 MRD X-band microwave bridge. ESEEM experiments were performed in the Albert Einstein College of Medicine at Yeshiva University. For the detailed description of the pulsed EPR spectrometer, see McCracken et al. (1987).

RR spectra were obtained using lines from the Coherent Model K-2 Kr^+ ion laser (568.2 and 647.1 nm) by collecting front-scattering directly from the surface of a frozen protein solution held in vacuo on a liquid N_2 -cooled cold finger (Johnson & Czernuszewicz, 1983). Conventional scanning Raman instrumentation equipped with a Spex 1403 double monochromator and a cooled Hamamatsu 928 photomultiplier tube were used to record the spectra under the control of a Spex DM3000 microcomputer system (Czernuszewicz, 1993). The spectral slit width was $3\text{--}4 \text{ cm}^{-1}$. Typical laser power was 150–200 mW, and average of 3–6 scans at 1-s/point and $0.2\text{--}1 \text{ cm}^{-1}/\text{s}$ increments were taken to improve signal to noise ratios. Spectra were calibrated using the known vibrational frequencies of CCl_4 and ice.

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