THE AMINO ACID SEQUENCES OF TWO PUTATIVE COPPER-SITE PEPTIDES FROM THE "BLUE" COPPER PROTEIN, STELLACYANIN*

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SUMMARY: The sequences of a thirteen residue glycopeptide containing the sole cysteine residue of stellacyanin and a pentapeptide containing histidine were determined by the dansyl-Edman method. There is relatively little homology between stellacyanin and plastocyanin or azurin in the cys region and the adjacent histidine proposed as a ligand to Cu in plastocyanin and azurin is absent in stellacyanin. There are homologies between the Cu subunit of cytochrome oxidase and these "blue" copper proteins, in this region. The his peptide shows homologies with the sequence around an invariant his in plastocyanin.

INTRODUCTION: On spectroscopic and chemical grounds, there is general agreement that the interaction of a cysteine residue with a copper atom is the basis for the intense blue colour of copper proteins such as plastocyanin, azurin, stellacyanin, laccase and ceruloplasmin. The amino-acid sequence of azurins and plastocyanins from several sources, summarised in (1), show that these proteins are homologous in the regions around their sole cysteine (residue 89 in plastocyanin) and around a histidine (residue 39 in plastocyanin). A model for the copper site comprising these two residues, plus a second invariant histidine (residue 92 in plastocyanin) near the cysteine and a nitrogen from the peptide backbone has been postulated (2). However, a tertiary structure for plastocyanin proposed from the sequence (3) places the second histidine away from the site. Additionally, methionine has been proposed as a ligand (4) but stellacyanin has no methionine residues (5). Sequence data for other blue copper proteins may help

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clarify models for the site, and to this end we determined the sequences in stellacyanin around the sole cysteine residue and around a histidine homologous to residue 39 of plastocyanin.

MATERIALS AND METHODS: Rhus vernicifera stellacyanin was prepared by the method of Osaki and Walaas (6). Porcine pepsin and TPCK-trypsin were obtained from Worthington Biochemical Corporation. Pencillocarboxypeptidase-S1 was kindly given by Dr. T. Hofmann, University of Toronto. Amino acid analyses were performed on either a Durrum D-500 or Beckman 120-C analyzer (7).

^{14}C-carboxymethylation of stellacyanin: Apostellacyanin was prepared by the method of Morpurgo et al. (8) and converted to the CM-cysteine derivative by incubating under N_2 with [^{14}C]-iodoacetic acid (New England Nuclear) in 6M guanidinium-HCl and 10 mM EDTA in 0.1M Tris-HCl buffer, pH 8.5, for one hour. After extensive dialysis against water, the product was freeze-dried.

Preparation of the tryptic peptides: About 1.5 μmole (30 mg) of ^{14}C-CM-stellacyanin was dissolved in 5 ml of 0.05M formic acid pH 2.0 and digested at 37° for 24 hrs with 1 mg of pepsin. The peptide mixture was taken to dryness, dissolved in 5% formic acid and applied to a Sephadex G-25 column (1.5 x 100 cm) in 5% formic acid. Material in a peak at the void volume containing nearly all the radioactivity was recovered by freeze-drying, dissolved in 0.1M N-ethyl morpholine buffer pH 8.0 and digested at 37° for 24 hr with 1 mg of TPCK-trypsin. The tryptic peptides were purified by gel-filtration on Sephadex G-25 and high-voltage electrophoresis on Whatman 3 MM paper at pH 6.5 and thin-layer chromatography. The cys peptide eluted at the void volume followed by the his peptide.

Sequence determination: Using 150 nmoles of cys peptide and 40 nmoles of his peptide the amino acid sequences were determined by the dansyl-Edman method (9), except that butyl chloride was used instead of butyl acetate. Dansyl amino acids were identified by thin-layer chromatography on polyamide sheets (10). Amides were identified by thin-layer chromatography of the phenylthiohydantoins. The dansyl identifications were all confirmed by amino-acid analyses of the products from hydrolysis of the anilinothiazolines in 5.7N HCl containing 0.1% $SnCl_2$ at 150° for 4 hr in sealed tubes (11). The C-terminal sequences were determined by the use of penicillocarboxypeptidase-S1 in 0.2M pyridine-formate buffer, pH 4.2 (12). A column of Dowex 50x2 (H^+), 0.3 ml volume, was used to isolate the PTH products of cycles 6 and 7 of the cys peptide for carbohydrate assignment. The PTH-products eluted with 2 ml of water and the adsorbed peptide with 1.5 ml of M ammonium formate pH 6.5.

Pronase digestion of the cys peptide: 120 nmoles of the cysteine peptide were digested at 37° for 24 hr with 0.15 mg of pronase, in pH 8.0 tris-HCl buffer, 1 mM $CaCl_2$. Gel-filtration of the digest on Sephadex G-25 gave two radioactive peaks, one at the void volume, which was separated by paper electrophoresis into one radioactive (pp1) and one non-radioactive component (pp2), and one near the bed volume (pp3) which was separated by paper electrophoresis from free amino acids.

TABLE 1: Peptide Compositions

	Histidine peptide	Cysteine peptide	Pronase peptides ppl	pp2	pp3
asp	2.0	3.0	1.8	1.8	
thr		2.0	1.8	1.7	
ser		2.0			1.0
glu		1.0			1.0
pro		1.0	1.0	1.0	
CM-cys		0.9		0.6	0.9
ala		1.0			
ile		1.0			
tyr		0.8			
glucosamine		2.7	2.9	2.8	
val	1.0				
phe	1.0				
his	1.0				
N-terminal residue	phe	asn	CHO-asn	CM-cys	gln
Mobility[1]	0.15	0.28	0.23	0.33	0.72

[1]with respect to aspartic acid at pH 6.5

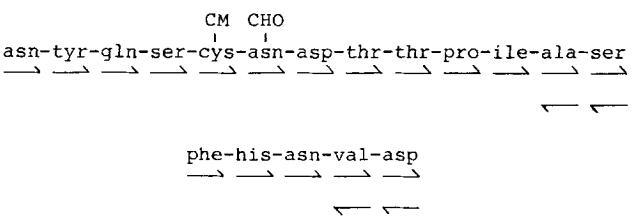

FIGURE 1: Sequences of stellacyanin cysteine and histidine peptides. Residues determined by dansyl-Edman method, ——→ ; by penicillocarboxypeptidase, ←—— .

RESULTS: The protein contained 0.9 moles of thiol per mole by the 5,5'-dithio-bis-(2-nitrobenzoic acid) method (13) and incorporated 0.8 mole of radiolabel per mole. Radioactivity was associated with a single component throughout the purification. The cys peptide was purified with an overall yield of 35% and the his peptide 15% and they had the compositions shown in Table 1. The sequences and their determination are summarised in Fig. 1. The cys peptide had attached

carbohydrate, the amino acid analyser data indicating at least three residues of glucosamine, presumably N-acetylated in the native peptide (the conditions for amino acid analysis not being optimal for recovery of glucosamine, three is a minimum estimate of the glucosamine). This type of glycopeptide is normally attached to aspargine residues in sequences of the type asn-X-ser or asn-X-thr. The assignment of the point of attachment to residue 6 rather than 7 (or even 1) was verified by a) the composition of the glycopeptides produced by pronase digestion b) the nature of the PTH-derivative from cycle 6 - a glucosamine - containing product which was separable from the remaining peptide by ion-exchange chromatography, not PTH-asp nor PTH-asn, and c) identification of the products from cycles 7 (and 1) as PTH-asp not PTH-asn and absence of glucosamine in these products.

Identification of residue 5 as the labelled cysteine residue was confirmed by the presence of more than 90% of the initial radioactivity of the peptide in the PTH derivative.

DISCUSSION: It has recently been shown (data of C. Bergman and L. Strid in (1)) that the N-terminal sequence of stellacyanin is quite similar to that of the plastocyanins and umecyanin. The his peptide also shows homology with the invariant histidine region but the sequence around the sole cysteine residue of stellacyanin shows much less apparent homology when aligned with representative plastocyanin and azurin sequences (Fig. 2). Thus, though the cys regions are almost certainly key parts of the plastocyanins and stellacyanin, they are less homologous than the N-terminal regions, which suggests the latter may be functionally important. The N-terminal regions of the azurins and the plastocyanins while not directly homologous, do have hydrophobic residues in similar positions (1).

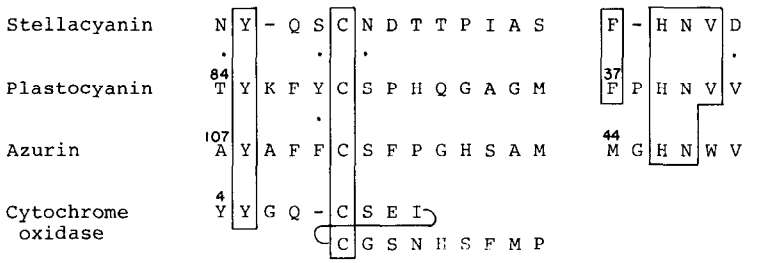

FIGURE 2: Alignment of stellacyanin peptide sequences with portions of plastocyanin from the elder, Sambucus niger (23), azurin from Pseudomonas denitrificans (R.P. Ambler, quoted in (1)) and a 36 residue peptide from the copper subunit of bovine cardiac cytochrome oxidase (22). Invariant residues are in boxes and dots indicate some residues relatable by single base changes. The one-letter code is described in ref. 24.

The tyr-2 of the stellacyanin cys peptide appears homologous to tyr-85 of plastocyanin and tyr-109 of azurin (using the numbering of ref. 1). There is NMR (14,15) and fluorescence (16) evidence for a tyrosine being sensitive to the status of the copper site in azurin and plastocyanin. No tyrosine vibrations were seen in their resonance Raman spectra (17), however, so direct coupling to the copper atom is unproven. The lack of other aromatic residues between tyr and cys in the stellacyanin peptide is striking.

In its C-terminal region, there are obvious differences between the cys peptide and the other blue proteins, particularly in the absence of histidine. The histidine residue in this region in plastocyanin and azurin has been proposed as a ligand to copper in a model of the copper site. Either stellacyanin has only one histidine in its copper site as recent EPR evidence suggests (18) or a histidine from another region of the molecule replaces the one near cys (stellacyanin has four histidines (5)). NMR evidence favours the participation of two histidines in the copper sites (14,15) of

plastocyanin and azurin though an attempt to deduce the three-dimensional structure of plastocyanin from its sequence placed his 92 away from the cysteine (3). The sequence of the stellacyanin his peptide shows considerable homology with the his 39 region suggesting this region is highly conserved in all three proteins. We propose therefore that the resonance Raman (17) and other spectral (2), redox potential (19) and kinetic properties (20) of stellacyanin differ from those of plastocyanin and azurin because part of its copper site is constructed differently rather than there being a common site in the three proteins uniquely distorted in stellacyanin.

It is surprising to find the residue adjacent to the important cysteine substituted with a bulky carbohydrate group. This provides additional support for this region not being of importance in the construction of the copper site in stellacyanin. The sequence asn-asp-thr-thr is noteworthy since it also occurs in a glycopeptide from another blue copper protein, ceruloplasmin (21) though not in that case adjacent to a cysteine residue.

Finally, it is interesting to compare the stellacyanin, plastocyanin and azurin sequences with a peptide containing two cysteines from a copper-binding subunit of cytochrome oxidase (22). If one postulates a small internal duplication the alignment shown in Fig. 2 can be made. In the NH_2-terminal region it resembles stellacyanin. A tyrosine corresponding to tyr-2 of the stellacyanin peptide is present and it lacks the aromatics found in plastocyanin. A glutamine residue can also be aligned. Following the first cysteine is a serine residue in homology to plastocyanin and azurin, and following the second cysteine, a region containing histidine and methionine similarly placed to those in plastocyanin and azurin. The EPR spectrum of the copper in cytochrome oxidase (25) is partly

similar to that of the blue copper proteins, and the 830 nm feature (26) of the visible spectrum (ε, 1400) resembles that of the blue copper proteins. The cytochrome f-plastocyanin relationship in the photosynthetic system may have parallels in the subunits of the mitochondrial electron-transport protein, cytochrome oxidase.

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