THE AMINO ACID SEQUENCE OF STELLACYANIN* FROM THE LACQUER TREE

Claes Bergman, Eva-Karin Gandvik, Per Olof Nyman and Lars Strid
Department of Biochemistry, University of Göteborg and Chalmers Institute of Technology, Fack, S-402 20 Göteborg 5, Sweden

Received June 23,1977

SUMMARY: The complete amino acid sequence of stellacyanin, a blue copper-containing glycoprotein, is presented. The protein has been enzymatically cleaved by trypsin, chymotrypsin, and by the protease from the *Staphylococcus aureus* strain U8. Stellacyanin consists of a single polypeptide chain of 107 amino acid residues. The protein contains one SH-group and one disulfide bond. The carbohydrate moieties, which are not yet characterized, are attached to the protein at three different positions, all having the characteristic sequence Asn-X-Thr.

INTRODUCTION: Copper-containing proteins are widely distributed among living organisms where some of them participate in the fundamental biochemical reaction of reducing molecular oxygen (cytochrome *c* oxidase). Copper enzymes that have been frequently studied in recent years are laccases of fungal origin (*Polyporus versicolor*) (1) as well as those from the Japanese lacquer tree (*Rhus vernicifera*) (2). Laccase is considered to be a good model for elucidating the mechanism of molecular oxygen reduction. This enzyme contains four copper ions which have been shown to be involved in the catalytic reaction. The groups in the protein liganding the metal ions have, however, not yet been identified.

The present investigation deals with the amino acid sequence of a low-molecular-weight copper protein known as stellacyanin. This protein is obtained from *Rhus vernicifera* (2), i.e. the same source as for one of the laccases. Stellacyanin contains a single copper ion which has been spectroscopically characterized as being bound in a similar manner as one of the

*Issued as N.R.C.C. No 15573

ISSN 0006-291X

copper ions in laccase, namely the so-called Type 1 copper (3). Low-molecular-weight copper proteins from other plant sources have previously been characterized with respect to primary structure, for example azurins from bacteria (4) and plastocyanins from higher plants and algae (5), the latter protein being believed to participate in photosynthesis.

Azurins, plastocyanins, and stellacyanin have certain features in common: they possess a single SH-group, their molecular weights are 15,000-20,000, they contain a single copper ion, and the coordination around the metal appears to be very similar judging by spectroscopic studies. It has been suggested that the copper ion is liganded to the protein by the SH-group and histidine side chains (6). It would therefore be of interest to compare sequences in the proximity of these residues in different copper proteins. This paper presents a proposal for the complete amino acid sequence of stellacyanin. A preliminary report of the work has been given earlier (7). Furthermore, the sequences of two peptides, one of them containing the SH-group of the protein, have recently been published from another laboratory (8).

MATERIALS AND METHODS: Stellacyanin from *Rhus vernicifera* was prepared according to Reinhammar (2). Carboxypeptidases A and B and N-tosyl-phenylalanylchloromethyl ketone-treated trypsin were obtained from Worthington Biochemical Corp. The protease from *Staphylococcus aureus* strain U 8 was purchased from Miles Corp., thermolysin was obtained from Kasei K.K., Osaka, Japan, and chymotrypsin from Miles-Seravac (England). Amino acid analysis were performed according to Moore and Stein (9) on a Beckman 120-B analyser.

Carboxymethylation of stellacyanin: The protein was first dissolved in 6 M guanidinium-chloride with 0.1 M Tris-HCl, pH 8.2, containing 25 mM EDTA, and then heated on a boiling water-bath for complete denaturation before reducing with Cleland's reagent and subsequent conversion to the S-carboxymethyl derivative with iodoacetic acid (10).

Labelling of the free SH-group: Stellacyanin was carboxymethylated with [^{14}C]-iodoacetic acid (Amersham, England) using the

same conditions as above but without reduction of the S-S bond (11). The same reaction was also performed on apostellacyanin prepared according to Morpurgo (12). After tryptic digestion, the peptide mixture was subjected to fingerprinting on cellulose thin-layer plates (Polygram 300) (13) and the radioactive spot was detected using autoradiography (14).

Enzymic digestion: Enzymic digestion with trypsin, chymotrypsin, and thermolysin were carried out by methods commonly used in primary structure investigations (15). Digestion with the protease from *Staphylococcus aureus* was achieved in 0.1 M sodium-phosphate buffer, pH 7.8, 37°C, 18 h, ratio 1:30 (w/w) (16). Peptides from the proteolytic digests were purified by gel filtrations on Sephadex, preparative high-voltage paper electrophoresis (17), and paper chromatography using pyridine, n-butanol, acetic acid, and water in the ratio 50:75:15:60 (v/v) (18) as the solvent.

Sequence determination: The sequence of the amino-terminal region of the protein was determined by means of the direct phenylisothiocyanate method as described by Iwanaga et al. (19). The phenylthiohydantoin derivatives were identified by thin-layer chromatography (20). The carboxy-terminal amino acids were determined using carboxypeptidases A and B (21). Sequence studies on the tryptic peptides were done using the dansyl-Edman procedure (22). Chromatography of the dansyl amino acid derivatives was performed on polyamide plates (23). Mass spectrometry for sequence determinations of peptides was used to a limited extent (24).

RESULTS AND DISCUSSION: The results are summarized in Fig. 1. The protein contains 107 amino acid residues in a single polypeptide chain and has a composition of Trp_3 His_4 Lys_{10} Arg_4 Cys_3 Asp_{18} Thr_9 Ser_5 Glu_4 Pro_3 Gly_8 Ala_3 Val_{13} Ile_5 Leu_3 Tyr_7 Phe_5. Stellacyanin has previously been reported to lack Val (26), a statement contradicted by the results obtained here. The same authors give a half-cysteine content of six residues compared to our finding of three residues. The molecular weight of the glycoprotein has been reported as 20,000 (25). The amino acid composition corresponds to a molecular weight for the protein moiety of 11,300, i.e. 60% of the total weight.

The carbohydrate-containing peptide 55-68 was isolated in good yield from the tryptic digest but cannot be a result of a legitimate tryptic cleavage. When performing the separation of the tryptic digest using gel filtration in 1 M acetic acid,

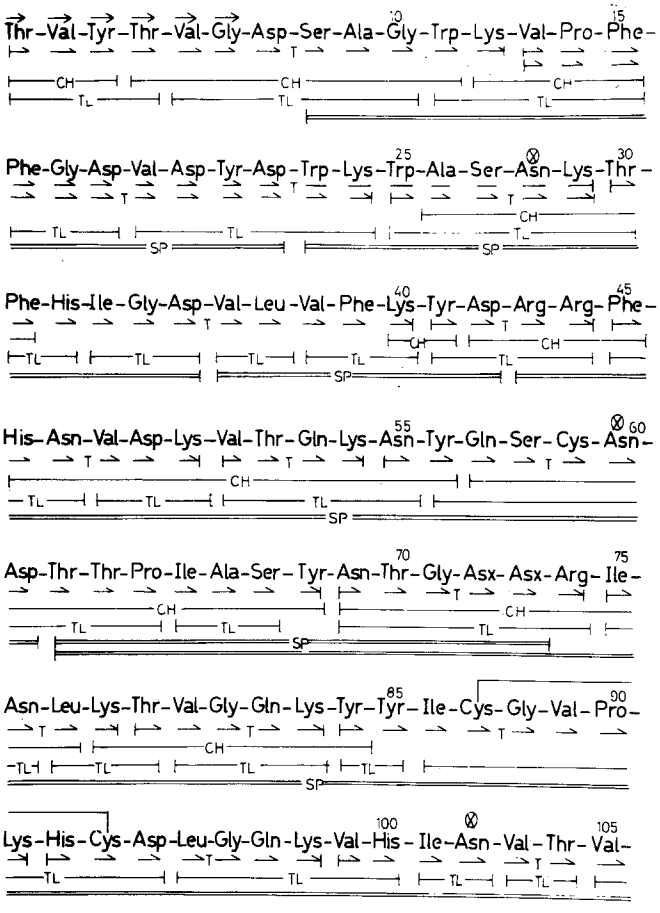

Fig. 1. Amino acid sequence proposed for stellacyanin from *Rhus vernicifera*.
(T) tryptic, (CH) chymotryptic, (TL) thermolytic, and (SP) *Staphylococcus aureus* protease peptides.
⊗ attachment of carbohydrates to residues 28, 60 and 102.
→ dansyl-Edman, ⟶ direct Edman
S--S bridge between residues 87 and 93
Free SH-group at position 59

The sequence is based principally on dansyl-Edman degradation of tryptic peptides as indicated in the figure. Additional evidence derived by sequencial degradations of peptides produced by other fragmentation methods has been obtained but is not included in the figure.

peptide 30-40 was obtained as a precipitate in the test tubes used for collecting the fractions. The hydrophobic character of this tryptic peptide would perhaps explain this behavior.

We want to stress the good results in peptide cleavage achieved with the protease from *Staphylococcus aureus*. Since stellacyanin lacks glutamyl residues, hydrolysis was encountered exclusively at aspartyl peptide bonds in accordance with the specificity for the protease reported in the literature (16, 27).

Mass spectrometry was a helpful tool in establishing the sequences of some of the tryptic peptides, especially the peptides 51-54, 75-58, which, due to their similar amino acid compositions, did not separate upon electrophoresis. Since mass spectrometry technique does not differentiate between Leu and Ile, their positions in the two peptides were established by the conventional dansyl-Edman procedure which, when applied to the peptide mixture, resulted in two answers at each step.

Carbohydrates were found to be attached at three different sites in the sequence. All have the sequence Asn-X-Thr, the carbohydrate moiety being attached to the Asn. This is one of the characteristic sequences involved in the binding of carbohydrates as prosthetic groups in proteins (28). We have confirmed that the carbohydrate is bound to the Asn by hydrolysis and amino acid analysis of the Edman derivative as well as by the determination of the composition of the residual peptide. For detection of alkali-labile bonds which would suggest that the carbohydrate group is attached to the Thr-residue, the carbohydrate peptides were subjected to alkaline treatment (29). No destruction of Thr was observed which is in consistency with Asn being the only amino acid residue involved in binding of the carbohydrate group.

When performing carboxymethylation of stellacyanin in guanidinium-chloride with radioactive iodoacetic acid followed by

trypsination and separation of the tryptic digest on cellulose thin-layer plates, essentially one tryptic peptide became radioactively labelled as judged by the autoradiography. The peptide 55-68 was shown to contain S-carboxymethyl cysteine which we concluded was derived from a single SH-group present in stellacyanin. No difference was observed when the reaction was carried out with apostellacyanin. In the native stellacyanin, however, the SH-group could not be modified by carboxymethylation suggesting that the group is buried in the protein or blocked. The closest neighbour to the Cys is an Asn carrying a bulky carbohydrate group. The presence of a carbohydrate side chain close to the Cys would suggest that the SH-group is situated near the surface of the protein molecule. The observation that native stellacyanin cannot be modified with iodoacetic acid is, therefore, somewhat surprising. One explanation might be that the cysteine residue participates in metal binding, as proposed by several authors (6, 30).

Very little homology in the vicinity of the SH-group is observed upon comparison of the sequence of stellacyanin with those of other small blue copper-containing proteins (31). The positions of the histidine residues are also of special interest for the metal coordination. We would like to stress the presence of the invariant His-92 in plastocyanin and stellacyanin and His-46 in azurin and stellacyanin. The sequence adjacent to His-46 in stellacyanin shows some similarities with the sequence adjacent to His-39 in plastocyanins as shown in Fig. 2 (8).

The histidine residues 92 and 39 in plastocyanin (the same numbering as in ref. 30 is used) have been proposed to be two of the ligands to the copper ion (6). It has recently been suggested that methionine is one of the ligands to the copper

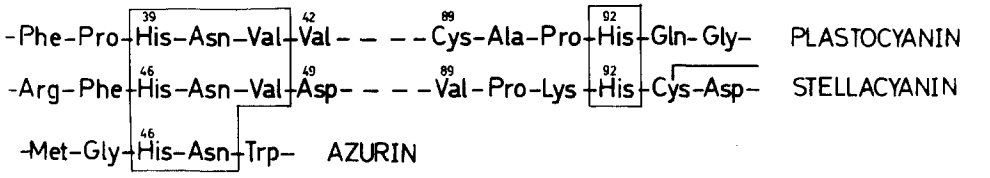

Fig. 2. Comparison between amino acid sequences around histidine residues in stellacyanin, azurin, and plastocyanin. The numbering used is the same as in reference 30.

ion in azurin and plastocyanin (32), but this cannot be the case in stellacyanin, since the protein is devoid of methionine.

ACKNOWLEDGEMENTS: This investigation has been aided by grants from the Swedish Medical and Natural Science Research Councils.

REFERENCES

1. Fåhraeus, G. and Reinhammar, B. (1967) Acta Chem. Scand. 21, 2367-2378
2. Reinhammar, B. (1970) Biochim. Biophys. Acta 205, 35-47
3. Vänngård, T., in Biological Applications of Electron Spin Resonance (Swartz, J.R., Bolton, J.R., and Borg, D.C., eds.) Wiley, New York, (1972), p. 411-447
4. Ambler, R.P. (1963) Biochem. J. 89, 341-349
5. Katoh, S. (1960) Nature 186, 533-534
6. Solomon, E.I., Hare, J.W., and Gray, H.B. (1976) Proc. Natl. Acad. Sci. USA 73, 1389-1393
7. Bergman, C., Nyman, P.O., and Strid, L. (1976) Abstracts Tenth International Congress of Biochemistry in Hamburg, July 25 to 31, p. 201
8. Wang, T.T., and Young, M.N. (1977) Biochem. Biophys. Res. Comm. 74, 119-125
9. Moore, S., and Stein, W.H., in Methods in Enzymology Vol 6,(Colowick, S.P., and Kaplan, N.O., eds.), Academic Press, New York and London, (1963), p. 819-831
10. Crestfield, A.M., Moore, S., and Stein, S. (1963) J. Biol. Chem. 238, 622-627
11. Jörnvall, H., Petterson, U., and Philipson, L. (1974) Eur. J. Biochem. 48, 179-192
12. Morpurgo, L., Finazzi-Agro, A., Rotilio, G., and Mondovì, B. (1972) Biochim. Biophys. Acta 271, 292-299
13. Cheng, R. (1976) Hoppe-Seyler's Z. Physiol. Chem. 357, 873-886
14. Smith, I. (1960) Chromatographic and Electrophoretic Techniques Vol 1, p. 491-497, 2:nd ed., William Heinemann Medical Books, Ltd., London, Interscience Publishers Inc., USA

15. Smyth, D.G., in Methods in Enzymology, Vol 11 (Hirs, C.H.W., ed.) Academic Press, New York and London, (1967) 214-231

16. Drapeau, G. (1972) Proc. Natl. Acad. Sci. USA 69, 3506-3509

17. Smillie, L.B., and Hartley, B.S. (1966) Biochem. J. 101, 232-241

18. Waley, S.G., and Watson, J. (1953) Biochem. J. 55, 328-337

19. Iwanaga, S., Wallén, P., Gröndahl, N.J., Henschen, A., and Blombäck, B. (1969) Eur. J. Biochem. 8, 189-199

20. Jeppson, J.O., and Sjöquist, J. (1967) Anal. Biochem. 18, 264-269
Brenner, M., Niederweiser, A., and Pataki, G. in Dünnschichtschromatographie (Stahl, E., ed.), Springer-Verlag, West Berlin, (1962) p. 443-445

21. Ambler, R.P., in Methods in Enzymology Vol 25, (Hirs, C.H.W., and Timasheff, S.N., ed.), Academic Press, New York and London, (1972), p. 143-145

22. Gray, W.R., in Methods in Enzymology Vol 11, (Hirs, C.H.W., ed.), Academic Press, New York and London, (1967), p. 469-475

23. Gray, W.R., in Methods in Enzymology Vol 25, (1972), p. 121-138

24. Morris, H.R., Williams, D.H., and Ambler, R.P. (1971) Biochem. J. 125, 189-201

25. Omura, T. (1961) J. Biochem. (Tokyo) 50, 394-399

26. Peisach, J., Levine, W.G., and Blumberg, W.E. (1967) J. Biol. Chem. 242, 2847-2858

27. Austen, B.M., and Smith, E.L. (1976) Biochem. Biophys. Res. Comm. 72, 411-417

28. Neuberger, A., Gottschalk, A., Marsall, R.D., and Spiro, R.G., in Glycoproteins (Gottschalk, A., ed.), 2:nd ed., Elsevier Publishing Comp., Amsterdam, (1972), p. 464-470

29. Roberts, G.P. (1974) Eur. J. Biochem. 50, 265-280

30. Siiman, O., Young, N.M., and Carey, P.R. (1976) J. Amer. Chem. Soc. 98, 744-748
Solomon, E.I., Wang, R.H., McMillin, D.R., and Gray, H.B. (1976) Biochem. Biophys. Res. Comm. 69, 1039-1042

31. Rydén, L., and Lundgren, J.-O. (1976) Nature 261, 344-346

32. McLendon, G., and Martell, E. (1977) J. inorg. nucl. Chem. 39, 191-193