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STRUCTURAL STUDIES AROUND CYSTEINE AND CYSTINE RESIDUES IN THE "BLUE" OXIDASE FUNGAL LACCASE B. SIMILARITY IN AMINO ACID SEQUENCE WITH CERULOPLASMIN

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SUMMARY. Fungal laccase B from *Polyporus versicolor* is a "blue" oxidase containing four copper ions. It consists of a single polypeptide chain of about 545 amino acid residues. The enzyme has been hydrolyzed with pepsin, pronase and thermolysin, and peptides containing the single sulfhydryl group and one of the disulfide bridges have been isolated and characterized. The results show that there is some similarity in amino acid sequence on both sides of the disulfide bridge indicating an internal homology in the laccase molecule. The structure around the single cysteine residue (Leu-His-Cys-His-Ile-Asx-Phe) differs considerably from the cysteine region in low-molecular-weight "blue" proteins like plastocyanin and azurin which contain a single copper ion. However, it shows a pronounced similarity with the sequence around a cysteine residue in human ceruloplasmin suggesting that this structure has an important role in the multi-copper oxidases that is absent or different in the small "blue" proteins. We propose that this role is to constitute a bridge between different copper ions in the molecule and mediate the specific interaction between those which is a crucial feature in the catalytic action of the multi-copper oxidases.

INTRODUCTION. The reduction of molecular oxygen to water is a fundamental biochemical reaction the mechanism of which is still insufficiently known. Enzymes that can catalyze this reaction are cytochrome *c* oxidase (1,2) and the so-called "blue" oxidases (3-5). These enzymes contain four or more redox centers that are metal ions bound in unique coordinations. These redox centers partake in the catalytic process by interacting with each other in a complicated manner undergoing valency changes. To fully understand the mechanism of the catalyzed reaction would require the determination of the structure of the enzymes. Of particular interest would be to know the structures of the metal-binding regions and how these interact.

This investigation is concerned with one of the "blue" oxidases, namely laccase. It is the most studied of the "blue" oxidases with respect to kinetic and spectroscopic properties (3-5). We have used form B of the fungal laccase from *Polyporus versicolor*. It consists of a single polypeptide chain of around 545 amino acid residues (6) containing two disulfide bridges and a single

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sulfhydryl group (7). Fungal laccase contains four copper ions per molecule acting as redox centers in the enzymic reaction. The enzyme catalyzes the oxidation of p-diphenols and related substances under reduction of molecular oxygen to water. According to physical measurements the copper ions are of three distinct types. Of particular interest is the so-called type 1 copper ion which has the unique coordination providing the intense blue color.

Of considerable interest for the understanding of the properties of the "blue" copper site in the multi-copper oxidases is a group of smaller "blue" proteins comprising azurins from bacteria, plastocyanins from photosynthetic cells, and stellacyanin and umecyanin from higher plants. They have molecular weights of 15000-20000 and contain only a single copper ion which has type 1 character. X-ray diffraction studies have identified the metal ligands of the type 1 copper site in plastocyanin (8) and azurin (9). It was found that the single sulfhydryl group in these proteins partakes in the metal binding which confirmed previous suggestions from other approaches (10,11).

Amino acid sequence determinations have been carried out on a great number of the small "blue" proteins and have been summarized in a recent discussion on homology among "blue" proteins (12). For the multi-copper oxidases, on the other hand, only a limited amount of sequence information is available. For ceruloplasmin a histidine-rich carboxyl-terminal fragment of 159 amino acid residues has been sequenced (13). The fragment contains a cysteine residue (Cys) and on the basis of sequence homology with the small "blue" proteins this Cys has been proposed to partake in the binding of one of the type 1 copper ions in ceruloplasmin (14). Information about another Cys region in ceruloplasmin has been provided by Rydén and Lundgren (12).

We have studied the amino acid sequences around the single Cys and the disulfide bridges in fungal laccase B. The Cys region in laccase B was found to be very similar to that of the ceruloplasmin fragment studied by Kingston et al. (13,14). We suggest that this Cys region in the multi-copper oxidase has an additional function compared to that in the small "blue" proteins. This function could be to form a link from the type 1 copper to one of the other copper ions present in the molecule, presumably type 2.

MATERIALS AND METHODS. Fungal laccase B from *Polyporus versicolor* was prepared according to Fåhreaus and Reinhammar (15).

Digestion of heat-denatured laccase with thermolysin (Kasei K.K., Osaka, Japan) was performed at low pH in 0.15 M citrate-phosphate, pH 6.5, containing 2 mM $CaCl_2$ to minimize disulfide interchange (16). Digestions with pepsin (Worthington Biochemical Corp.) or Pronase B (Calbiochem) were carried out as generally recommended (17).

Peptides were separated by high-voltage paper electrophoresis (18). Peptides containing cystine or the single Cys of fungal laccase B were isolated using the disulfide diagonal method of Brown and Hartley (19). One of the

peptides containing the Cys (P 3 in Fig. 1B) was isolated from a peptic digest of a laccase sample where the sulfhydryl group had been reacted with iodo [2-^{14}C] acetic acid (see below). The radioactive peptide was purified by paper electrophoresis at pH 6.5 followed by electrophoresis at pH 2 and localized by autoradiography (20).

Amino acid analyses were performed with a Beckman 120-B or a Unichrom analyzer (21).

Sequential degradations of peptides were carried out by the DNS-Edman procedures (29). The DNS derivatives were chromatographed on polyamide plates (23). DNS cysteic acid was identified by electrophoresis (23) using a Millipore Phoro Slide system.

Labelling of the cysteine residue in laccase B. Iodo [2-^{14}C] acetic acid (Radiochemical Centre, Amersham, England) was dissolved in an equivalent amount of NaOH. An aliquot of 0.33 ml (16.5 µmol) was added to 6.6 ml of 7.5 M guanidine hydrochloride in 0.125 M Tris-HCl, pH 8.2, containing also 312 mM EDTA. Laccase B (1.65 µmol) in 1.65 ml of water was rapidly added and allowed to react in the dark under nitrogen for 1 h at room temperature. Glacial acetic acid (8.3 ml) was then added and the sample was dialyzed extensively in the cold room against 1 M acetic acid and water and lyophilized. The [^{14}C]-content of the protein was estimated using a Packard Model 3255 liquid scintillation counter and carboxymethylcysteine was determined by amino acid analysis. Both methods gave a Cys modification of about 50%.

RESULTS AND DISCUSSION. Fungal laccase B from *Polyporus versicolor* consists of a polypeptide chain of about 545 amino acid residues (6) with two internal disulfide bridges and one sulfhydryl group (7). We have determined the immediate structure around the sulfhydryl group and one of the disulfide bridges. The results are presented in Fig. 1.

Inspection of the disulfide peptide in Fig. 1A shows that there is a certain resemblance in structure on the two sides of the disulfide bridge. Both sides contain the sequence -Cys-Pro-Ile- and in addition there is an identity in the location of a Gly. This result would suggest that an internal homoloy exists in the laccase molecule. Recently, evidence for internal similarities

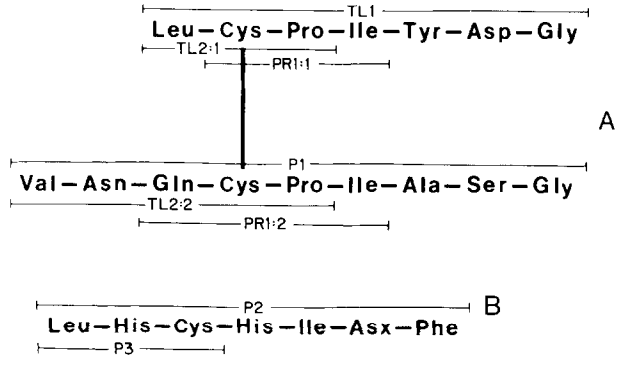

Fig. 1. Amino acid sequences around cysteine and cystine residues in fungal laccase B. The data are based upon peptide fragments derived from digestions of the laccase molecule with pepsin (P), thermolysin (TL), and pronase (PR).

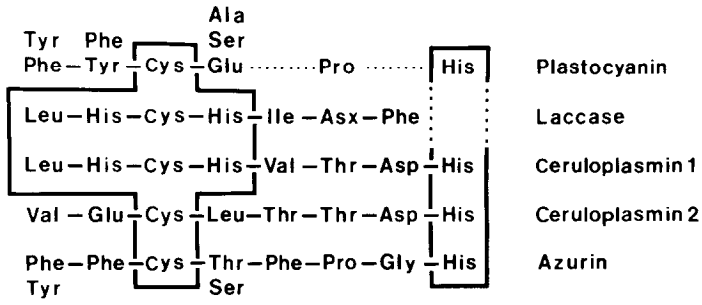

Fig. 2. Amino acid sequence around the single cysteine residue in fungal laccase B compared to previously reported sequences in ceruloplasmin (12,13), plastocyanin, and azurin. The data for the two last proteins are from Kingston et al. (14).

in sequence has also been obtained for ceruloplasmin (12). Together, these findings would suggest that internal homology may be a general feature in the structure of multi-copper oxidases.

The structure around the sulfhydryl group in laccase B is shown in Fig. 1B. The elucidation of the sequence was made using a heptapeptide and tripeptide, both derived after digestion with pepsin.

In Fig. 2 the amino acid sequence around the Cys in fungal laccase B is compared to Cys-containing sequences in other "blue" copper proteins like ceruloplasmin, plastocyanin and azurin. In all these sequences the Cys is probably a ligand to type 1 copper as will be discussed below.

X-ray diffraction studies carried out on crystallized poplar plastocyanin and azurin from Pseudomonas (7,8) have shown that the single Cys in these proteins is involved in the binding of the type 1 copper. The other ligands are a His and a Met located not far from the Cys and another His farther away in the sequence. The two Cys sequences from ceruloplasmin given in Fig. 2 (ceruloplasmin 1 and 2) are parts of longer sequences that have recently been determined (12,13). These sequences show homology with plastocyanin and azurin with respect to metal ligands (Cys, His, and Met) strongly suggesting that they both represent type 1 copper binding sites (12,13). Fungal laccase B contains just a single Cys and it appears likely that this residue should partake in the type 1 copper site present in laccase.

The data in Fig. 2 show that there is a striking similarity in the Cys sequence of fungal laccase B and one of those of human ceruloplasmin (ceruloplasmin 1). There is an identity of four residues (Leu-His-Cys-His) and the position in the sequence coming next has a conservative interchange (Val and Ile). Such a resemblance in sequence between two proteins from widely different sources would suggest that this particular region has an important functional role which is identical or similar in the two proteins. An interesting feature

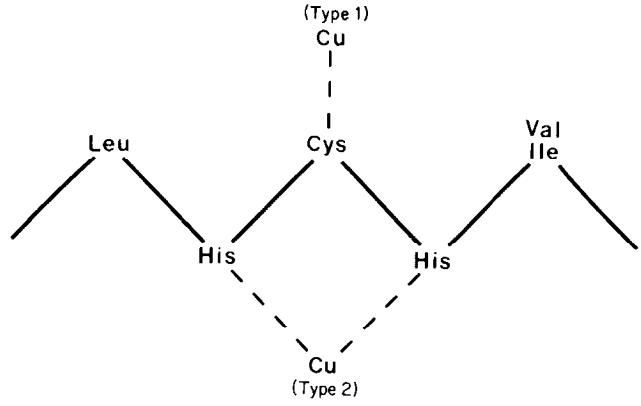

Fig. 3. A possible mode of interaction between copper ions proposed for fungal laccase B and human ceruloplasmin.

with respect to function is the two conserved histidine residues located adjacent to the Cys. They may have a crucial role, not present and asked for in the other type 1 binding structures shown in Fig. 2, and we propose that this role is to bind a second copper ion (see Fig. 3). Laccase and ceruloplasmin both contain several copper ions and one could imagine that while the Cys is part of a type 1 site the two histidine side chains partake in the binding of another of the copper ions in the molecule.

The role suggested here for the two histidine residues is supported by the findings with other metalloenzymes that histidine residues in a similar arrangement can act as metal ligands. Structural studies on carbonic anhydrase have shown that the histidine residues in the sequence -His-Gly-His-, being part of a pleated sheet structure, are ligands to the zinc ion (24). A similar situation has been encountered in superoxide dismutase (25) where the sequence -His-Val-His- is chelating a copper ion.

Kinetic and spectroscopic evidence shows that an interesting property of the multi-copper oxidases is an interplay between the various copper ions in the molecule (3-5). This interplay is essential in the catalyzed reaction where molecular oxygen becomes reduced to water by accepting electrons from redox centers in the protein molecule that are considered to be the copper ions which undergo valency change. Intra-enzyme electron transfer between the redox centers is believed to be part of the catalyzed reaction. According to the suggestion in Fig. 3 the histidine-containing Cys sequence in laccase and ceruloplasmin forms a link between two of the copper ions and it may well have a role in mediating an interplay between theses redox centers. Model building suggests that the distance between the two copper ions depicted in Fig. 3 would be around 8-10 Å which is the minimum distance between paramagnetic copper ions (types 1 and 2) in fungal laccase estimated from EPR measurements (3,4).

The role for the sequence -His-Cys-His- as a link between copper ions would predict that this structure is evolutionary conserved only in the multi-copper oxidases where there is a need for interplay between the metal ions. It should not be expected to be selected for among the small "blue" proteins which contain only a single copper ion in each molecule. Consistent with this prediction is the finding that among a great number of small "blue" proteins investigated none has been found to have the sequence -His-Cys-His- (see Fig. 2).

Spectroscopic studies strongly suggest that ceruloplasmin has two type 1, one type 2, and four type 3 copper ions in the molecule (3,5). The type 1 binding sites, identified by sequence analysis (12,13), are illustrated by the two Cys sequences for ceruloplasmin in Fig. 2. Of these, only one contains the two histidine residues also present in fungal laccase B.[‡] Fungal laccase contains one type 1, one type 2, and two type 3 copper ions. A similarity with ceruloplasmin is thus the occurrence of a single copy of type 2 copper. It is tempting to speculate that the copper suggested to be chelated by the histidine residues would be the single type 2 as indicated in Fig. 3. This idea would be consistent with kinetic studies of the sequential order in which the various types of copper ions become reduced and reoxidized (26). It has also been shown that binding of a fluoride ion at the type 2 site perturbs the CD spectrum of the type 1 site (27) and that removal of type 2 copper affects the EPR parameters of the type 1 copper as demonstrated for laccase from lacquer tree (28). The possibility to remove type 2 copper specifically (28,29) may be utilized to test experimentally the idea that type 2 copper is bound to the histidine residues adjacent to the Cys in the multi-copper oxidases by using group specific chemical modification.

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[‡]For a discussion of the differences in properties of the two type 1 sites in ceruloplasmin, see ref. 30.

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