A new assignment of the disulfide linkage in stellacyanin

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Which cysteine of apostellacyanine reacts with 4-vinylpyridine in 6 M guanidine–HCl depends upon the pH. We infer that a disulfide switch occurs at higher pH and that in the native protein the disulfide bridge occurs between Cys-59 and Cys-93. Thus Cys-87, which is homologous to the cysteine ligands of azurin and plastocyanin, is available to bind copper.

Stellacyanin *Cysteine* *Disulfide* *Blue copper* *Sequence homology* *Switching*

1. INTRODUCTION

The type 1 or blue copper center occurs in a number of proteins which exhibit a characteristic visible absorption around 600 nm with $\lambda_{max} \approx 5000\ M^{-1} \cdot cm^{-1}$ [1–3]. The major component of the absorption has been assigned to a charge-transfer transition involving a cysteine sulfur and Cu(II) [3–5]. In plastocyanin from *Populus nigra* [6,7] and the azurins from *Pseudomonas aeruginosa* [8] and *Alcaligenes faecalis* [9] crystallographic studies have revealed that the copper is bound in a distorted tetrahedral site with an N_2SS^* donor set provided by the side chains of two histidines, a cysteine and a methionine. In stellacyanine from *Rhus vernicifera*, spectroscopic studies indicate the metal is bound in a similar site [3]. In particular, ^{1}H-NMR studies have shown that the imidazole groups of two histidines bind the metal [10], while its characteristic charge-transfer absorption unambiguously implicates a cysteine thiolate donor [11]. The rest of the donor set in stellacyanine remains to be identified.

Sequence homology suggests that azurin and plastocyanin may be products of divergent evolution; i.e., they may have a common ancestor [12,13]. As can be seen from fig.1, sequence homology is also evident when stellacyanin [14] is included in the comparison. The two histidines and the cysteine which bind the metal in the crystallographically characterized proteins are conserved in all known plastocyanins and azurins as well as stellacyanine [15]. Methionine, the other ligand in azurin and plastocyanin, is not found in stellacyanin [14,16]. This point notwithstanding, the correlation between the structures is seriously weakened by the report that the conserved cysteine of stellacyanin, Cys-87, forms part of a disulfide bridge [14]. Moreover, the free cysteine, Cys-59, is found in a section of the peptide chain which shows little homology with azurins and plastocyanins. The correct placement of the disulfide bridge is the key to any structural comparisons involving stellacyanin. Since disulfide switching could have occurred under the conditions used in [14,17], we reexamined the nature of the disulfide linkage.

2. EXPERIMENTAL

2.1. *Materials*

Latex extract from the Chinese natural lacquer tree was obtained from Saito (Osaka, Japan). The *p*-hydroxymercuribenzoic acid (pmb) and trypsin, Type XI, were purchased from Sigma and used without further purification. 4-Vinylpyridine (Aldrich Chemical Co.) and citraconic anhydride (Pierce Chemical Co.) were vacuum distilled prior to use. All other chemicals used were reagent

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grade, and buffers were freed of metal ions with a Chelex-100 (Bio-Rad) column.

2.2. *Methods*

The stellacyanin was isolated as in [18]. The apoprotein was either prepared from the reduced protein by anaerobic dialysis against an aqueous solution of thiourea at pH 5 [19], or as in [20]. Aliquots of the apoprotein were titrated with pmb as in [21].

Three different procedures were used to label the free cysteine. Two differed only in the pH at which the protein was treated with 4-vinylpyridine. In the latter experiments about 40 mg of the apoprotein, prepared with thiourea, was dialyzed into 0.1 M phosphate buffer, at pH 7.0 or 7.5. The resulting solution was treated with excess 4-vinylpyridine for 1 h and then made 6 M in guanidine–HCl. After the solution was stirred for 1 h, it was desalted on a Sephadex G-25 column equilibrated with 0.1 M (pH 7) phosphate. The protein fraction was concentrated, dialyzed into 0.1 M (pH 8) phosphate and finally made 6 M in guanidine–HCl once again in preparation for the modification of the lysine residues. In a third experiment the apostellacyanin was prepared as in [20], and the protein was dialyzed into 0.1 M (pH 8.2) Tris buffer containing 25 mM Na_2H_2EDTA. The solution was made 6 M in guanidine–HCl, heated on a boiling water bath, and then exposed to excess 4-vinylpyridine for 90 min at 22°C.

In order to modify the lysines, excess citraconic anhydride was added to the labeled peptide in 6 M guanidine–HCl. The pH was maintained between 8 and 10 by the addition of 6 M NaOH. After the reaction was complete the solution was desalted on a Sephadex G-25 column equilibrated with 0.1 M ammonium bicarbonate (pH 8.2). About 1 mg trypsin was added to the eluate, and the solution was incubated at 37°C for 3 h. Then the pH was adjusted to 2 with concentrated formic acid, and the sample was lyophilized. Peptide fragments were separated on a Sephadex G-50 column which was equilibrated and eluted with 9% formic acid. Finally fractions were pooled, lyophilized and subjected to automated sequence analysis.

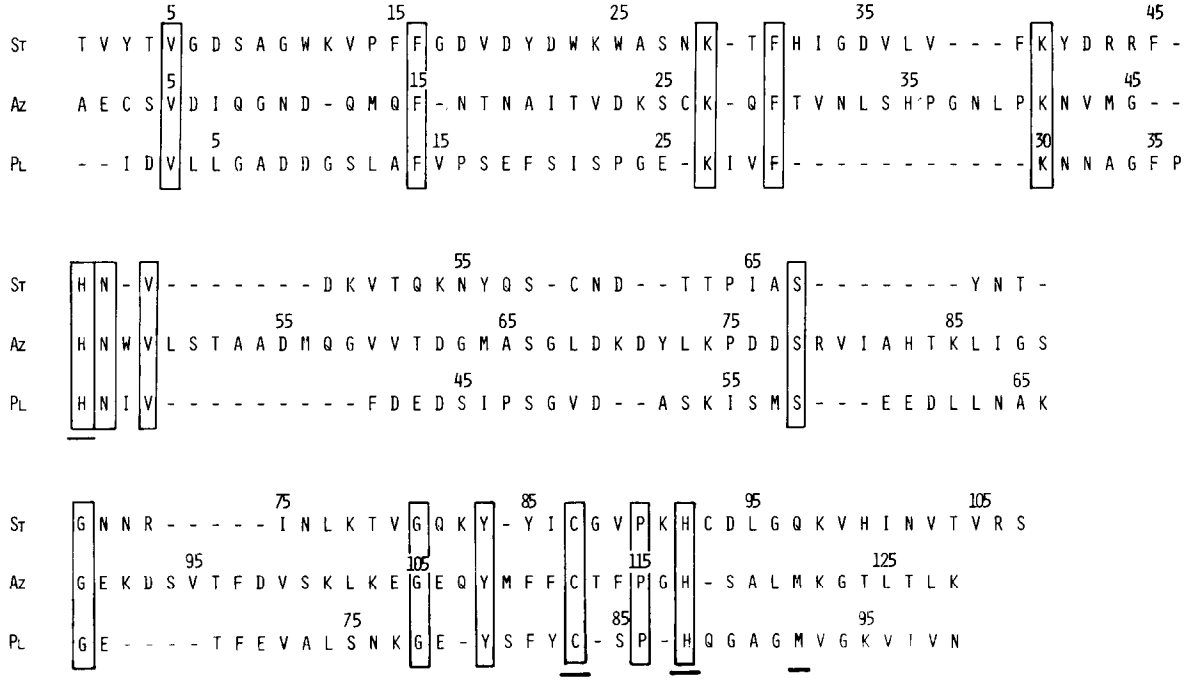

Fig.1. Amino acid sequence comparison among azurin (Az) from *P. aeruginosa*, plastocyanin (Pl) from *P. nigra* and stellacyanin (St) from *R. vernicifera*. The sequences and the one-letter symbols for the amino acids are compiled in [15]. The ligating residues of Pl and Az are underlined.

2.3. *Instrumentation*

Electronic absorption spectra were recorded on either a McPherson EU-700 or a Cary 17-D spectrophotometer. Fractionation was accomplished by either a Gilson Model FC-80K microfractionator or an ISCO Model 328. Peptide sequencing was done on a Beckman 890C or 890D automated sequencer, as in [22].

3. RESULTS

As determined by pmb titrations, apostellacyanin, prepared by dialysis against thiourea or cyanide, exhibited one free sulfhydryl group. In two separate experiments we verified that no titratable sulfhydryls were present after the label was applied.

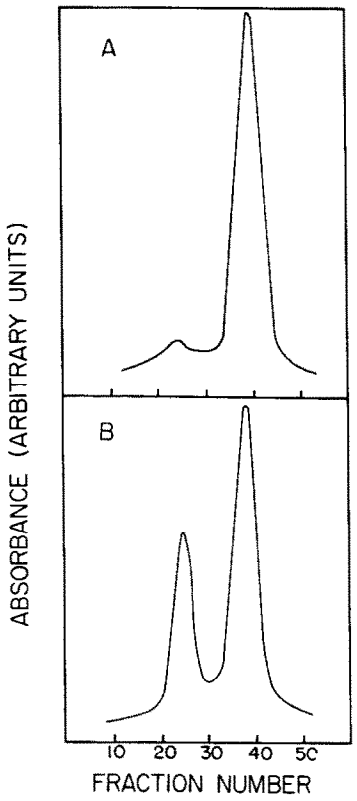

Fig.2. Gel elution profiles monitored by absorbance at 280 nm. (A) Fragment elution when the free cysteine was labeled at pH 8.2. (B) Fragment elution when the free cysteine was labeled at pH 7.0. The columns were eluted at 22°C with 9% formic acid.

The trypsin-induced scission of polypeptides is specific for Arg-X and Lys-X linkages, but in this case the lysines had been masked by the reaction with citroconic anhydride. Therefore, on the assumption that all arginyl bonds were cleaved, 3 polypeptides of roughly similar lengths were expected, the N-termini being Thr-1, Phe-45 and Ile-75 (fig.1). Sequence analyses confirmed that these peptides, denoted as fragments 1, 2 and 3, respectively, were present. We also found that the peptides fractionated differently on the G-50 column, depending on the conditions used in labeling the free cysteine.

Fig.2A shows the elution profile from the experiment where 4-vinylpyridine was applied at pH 8.2. In this case a single band eluted and was found to contain all 3 fragments in equal proportions. In contrast two polypeptide fractions were resolved for the samples which had been exposed to 4-vinylpyridine at pH 7.5 and 7.0 (fig.2B). The higher M_r fraction, which eluted first, was observed to be en equimolar mixture of fragments 2 and 3. All 3 fragments were found in the second fraction, although it was enriched in fragment 1. In the sample labeled at pH 7.5, 35–40% (by wt) of the total of fragments 2 and 3 eluted in the high M_r fraction. In the sample labeled at pH 7 this fraction contained about 80% of fragments 2 and 3, and sequence analysis revealed that the 4-vinylpyridine was bound at Cys-87.

4. DISCUSSION

4.1. *Fragment elution patterns*

If the disulfide linkage is internal to fragment 3, as assigned in [14], each fragment should migrate independently on the gel column. The elution profile depicted in fig.2A is consistent with this expectation. The lack of separation is attributable to the fact that the fragments are of similar size: each has roughly the same length, and each carries carbohydrate. (Carbohydrate is attached to stellacyanin via Asn-28, Asn-60 and Asn-102 [14].) These results confirm the previous reports that Cys-59 acts as the free cysteine when apostellacyanin is labeled at pH $\geqslant$ 8.2 in 6 M guanidine–HCl [14,17].

Quite different results are obtained when the free cysteine is labeled at pH 7. Most importantly,

the bulk of fragments 2 and 3 elute together with a retention time characteristic of a higher M_r fraction. (Note that the fractions were quantified by weight, not the absorbance at 280 nm since the aromatic residues are distributed unevenly among the fragments. In particular, fragment 1 contains 10 of the 15 aromatic residues including all 3 Trp residues [14].) The fact that the 4-vinylpyridine label was found at Cys-87 together with the chromatographic results implies that fragments 2 and 3 are linked by a disulfide bridge between Cys-59 and Cys-93.

4.2. *Rationale for the disulfide switch*

It is evident from the above that the disulfide linkage of stellacyanin can switch depending on conditions. The most probable assignment for the disulfide linkage in the native protein is 59–93 because disulfide switching is favored at high pH where the deprotonated sulfhydryl group can act as a nucleophile (eq.1):

$$RSH \rightleftharpoons RS^- + H^+ \quad (1a)$$

$$RS^- + R'SSR'' \rightleftharpoons RSSR' + R''S^- \quad (1b)$$

$$RS^- + R'SSR'' \rightleftharpoons RSSR'' + R'S^- \quad (1c)$$

In general, a random assortment of isomers might be expected; however, Cys-87 and Cys-93 are near neighbors in stellacyanin so the bridge between them is statistically likely to be most stable in the unfolded protein [23].

While it is conceivable that peculiar structural requirements of the peptide chain could confound the argument given above, the chemical reasoning behind our analysis is so fundamental that this is unlikely. The cucumber basic blue protein contains cysteines homologous to those of stellacyanin and has been crystallized [24]. The solution of its structure may shed additional light on the question of the disulfide assignment of stellacyanin. Our assignment is pleasing in that the conserved cysteine is available for binding to copper, as would be expected if stellacyanin is homologous with the others [25]. As a final point we note that it has been suggested [14,26], although not proven, that a disulfide group of stellacyanin may replace the methionine donor of azurin and plastocyanin. Such proximity between the cysteine and cystine groups in the folded protein could encourage disulfide switching.

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