

Preparation and characterization of a ruthenium-labeled *Rhus* stellacyanin

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Reaction of Cu(II)-stellacyanin with excess $(\text{NH}_3)_5\text{Ru}(\text{H}_2\text{O})^{2+}$ yields a protein to which $(\text{NH}_3)_5\text{Ru}(\text{III})$ is coordinated. The binding stoichiometry of Ru:Cu is $(2.1 \pm 0.1):1$ as shown by ICP atomic emission measurements. In the ^1H -NMR spectrum of the Ru(III)-labeled protein, the signals from the C-2H and C-4H protons of His-32 and His-100 are absent. The ESR spectra of the native oxidized Cu(II)-stellacyanin and of the Ru(III)-labeled protein are identical in spite of the $(\text{NH}_3)_5\text{Ru}(\text{His})^{3+}$ ions being a low-spin d^5 system. These data are interpreted as being the result of binding of two Ru(III) ions to the modified stellacyanin at two proximal imidazole side-chains which are within a separation distance that allows for their electronic coupling.

Blue copper protein; Ruthenium labeling

1. INTRODUCTION

Rhus vernicifera stellacyanin is a blue single-copper protein. So far no crystals of this protein have been grown. Hence, we have constructed a hypothetical three-dimensional model of St by energy minimization calculations based on sequence homology and an assumed structural relationship with plastocyanin [1]. The model suggests the presence of two proximal histidines (nos 32 and 100) on the surface of St, at a distance of ~ 20 Å from the copper center. This observation provided the basis for experiments described here aiming at specific modification of this protein with ruthenium, since solvent-exposed imidazole side chains of histidines were previously shown to react selectively with $[\text{a}_5\text{Ru}(\text{H}_2\text{O})]^{2+}$. Thus, derivatives of azurin, myoglobin, and mammalian cyto-

chrome *c* have been synthesized, characterized and employed in studies of electron transfer within these modified proteins [2].

We report here the preparation and initial characterization of a derivative of stellacyanin in which two a_5Ru -complexes are coordinated to the two free imidazoles of His-32 and His-100 in this protein.

2. EXPERIMENTAL

2.1. Preparations

Stellacyanin was prepared from the Japanese *R. vernicifera* lacquer tree according to the method of Reinhammar [3]. $[\text{Ru}(\text{NH}_3)_5\text{Cl}]\text{Cl}_2$ was a generous gift from Dr H.B. Gray. $[\text{a}_5\text{Ru}(\text{H}_2\text{O})]^{2+}$ was generated in Ar-saturated solutions by reduction of the former Ru complex with freshly amalgamated mossy Zn. A 10–50-fold excess of this complex was allowed to react with St[Cu(II)] for 1–4 h at room temperature; typically 0.1 mM protein, pH 7.5, 0.1 M phosphate buffer. The labeling reaction was terminated by separating excess Ru complex on a G-25 column (0.1 M phosphate, pH 6). During the separation, the Cu(I) of St was fully reoxidized by O_2 . Using a linear gradient of phosphate buffer (0.1–0.5 M, pH 6.0) unreacted and modified stellacyanin could be separated on a Whatman CM-52 ion-exchange column. The product was then dialyzed extensively against triply distilled water until it became free of buffer. The yield of labeled protein was $>90\%$.

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Abbreviations: ICP, inductively coupled plasma; St, stellacyanin; a, NH_3

All other reagents employed were of analytical grade, and were used without further purification.

The $^1\text{H-NMR}$ spectra were measured on a Bruker WH-270 spectrometer at room temperature. Ru and Cu contents were determined on a Perkin-Elmer Plasma-II ICP atomic emission spectrometer belonging to the Danish National Institute of Occupational Health.

3. RESULTS AND DISCUSSION

Atomic emission ICP measurements of the modified stellacyanin from four different preparations demonstrate that a 2.1 ± 0.1 Ru-St complex is the single product of the above-described procedure, while a 1:1 complex could not be synthesized by this method. Using shorter labeling time or lower concentration ratios than those given in section 2 yielded only mixtures of unlabeled and doubly labeled stellacyanin. The absorption spectrum of the purified, Ru-St complex in the 300–700 nm region is shown in fig.1. It is seen that each Ru(III) ion contributes an extinction coefficient of $\sim 1910 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 310 nm while the absorption of the blue copper site is identical to that of the native protein, with $\epsilon_{604} = 4030 \text{ M}^{-1} \cdot \text{cm}^{-1}$ [4]. The $^1\text{H-NMR}$ spectrum of native stellacyanin is identical to that reported by Hill and Lee [5], while the aromatic region (6–9 ppm) undergoes a significant broadening upon ruthenium modification of St, and the peaks belonging to the C-2H and C-4H protons from His-32 and His-100 disappear (see fig.2). This result is in line with all earlier protein-labeling experiments using $[\text{a}_5\text{Ru}(\text{H}_2\text{O})]^{2+}$, where a solvent-exposed imidazole ring of a histine is exchanged for the ligand water in the $\text{a}_5\text{Ru}(\text{H}_2\text{O})$ complex [2]. ESR measurements of the Ru(III)-

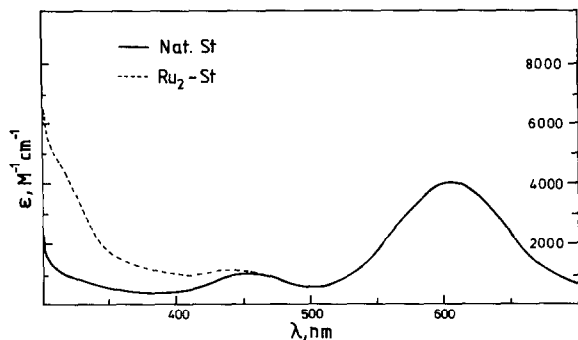


Fig.1. Absorption spectrum of native (—) and ruthenium-(III)-labeled (---) oxidized *Rhus* stellacyanin.

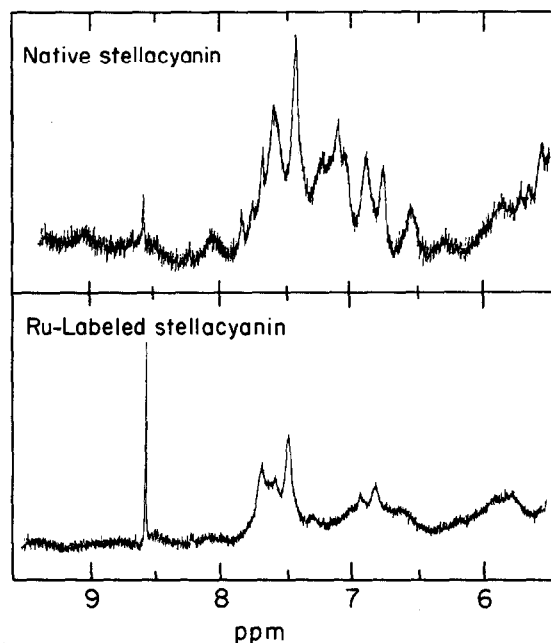


Fig.2. 270 MHz $^1\text{H-NMR}$ spectra of (upper) native oxidized stellacyanin and (lower) Ru(III)-labeled stellacyanin; 99.6% D_2O , 0.05 M phosphate buffer, pH* 7.0. The peaks belonging to C-2H and C-4H of His-32 and His-100 are seen in the spectrum of native stellacyanin at 7.1, 7.8 and 8.0 ppm (cf. [5]).

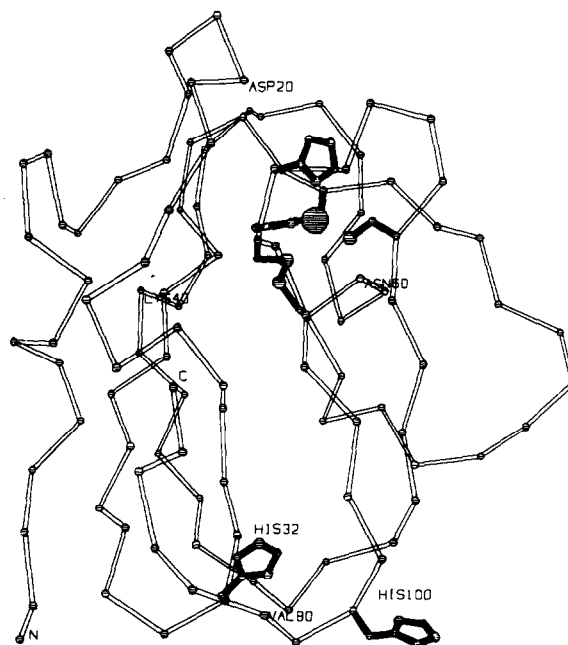


Fig.3. A hypothetical three-dimensional model of stellacyanin [1]. The copper ion (large shaded circle), the putative copper ligands (His-46, His-92, Cys-87 and S-93 of cystine-59/93) as well as the positions of His-32 and His-100 are shown.

St[Cu(II)] derivative give spectra which are identical to that of the native Cu(II)-stellacyanin, while the expected signal from the low-spin d^5 Ru(III) ions is missing. One possible rationale for this observation is that the two Ru(III) ions are proximal allowing for electronic coupling between them.

As mentioned above, based on an assumed structural relationship with plastocyanin we have constructed a hypothetical three-dimensional model of stellacyanin by energy minimization [1]. This model indeed shows that two histidine side chains (nos 32 and 100) are exposed to the solvent and sufficiently close to allow for hydrogen bond formation between them (see fig.3). The present experimental data thus fully support the predicted position of these histidine residues in the protein. The distance from N_δ of His-32 to the copper center as calculated from the model is 18.6 Å.

This substitution-inert modified form of stellacyanin with redox-active metal centers [E° (a_5 Ru(His) $^{3+/2+}$) = 80 mV; E' (stellacyanin-Cu(II)(I)) = 184 mV, pH 7.1] provides a suitable system for kinetic studies of electron transfer pro-

cesses between Ru(II) and Cu(II) in the semi-synthetic protein where the two centers are at fixed distances. This is the subject of the following paper.

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