Preparation and Characterisation of a Penta-ammineruthenium(III) Derivative of Plastocyanin and the Kinetics of Long Distance Electron Transfer

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Attachment of $Ru^{III}(NH_3)_5$ to the His59 of *Anabaena variabilis* plastocyanin, reduction of the Ru^{III} to Ru^{II} by pulse radiolysis techniques, and determination of the rate constant (0.3 s^{-1}) for intramolecular electron transfer $Ru^{II} \rightarrow Cu^{II}$ (~11.9 Å) is described.

Plastocyanin (M. Wt. ~10,500; ~100 amino acids) is a single (type 1) Cu protein involved as the PCu^{II} and PCu^{I} states in photosynthetic electron transport. Intermolecular electron-transfer reactions with small inorganic (and metalloprotein) redox partners have been extensively studied.[1] Still requiring much more detailed study and understanding is the way in which electrons are transferred between the redox partner at a binding site on the protein surface and the active site, and factors influencing such rate processes.

As an aid to such understanding the properties of cytochrome c with $Ru^{III}(NH_3)_5$ attached[2] has been explored by the groups of Gray[3] and Isied.[4] It is known that surface unco-ordinated histidine residues are most readily modified by reaction with $[Ru(NH_3)_5H_2O]^{2+}$.[5] Intramolecular $Ru^{II} \rightarrow Fe^{III}$ electron transfer over a fixed distance can be monitored. More recently azurin[6] and myoglobin[7] have been similarly studied. So far plastocyanin has not been a candidate for such studies, since from higher plant sources it has only two histidines (at residues 37 and 87), both co-ordinated to the Cu and not therefore available for Ru modification. However, plastocyanin from the prokaryotic blue–green alga *Anabaena variabilis* has been sequenced,[8] and is known to have an additional histidine at residue 59. We therefore set out to isolate and modify plastocyanin from this source. Highly relevant to such studies is the crystal structure information which, for electron transport proteins cytochrome c and more recently plastocyanin,[9] has reached an advanced stage of refinement.

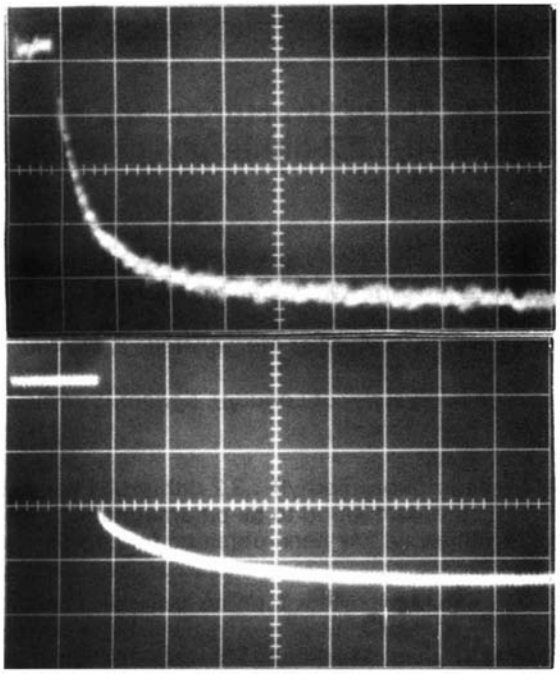

Figure 1. The top picture shows the rapid decay in Cu^{II} absorbance at 597 nm for the reaction of $CO_2^{\bullet -}$ with $Cu^{II}Ru^{III}$, time-base 0.1 ms per division, *y*-axis % absorption per division 0.94. Typically a radiation dose of 1.09 krad giving a radical concentration of 8 μM was used. Rapid reduction at the Ru^{III} centre gives rise to the subsequent slow $Cu^{II}Ru^{II} \rightarrow Cu^{I}Ru^{III}$ stage, bottom picture, time-base 0.2 s per division, *y*-axis % absorption per division 2.68.

A. variabilis was grown under conditions described by Kratz and Meyers.[10] Plastocyanin was isolated by the method of Ellefson *et al.*[11] to give PCu^{II} with a u.v.–visible absorbance peak ratio $A_{278}:A_{597}$ of 1.3:1, and ε of 4650 M^{-1} cm^{-1} at 597 nm in agreement with 4500 M^{-1} cm^{-1} for higher plant plastocyanins. N.m.r. studies have indicated extensive structural similarity to higher plant plastocyanins.[12,13] The complex $[Ru(NH_3)_5(H_2O)](PF_6)_2$ was prepared by a published method, and composition confirmed by analysis.[14] In a typical procedure, PCu^{II} (21 mg, 0.2 mM) was reacted with $[Ru(NH_3)_5(H_2O)]^{2+}$ (50 mg, 10 mM) in 10 ml of solution buffered at pH 7.5 (Tris/HCl), I = 0.10 M (NaCl), for 14 h at 20 °C under an argon atmosphere. Protein was separated from unreacted $[Ru(NH_3)_5(H_2O)]^{2+}$ on a Sephadex G25 column (3 × 25 cm), previously equilibrated with phosphate buffer (1 mM, pH 7), under argon. The eluate was collected until the yellow Ru band had moved ~80% down the column. Eluted protein was immediately oxidised with a slight excess of $[Fe(CN)_6]^{3-}$, and adsorbed onto a CM52 cation exchange column (1.5 × 7 cm) equilibrated in 1 mM phosphate buffer. Using a phosphate gradient (20—200 mM) at pH 7 the tight blue band was resolved into four fractions. The first band (7.9 mg) contained unmodified PCu^{II}, and the fourth band (2.1 mg) a more extensively modified protein fraction. The third blue band (4.5 mg) gave a shoulder at ~300 nm characteristic of the $[Ru(NH_3)_5(His)]^{3+}$ chromophore.[15]† The peak for PCu^{II} at 597 nm is unperturbed by modification. Isoelectric focusing of a fully oxidised sample gave an isoelectric point of 8.85 compared to 8.50 for unmodified protein. Analysis by inductively coupled plasma (ICP) atomic emission gave a Ru:Cu ratio of 1.05:1 (4 determinations). The second band product (1.8 mg), which also contains a single Ru, has a peak at 375 nm in addition to 597 nm. This product has not yet been characterised. The total protein recovery was ~80%.

Pulse radiolysis studies were with N_2O saturated solutions, 0.10 M in phosphate (pH 7.0), and in the presence of 0.10 M sodium formate to generate $CO_2^{\bullet -}$ (formate) radicals on pulsing. The $e_{aq.}^-$ generated react with N_2O to give OH radicals, and OH and H then react with formate to give $CO_2^{\bullet -}$. The total yield of OH and H was G = 7.1 molecules

† *Note added in proof.* Additional evidence for Ru attachment at His59 has been obtained. Thus at pH 7 (50 mM phosphate), 25 °C, the His59 of native *A. variabilis* plastocyanin is readily modified by diethyl pyrocarbonate, $(C_2H_5OCO)_2O$, Δε = 3200 M^{-1} cm^{-1} at 240 nm (E. W. Miles, *Methods Enzymol.*, 1977, **47**, 431). No modification is observed however when the protein sample (band 3) with Ru attached is used. On tryptic digest of the Ru modified band 3 (work with Dr. A. Aitken) the pentapeptide containing His59 moves into the h.p.l.c. profile.

per 100 eV.[16] We first prepared a sample of azurin with $Ru^{III}(NH_3)_5$ attached at His83 and for a protein concentration of 15 μM obtained a rate constant of ~2.0 s^{-1} (17 °C) for the Ru^{II} to Cu^{II} intramolecular electron transfer, in agreement with the value 1.9 ± 0.4 s^{-1} (22 °C) obtained by Gray and colleagues using flash photolysis techniques. It is known that there is little or no temperature dependence for this reaction. Reaction of modified *A. variabilis* plastocyanin with $CO_2^{\bullet-}$ occurred at both the Cu^{II} (65%) and Ru^{III} (35%) sites. Rapid reduction of the Cu^{II} monitored at 597 nm gave a rate constant of 7×10^8 M^{-1} s^{-1}. The $Cu^{II}Ru^{II}$ product gave $Cu^{I}Ru^{III}$ in a slow electron-transfer step, see Figure 1. Contributions from bimolecular decay of $Cu^{II}Ru^{II}$ were allowed for by varying the concentration of modified protein 1.9—23 μM. The rate constant for the intramolecular rate process is 0.30 ± 0.20 s^{-1}. Unmodified PCu^{II} showed the same rapid reduction but no subsequent slow process. Relevant reduction potentials are for native *A. variabilis* PCu^{II}/PCu^{I} 350 mV, and for $[Ru(NH_3)_5His]^{3+/2+}$ 80 mV,[3b] both against normal hydrogen electrode. The distance of the imidazole unco-ordinated N-atom from the active site co-ordinated S-atom of Cys84 (which is the most likely electron transfer lead-in group) has been estimated from the crystal structure co-ordinates of poplar plastocyanin.[17] The Glu59 residue of poplar was replaced by His59 giving a distance to the unco-ordinated N of the imidazole of 11.9 Å. There are no aromatic residues in or near the path of electron transfer.

Modification of the His59 plastocyanin from green algal *Scenedesmus obliquus* has also been carried out, and the rate constant for intramolecular Ru^{II} to Cu^{II} electron transfer is being determined by a similar procedure.

We thank Johnson Matthey for support of this work.

Received, 3rd April 1986; Com. 444

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