Volume 244, number 2, 379–382 FEB 06846 February 1989

Long-range intramolecular electron transfer in *Rhus vernicifera* stellacyanin: a pulse radiolysis study

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Received 8 December 1988

The $(NH_3)_5$Ru-modified stellacyanin Ru(III)$_2$-St[Cu(II)], where two ruthenium ions are coordinated to imidazole residues of His-32 and 100, has been synthesized. CO_2^- radicals, generated by pulse radiolysis, react with the modified protein yielding an intermediate Ru(II)-St[Cu(II)]. The rate of intramolecular electron transfer within this complex Ru(II)-St[Cu(II)] $\xrightarrow{k_u}$ Ru(III)-St[Cu(I)] has been determined, $k_u = 0.05\ s^{-1}$ at 18°C, pH 7.0. The Ru(His-32/100) to Cu(II) separation distance is relatively large (~18 Å), and the driving force is low (10 kJ·mol^{-1}), hence the correspondingly slow rate. This result is compared with those obtained in earlier studies of intramolecular fixed-distance electron transfer reactions in blue copper proteins.

Blue copper protein; Stellacyanin; Pulse radiolysis

1. INTRODUCTION

It is well established by now that electron transfer in proteins can taken place over long distances (>10 Å) at considerable rates [1]. In order to understand the factors which control these intra-protein processes, it is important to study the role of the protein matrix that intervenes between the electron donor and acceptor.

H.B. Gray and co-workers [2–7] have employed ruthenium(II) complexes as reagents binding to histidyl residues on protein surfaces, thereby forming semi-synthetic systems containing two or more redox centers that are amenable to studies of intramolecular electron transfer. Thus, several research groups have adapted this methodology and have investigated intramolecular electron transfer in Ru-modified cytochrome *c* [8–10], and in Ru-modified plastocyanin [11].

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Abbreviations: St, stellacyanin; a, NH_3

Rhus stellacyanin is a blue copper protein containing two histidines which according to our model calculations [12] are solvent exposed, and indeed it is possible to coordinate Ru(III) to the imidazole side chains of these residues [13]. This provided the possibility to examine the intramolecular electron transfer in this system. The present paper reports the kinetic study of electron transfer from Ru(II) to Cu(II) initiated by pulse radiolysis.

2. MATERIALS AND METHODS

Stellacyanin was prepared by the method of Reinhammar [14]. Ru-labeled protein was produced as described in the previous paper [13]. Pulse radiolysis experiments were performed using the 6 MeV Varian V-7715 linear accelerator of the Hebrew University in Jerusalem. Pulse lengths were in the range of 0.1 to 1.5 μs. The redox states of the metal ions were monitored both at 605 nm for Cu(II) (ϵ_{604} = 4030 M^{-1}·cm^{-1} [15]) and at 310 nm where each Ru(III) ion coordinated to stellacyanin contributes an extinction of 1910 M^{-1}·cm^{-1} [13]. Ru(II) (as well as Cu(I)) does not absorb significantly in the 300–700 nm region. The data acquisition system consisted of a Biomation 8100 transient recorder and a Nova 1200 minicomputer.

All experiments were performed in N_2O saturated, buffer-

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00145793/89/$3.50

free, 0.1 M sodium formate solutions, adjusted to pH 7.0. Pulse generated solvated electrons, e^-_{aq}, react with N_2O to give OH radicals [16]. When OH or H radicals react with formate, the reducing radical ion CO_2^- ($E^\circ = -1.2$ V [17]) is subsequently produced as the sole reducing agent:

$$OH(H) + HCOO^- \longrightarrow CO_2^- + H_2O(H_2).$$

Yields of CO_2^- radicals were determined by following the reduction of $Fe(CN)_6^{3-}$ or Fe(III)-cytochrome *c*, in control experiments and typical concentrations were in the range 0.2–8 μM. The temperature was 18°C.

3. RESULTS AND DISCUSSION

Native oxidized stellacyanin reacts with CO_2^- in a fast bimolecular reaction:

$$CO_2^- + St[Cu(II)] \longrightarrow St[Cu(I)] + CO_2.$$

The reaction could be monitored at 605 nm and the rate constant at 18°C is $(8.3 \pm 0.5) \times 10^8$ $M^{-1} \cdot s^{-1}$. In the modified protein, both the Ru(III) site (55%) and the Cu(II) site (45%) are reduced by CO_2^- (fig.1). Most experiments were performed under pseudo-first order conditions with typically a 10-fold excess protein over the reducing radical concentration. At both 310 nm and 605 nm a linear relationship between the apparent first order rate, k_{obs} and protein concentration was observed. For the direct reduction of the Cu(II) site by CO_2^- (see fig.1) a bimolecular rate constant, $k_{b2} = (8.5 \pm 0.5) \times 10^8$ $M^{-1} \cdot s^{-1}$ (scheme 1), was calculated which, within experimental error, is the same as the above determined value for reduction of native oxidized St by CO_2^-. The rate of reduction of the Ru(III) site in modified St is slightly faster, $(1.0 \pm 0.1) \times 10^9$ $M^{-1} \cdot s^{-1}$; k_{b1} in scheme 1).

Following the fast reduction step, a slow reduction phase was observed at 605 nm (fig.2A). The rate of this process was examined over a wide protein and radical concentration range, and the rate

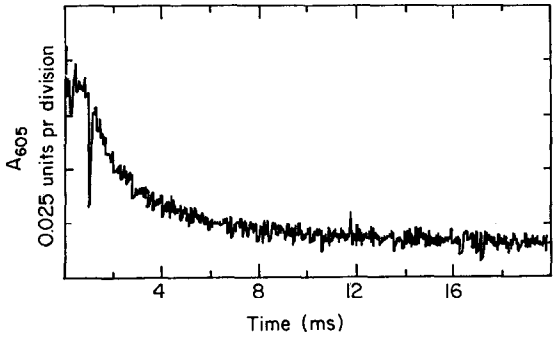

Fig.1. Fast decay in the absorption of Cu(II) at 605 nm due to the direct reduction by CO_2^- in ruthenium modified stellacyanin. Protein concentration, 20 μM; 2.0 μM CO_2^-; pH = 7.0; temperature, 18°C; optical path-length, 12.3 cm.

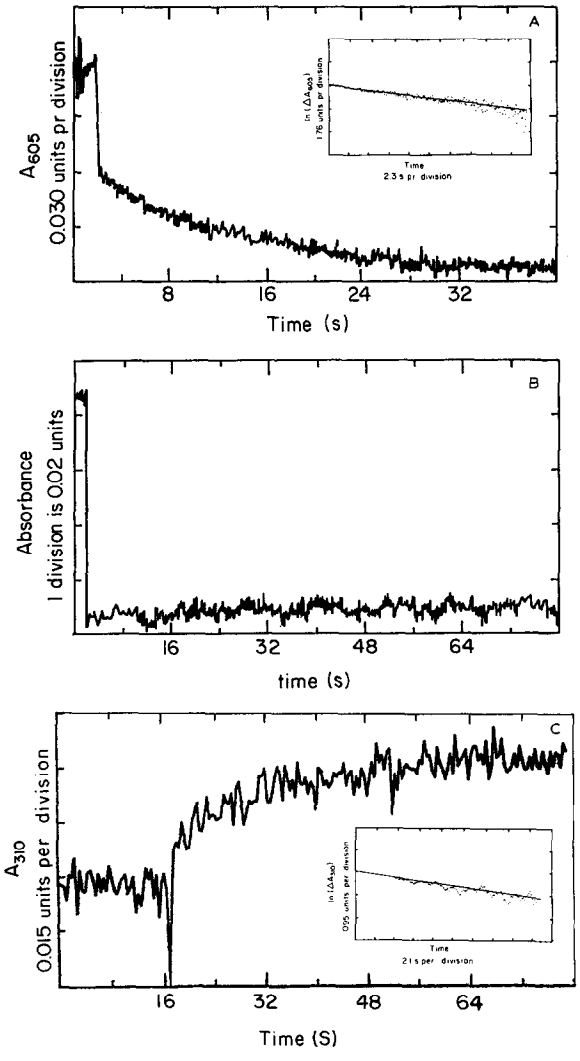

Fig.2. (A) Slow decay in the Cu(II) absorption at 605 nm due to an intramolecular reduction by Ru(II) in ruthenium labeled stellacyanin. Other experimental conditions are as in fig.1. Inset: Logarithmic plot of the data. (B) Reduction of native stellacyanin by CO_2^- followed at 605 nm at the slow time scale. Protein concentration: 8.0 μM. Other experimental conditions as above. (C) Slow recovery of the Ru(III) absorbance at 310 nm due to intramolecular reoxidation of Ru(II) by Cu(II) in ruthenium labeled stellacyanin. Other experimental details as in fig.1. Inset: Logarithmic plot of the data.

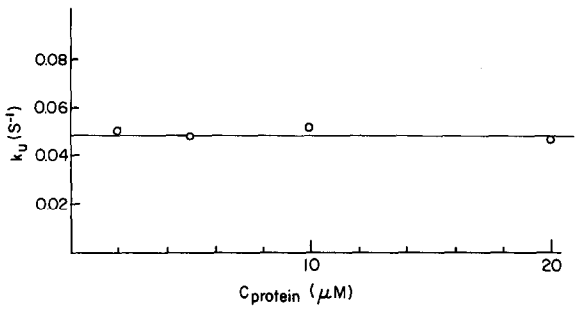

Fig.3. Concentration dependence of the slow rate on protein concentration. Each point is the average of results from 5 or more individual experiments.

constant was found to be $k_u = 0.05\ s^{-1}$ (scheme 1), independent of the respective reagents concentrations employed (see fig.3). The Ru(III) formation, monitored at 310 nm, occurs concomitantly with the reduction of Cu(II) (fig.2C). The same time range was checked following reduction of the native protein by CO_2^-, but no absorption change could be detected (fig.2B). We can therefore conclude that a slow intramolecular electron transfer from Ru(II) to Cu(II) takes place in this bicentered metalloprotein. The yield of Ru(II) and Cu(I) wa relatively high: with a 10-fold excess of modified protein over radical concentration, more than 90% of the reducing equivalents were used up on reduction of the metal ions.

At the above concentrations only one of the two Ru(III) ions bound to a St-molecule is expected to be reduced by CO_2^-. For simplicity therefore, only one ruthenium ion is presented in reaction scheme 1:

$$CO_2^- + \text{Ru(III)-St[Cu(II)]} \xrightarrow{k_{b1}} \text{Ru(II)-St[Cu(II)]} + CO_2$$

$$\text{Ru(II)-St[Cu(II)]} \xrightarrow{k_u} \text{Ru(III)-St[Cu(I)]}$$

$$CO_2^- + \text{Ru(III)-St[Cu(II)]} \xrightarrow{k_{b2}} \text{Ru(III)-St[Cu(I)]} + CO_2$$

Scheme 1.

The ratio of k_{b1}/k_{b2} is 1.2 which is in good agreement with the relative yield of Ru(II)/Cu(I) in the fast step (55% vs 45%). The yield of Cu(I) in the slow intramolecular step corresponds to the concentration of Ru(II) formed in the fast bimolecular reaction, b1 (scheme 1). Thus, a possible interprotein electron transfer from Ru(II) in one molecule to Cu(II) in another is negligible.

Fig.4 shows details of the possible pathway from His-32/100 to the copper site, based on our model building of stellacyanin [12]. The shortest distance from the Ru-binding site to the copper redox site (16.1 Å) is from Nδ of His-32 to S of the copper ligating Cys-93. It is noteworthy that between His-32 and Cys-93 we find Tyr-84 (Tyr-85 is solvent exposed and points out of the figure plane).

A tentative pathway for electron transfer to the Cu(II) site from Ru(II) coordinated to His-32/100 is through a π-orbital system coupled by superexchange interactions through the intervening aromatic rings of His-32/100 and Tyr-84 to S(Cys-93). Similar π-electron transfer has been implied in earlier studies [18–20]. One reason for the

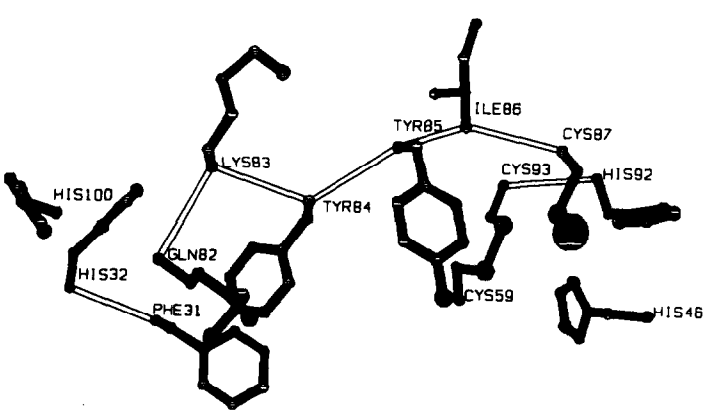

Fig.4. View of the electron transfer pathway from His-32/100 to Cu(II) in the hypothetic stellacyanin model [12]. Tyr-85 is pointing out of the plane and, thus, does not interfere with the proposed electron transfer pathway.

relatively slow electron transfer observed in the present case may be the rather large distance separating the π-system in Ru-St. Also, the driving force of the reaction ($\Delta E \sim 100$ mV or 10 kJ · mol^{-1}) is smaller than for other proteins examined in similar studies. Thus, in Ru-modified azurin the long range (11.8 Å) transfer is much faster ($k = 2\ s^{-1}$) [4] while in modified plastocyanin where the intramolecular electron transfer proceeds over a similar distance (11.9 Å) the rate is 0.3 s^{-1} [11]. For these two reactions ΔE is 240 and 270 mV, respectively. Clearly, the low driving force combined with a larger separation distance between the donor and acceptor leads to an extremely weak donor-acceptor electronic coupling, and hence to the observed slow rate of intramolecular electron transfer in Ru-modified stellacyanin.

Acknowledgements: We are grateful to Professor Gideon Czapski, Department of Physical Chemistry, Hebrew University, Jerusalem, for generously allowing us to use the Linear Accelerator Laboratory. The ruthenium complex used in this investigation was a kind gift from Professor H.B. Gray, California Institute of Technology, Pasadena. The technical assistance of Arieh Licht is gratefully acknowledged. The research was supported by a short term EMBO fellowship (ASTF5477) to one of us (O.F.).

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