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Photoinduced reduction of the medial FeS center in the hydrogenase small subunit HupS from *Nostoc punctiforme*

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ABSTRACT

The small subunit from the NiFe uptake hydrogenase, HupSL, in the cyanobacterium *Nostoc punctiforme* ATCC 29133, has been isolated in the absence of the large subunit (P. Raleiras, P. Kellers, P. Lindblad, S. Styring, A. Magnuson, J. Biol. Chem. 288 (2013) 18,345–18,352). Here, we have used flash photolysis to reduce the iron-sulfur clusters in the isolated small subunit, HupS. We used ascorbate as electron donor to the photogenerated excited state of Ru(II)-trisbipyridine (Ru(bpy)$_3$), to generate Ru(I)(bpy)$_3$ as reducing agent. Our results show that the isolated small subunit can be reduced by the Ru(I)(bpy)$_3$ generated through flash photolysis.

1. Introduction

Hydrogenases are metalloenzymes that catalyze the reversible oxidation of H_2:

$$H_2 \rightleftharpoons 2H^+ + 2e^-.$$

Hydrogenases belong to either one of three categories: the [NiFe] hydrogenases, containing an inorganic nickel–iron complex in the catalytic center, the [FeFe] hydrogenases, containing a binuclear iron complex, and the [Fe] hydrogenases, containing a mononuclear iron center [1]. The [NiFe] hydrogenases consist of two protein subunits, referred to as the large and small subunit respectively [2,3]. The large subunit contains the active site where H_2 oxidation or production is catalyzed by the [NiFe] complex.

The typical electron transfer motif in the small subunit is three iron–sulfur (FeS) clusters: a proximal [4Fe–4S] cluster closest to the active site, a medial [3Fe–4S] and a distal [4Fe–4S] cluster. The three FeS clusters are aligned so that they connect the protein surface with the active site in the large subunit, and it is generally believed that electron transfer in the small subunit goes via this array of FeS clusters. Depending on whether the hydrogenase catalyzes proton reduction or hydrogen oxidation, the electrons are transported to, or away from the active site.

Cyanobacteria are phototrophic microorganisms that can produce H_2 from solar energy and water. They are therefore attractive targets for efforts to improve their productivity via genetic engineering. All cyanobacteria with known gene sequence possess at least one hydrogenase copy. In cases where more than one copy exists within a genus, they usually differ in metabolic purpose and activity [4]. In nitrogen-fixing strains, an uptake hydrogenase is usually present which recycles H_2 that is evolved in the nitrogenase reaction. Other, bidirectional, hydrogenases are known to perform both hydrogen evolution and uptake under different metabolic conditions. Only one cyanobacterial hydrogenase, the bidirectional hydrogenase from *Synechocystis* PCC 6803, has so far been isolated and characterized [5].

The filamentous, heterocystous cyanobacterium *Nostoc punctiforme* ATCC 29133 has only one hydrogenase, the [NiFe] uptake hydrogenase HupSL. We have previously isolated the small subunit, HupS, in the absence of the large subunit by expressing it in *Escherichia coli* as a fusion protein, from now on referred to as f–HupS [6]. This enabled us to characterize the FeS centers in the small subunit without spectroscopic or magnetic interference from the active site. Since HupSL is a H_2-oxidizing enzyme, the electron transfer in HupS is expected to be directed away from the active site. Due to the linear arrangement of the FeS clusters in HupS, the electron transfer route from the active site is believed to be first to the proximal cluster, then to the medial and distal clusters, and finally to the native redox partner.

The relative reduction potentials of the three FeS clusters have been suggested to play an important role in steering the electron transfer directionality in known NiFe hydrogenases [7,8]. The reduction potentials of the proximal and distal [4Fe–4S] clusters have been determined to −290 to −360 mV [7,9–13]. The medial [3Fe–4S] cluster on the other

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hand, is more oxidizing with a potential of −70–+100 mV in known cases. It might thus act as an electron trap in the electron transfer chain [14,15]. It remains unclear whether electron transfer proceeds via the medial cluster, or if the electron tunnels the ca. 17 Å distance from the proximal to the distal cluster avoiding the medial cluster entirely. All previous investigations of electron transfer reactions in NiFe hydrogenases have been performed in the intact enzyme including the large subunit. The spectroscopic and redox properties of the small subunit have therefore been affected by the presence of the nearby active site.

Our aim with this study is to investigate if the FeS clusters in the isolated f-HupS are accessible for photo-induced reduction from the surface of the protein. Flash photolysis using ruthenium-based photosensitizers, has been used for investigating electron transfer pathways in a number of metalloproteins [16–18] and recently to initiate turnover in hydrogenases [19–21]. We used ascorbate as electron donor to the photogenerated excited state of Ru(II)-trisbipyridine ($Ru(bpy)_3$), to generate $Ru(I)(bpy)_3$ as reducing agent, and monitored photoreduction of f-HupS using electron paramagnetic resonance (EPR) spectroscopy. Our results show that the isolated f-HupS can be reduced by flash photolysis.

2. Materials and methods

2.1. Protein expression and purification

The HupS protein was heterologously expressed and isolated as a fusion protein, f-HupS, with NusA, in *E. coli* BL21(DE3) as described previously [6]. Briefly: *E. coli* BL21(DE3) (Novagen), carrying the pET431HupS plasmid vector, were grown aerobically for about 20–24 h in autoinduction medium ZYP-5052 [22]. The cells were then collected by centrifugation, washed once in buffer W (100 mM Tris–HCl pH 7.5 containing 150 mM NaCl) and frozen at −20 °C until further use. The cells were broken by sonication after suspension in buffer W containing a glucose/glucose oxidase/catalase mixture and a protease inhibitor. A soluble fraction was obtained after addition of avidin, DNAse I and RNAse A and centrifugation of the resulting crude extract at 184,000 ×g. Protein purification was carried out by loading the soluble fraction on a Strep-Tactin column (IBA) in a glove box (MBraun) under an argon atmosphere. The Strep-tagged fusion protein, f-HupS, was eluted with three bed volumes of buffer W containing 5 mM desthiobiotin.

f-HupS was aliquoted either directly into EPR tubes (150 μL each), capped with rubber septa, removed from the glove box and frozen (and kept frozen) in liquid nitrogen; or into 2 mL screw-cap tubes capped with rubber septa, removed from the glove box and frozen at −80 °C. Fractions containing the highest concentration of pure f-HupS were used in EPR experiments. All solutions used in the purification step were deoxygenated by purging for at least 15 min with N_2 prior to use.

2.2. Sample preparation and flash photolysis

EPR samples were prepared from f-HupS that had either been directly added to EPR tubes immediately after purification in the glove box, or from stock solutions kept anaerobic outside the glove box as detailed above. In the latter case, transfer to the EPR tubes was performed under a flow of argon gas. $Ru(bpy)_3Cl_2$ (20 mM) and sodium ascorbate (2 M) stock solutions were prepared in buffer W, purged for at least 15 min with N_2 and kept in the dark until further use. $Ru(bpy)_3^{2+}$ and ascorbate were added anaerobically (under an argon gas flow) to each EPR tube, to a final concentration of 1.7 mM and 170 mM, respectively. All manipulations were at this stage performed under dim red light, chosen to avoid photoreactions by the sensitizer and electron donor. The final protein concentration in the EPR tubes was 13.6 μM. The samples were subjected to a train of laser flashes at room temperature and then immediately frozen at 77 K. Flashes were provided by a Nd:YAG laser (Spectra Physics, USA) at 532 nm, 850 mJ/pulse and a frequency of 1 Hz, or in some cases 5 Hz.

2.3. EPR spectroscopy

Samples were investigated by continuous wave X-band EPR directly as purified, after addition of $Ru(bpy)_3^{2+}$ and sodium ascorbate in the dark, and after flash photolysis. EPR measurements were performed on a Bruker ELEXYS E500 spectrometer using an ER049X SuperX microwave bridge, in a Bruker SHQ0601 cavity equipped with an Oxford Instruments continuous flow cryostat. Measurement temperature was 7 K, using an ITC 503 temperature controller (Oxford Instruments) and liquid helium as coolant. Signal processing and quantification was performed using the Xepr software package (Bruker).

3. Results and discussion

To achieve photoreduction of the isolated f-HupS protein, we used $Ru(bpy)_3^{2+}$ as photosensitizer and sodium ascorbate as electron donor. Scheme 1 illustrates the reactions involved in this system. When the photosensitizer is excited by a laser flash (Scheme 1, (1)) in the presence of an excess amount of ascorbate (ca. 100 times the concentration of $Ru(bpy)_3^{2+}$), the excited state $Ru(bpy)_3^{2+*}$ is reductively quenched by ascorbate (Scheme 1, (2)), generating $Ru(bpy)_3^{+}$ [23,24]. The reduction potential of $Ru(bpy)_3^{+}$ in aqueous solution at neutral pH is about −1.3 V *vs.* the standard hydrogen electrode (SHE) [25], which is theoretically well below the potentials of the FeS clusters in HupS and should therefore be sufficient to reduce all three clusters. The $Ru(bpy)_3^{+}$ then transfers an electron to the f-HupS protein (Scheme 1, (3)) where one of the FeS clusters is reduced. There is sufficient electron donor in the reaction mix to allow for this reaction cycle to occur several times on continued flashing.

FeS clusters are known to display changes in the UV-visible (UV–VIS) absorption spectrum upon reduction [26–28]. However, the presence of three FeS clusters with overlapping absorption spectra in the same protein makes the observation and assignment of reduction in the different clusters difficult. On the other hand, the spectral signatures of FeS clusters as observed by EPR spectroscopy are distinguishable from each other. In fact, we have previously observed the EPR signatures of all three FeS clusters in the isolated f-HupS [6]. We therefore utilized EPR spectroscopy to monitor the photoreduction of FeS clusters in f-HupS.

Fig. 1A shows the EPR spectrum of the medial, [3Fe–4S] cluster before and after flash photolysis of f-HupS at room temperature. When f-HupS is isolated from the cell culture, the medial cluster is in the oxidized form in a variable portion (15–45%) of the proteins, indicated by the presence of the medial cluster EPR signature (Fig. 1A, top spectrum). When $Ru(bpy)_3^{2+}$ and ascorbate were added to the protein in the dark, the EPR spectrum was virtually identical to that of the as-purified protein (not shown). This shows that, in the absence of light, the photosensitizer and in particular the electron donor were incapable of reducing the FeS clusters on their own, on the timescale of the experiment including freezing of the sample.

We then exposed samples containing f-HupS, $Ru(bpy)_3^{2+}$ and ascorbate to a train of 150 laser flashes at 532 nm, which efficiently excites $Ru(bpy)_3^{2+}$. The EPR signal from the [3Fe–4S] cluster then decreased in amplitude (Fig. 1A, middle spectrum). Fig. 1B shows a plot of the decrease in EPR signal intensity in five individual samples given different numbers of flashes. It shows how the EPR signal from the medial cluster decreased successively with higher number of flashes. After ca. 20 flashes, the signal amplitude was reduced by 50%, and the signal was completely absent after 190 flashes. This suggests that the reduction yield per flash is in the 5% range. A difference spectrum, obtained by subtracting the top and middle spectra in Fig. 1A, shows the EPR signal which had disappeared with a total number of 150 flashes (Fig. 1A, bottom spectrum). This $g = 2.023$ signal is typical of the oxidized [3Fe–4S]