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Photoinduced electron transfer from carboxymethylated cytochrome *c* to plastocyanin

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Abstract

Photoinduced electron transfer from cytochrome *c* to plastocyanin was investigated using a novel method. Reduced carboxymethylated cytochrome *c* (CmCyt *c*), with carbon monoxide bound to the heme iron, and oxidized plastocyanin were mixed. At 1 mM CO the reduced state of CmCyt *c* is stabilized by about 350 meV. After flash photolysis of CO the apparent redox potential of CmCyt *c* drops resulting in electron transfer to plastocyanin. The electron transfer characteristics were investigated at ~ 30 different wavelengths in the range 390–460 nm. A global fit of the data showed that the electron transfer rate is 960 ± 30 s^{-1} at pH 7.

Keywords: Flash photolysis; Kinetics; Photochemistry; Copper protein

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1. Introduction

Plastocyanin is a relatively small (10.4 kDa) water-soluble blue copper protein. Its main function is to carry electrons from the cytochrome b_6f complex, where it interacts with cytochrome *f*, to photosystem I in chloroplasts, where it donates the electron to P700 (for a review, see Ref. [1]). The interaction of plastocyanin with cytochrome *f* has been studied extensively. To investigate the electron transfer characteristics between plastocyanin and cytochrome *f*, cytochrome *c* is often used as a model because its structure is known in detail and its binding to plastocyanin is thought to be very similar to that of cytochrome *f* [2]. At low ionic strength, plastocyanin and cytochrome *c* form a complex due to noncovalent electrostatic interactions between the lysines surrounding the exposed heme edge of cytochrome *c* and the acidic amino acid residues in plastocyanin [3–5].

Electron transfer from reduced cytochrome *c* to oxidized plastocyanin can be studied by mixing the two proteins, but the reaction then also involves formation of the bimolecular complex, which may be rate limiting for the electron transfer. Electron transfer within the complex has been studied using a number of approaches including zinc-reconstituted cytochrome *c* [6,7], ruthenium-modified complexes [8,9], pulse radiolysis [10] and flavin [11], where

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electron transfer can be triggered by pulsed illumination. In the first case the driving force from the excited Zn–porphyrin to plastocyanin is about 1.2 eV and therefore the electron transfer rate is much faster than from the native cytochrome *c* [7]. In the latter cases the native cytochrome *c* is used, but it must first be reduced from an external source.

In this study a new method is presented which makes it possible to rapidly inject electrons photochemically from reduced cytochrome *c* to oxidized plastocyanin without the use of any external electron donors [12]. The sixth coordination ligand of the heme iron, methionine-80, was carboxymethylated [13–18], leaving a pentacoordinated iron ion [19] that can bind other ligands such as, e.g., CO. Only the ferrous form of carboxymethylated cytochrome *c* (CmCyt *c*) has a high affinity for CO and hexacoordinated heme proteins have a higher redox potential than their pentacoordinated forms. Therefore, upon binding of CO the apparent redox potential of the CmCyt *c* increases. This property was used to study electron transfer from CmCyt *c* to plastocyanin.

A complex of CmCyt *c*–CO and plastocyanin was formed by mixing the two proteins at low ionic strength. The CO ligand was dissociated by means of a short laser flash resulting in a drop of the apparent CmCyt *c* redox potential and electron transfer to plastocyanin. The electron transfer rate was found to be $960 \pm 30\ \mathrm{s}^{-1}$ at pH 7.

2. Materials and methods

2.1. Materials

Plastocyanin was prepared as described [20] and stored under liquid nitrogen. Carboxymethylated cytochrome *c* (CmCyt *c*) was prepared from horse heart cytochrome *c* (Sigma, type VI) as described earlier [18]. Cyanide was removed by reducing the protein with a small excess of sodium dithionite and subsequent passage through a Sephadex-G25 column equilibrated with 0.1 M potassium phosphate buffer at pH 7.0.

2.2. Sample preparation

Plastocyanin and CmCyt *c* were dissolved in a 3 mM MOPS buffer (pH 7.2) to concentrations of 70 μM and 6 μM, respectively. The solutions were made anaerobic by repetitive evacuation and replacement of air by N_2 on a vacuum line in modified anaerobic cuvettes. CmCyt *c* was reduced by dithionite at a final concentration of $\sim 80\ \mu$M (added from a 100 mM solution in 100 mM MOPS at pH ~ 7). The atmosphere in the cuvette was then replaced with CO and formation of the CmCyt *c*–CO complex was confirmed by its optical absorption spectrum. Optical absorption spectra were recorded on a Cary 4 UV/vis spectrophotometer from Varian. If necessary, the CO concentration in the CmCyt *c* solution was reduced by partially exchanging CO with N_2.

2.3. Time-resolved optical absorption spectroscopy

The protein solutions were transferred anaerobically to the drive syringes of a stopped-flow apparatus (Applied Photophysics, DX-17MV). In the stopped-flow apparatus they were mixed at a CmCyt *c*:plastocyanin ratio of 1:5. After mixing, the CmCyt *c* and plastocyanin concentrations were 5 and $\sim 12\ \mu$M, respectively. About 100 ms after mixing, CO was flashed off with a 10 ns, ~ 100 mJ laser flash at 532 nm (Nd-YAG laser from Spectra Physics) and electron transfer from CmCyt *c* to plastocyanin was followed at various wavelengths. The cuvette path length was 1.00 cm. The output from the photomultiplier was connected to a 3 MHz current-to-voltage converter and a preamplifier with a variable RC filter (Tektronix, model AM 502). The output voltage was recorded using a digital transient recorder (Nicolet, model 490). Rate constants were determined using a non-linear fitting algorithm on a personal computer.

3. Results and discussion

Flash photolysis of the CmCyt *c*–CO complex resulted in dissociation of CO, followed by recombination with a rate constant of $1.3 \times 10^3\ \mathrm{s}^{-1}$ at ~ 1 mM CO, consistent with the previously determined second order recombination rate constant of $1.6 \times 10^6\ \mathrm{M}^{-1}\mathrm{s}^{-1}$ [16]. In the experiments described below, the CO concentration was reduced to about 0.1 mM to slow the CO recombination (Fig. 1).

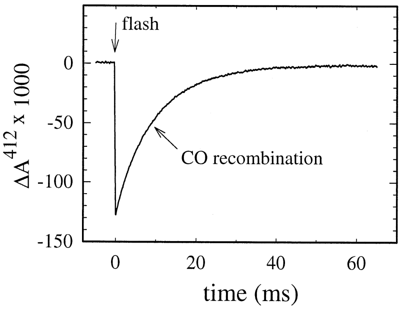

Fig. 1. Absorbance changes at 412 nm following flash-induced dissociation of CO (decrease in absorbance at $t=0$) from reduced CmCyt *c*. The recombination rate constant is 1.3×10^3 s^{-1}. Conditions: 1 μM CmCyt *c*, 3 mM MOPS, pH 7.2, [CO] ≅ 0.1 mM, 22 ± 1°C.

CmCyt *c* and plastocyanin were mixed in a stopped-flow device at a volume ratio of 1:5. A low ionic strength (see legend of Fig. 2) was used to ensure complex formation after mixing. About 100 ms after mixing, the sample was illuminated by a short laser flash, which resulted in a rapid increase in absorbance at 400 nm, associated with CO dissociation, followed by a slower increase in absorbance with a rate constant of 960 s^{-1} (Fig. 2, upper trace). The 960 s^{-1} phase was not observed after increase of the ionic strength to ∼300 mM KCl, which prevents formation of the CmCyt *c*–plastocyanin complex (Fig. 2, lower trace) and indicates that the slower increase in absorbance was associated with electron transfer from the reduced CmCyt *c* to the oxidized plastocyanin.

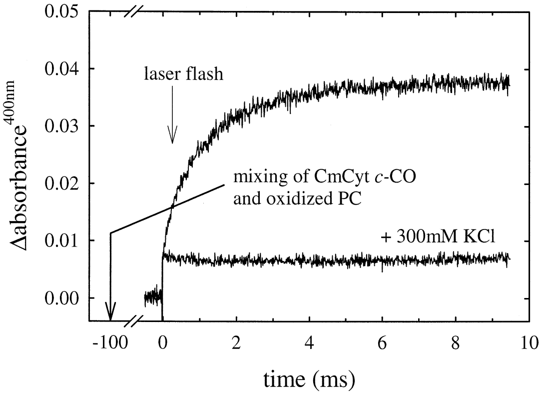

Fig. 2. Absorbance changes at 400 nm upon pulsed illumination of CmCyt *c*–CO about 100 ms after mixing with oxidized plastocyanin (PC). The rapid increase in absorbance is associated with dissociation of CO. The following, slower increase is associated with electron transfer from reduced CmCyt *c* to oxidized plastocyanin (PC) (upper trace). The lower trace shows the same experiment but in the presence of ∼300 mM KCl. Conditions after mixing: 5 μM CmCyt *c*, 12 μM plastocyanin, 60 μM dithionite, 3 mM MOPS, pH 7.2, [CO] ≅ 0.1 mM, 22 ± 1°C.

Measurements at 27 different wavelengths in the range 390 nm to 460 nm were done and the amplitudes of the kinetic components were determined from a multiwavelength exponential fitting. After filtering the absorbance data using Singular Value Decomposition (SVD), exponential functions were fitted to all wavelengths simultaneously. The absorbance change following CO dissociation was found to have a rate constant of 960 ± 30 s^{-1}.

Fig. 3A shows a kinetic difference spectrum of the rapid, unresolved CO-dissociation absorbance

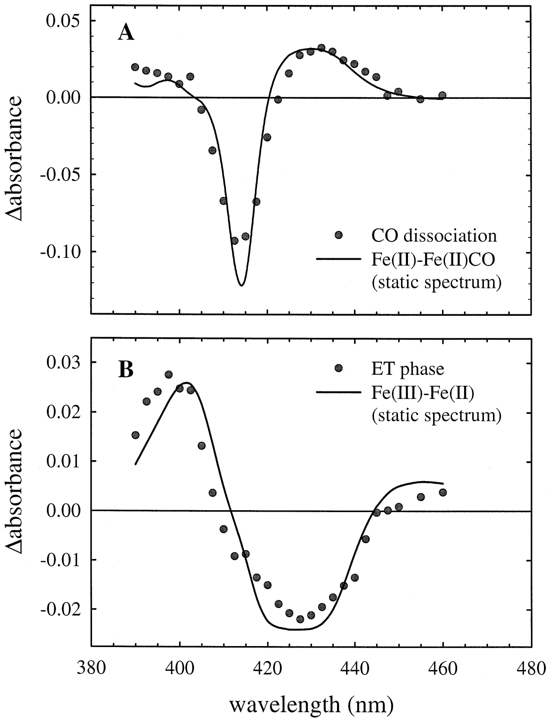

Fig. 3. (A) The initial rapid absorbance change upon pulsed illumination (filled circles, see Fig. 2) at different wavelengths, shown together with the static optical difference spectrum of ferro minus carboxy-ferro CmCyt *c* (solid line) that was scaled to a concentration of 1.2 μM. (B) Kinetic difference spectrum for the 960 s^{-1} phase following CO dissociation (solid circles, see Fig. 2) together with a static optical absorption difference spectrum of oxidized-minus-reduced CmCyt *c*.

change.[1] As shown in the figure it fits well with a static difference spectrum of the ferrous CmCyt *c* minus ferrous CmCyt *c*–CO complex, corresponding to 1.2 ± 0.1 μM CmCyt *c*. A kinetic difference spectrum of the 960 s^{-1} phase is shown in Fig. 3B. It was well fitted with a static difference spectrum of ferrous minus ferric CmCyt *c*, which also indicates that this phase was associated with electron transfer from ferrous CmCyt *c* to cupric plastocyanin after CO dissociation. From the spectrum in Fig. 3A the concentration oxidized CmCyt *c* was determined to be 1.3 ± 0.2 μM, which shows that the yield of electron transfer was ~100% from those CmCyt *c* in which CO was dissociated. Absorbance changes due to redox changes in plastocyanin between 390 nm and 460 nm are negligible compared to those from CmCyt *c*.

As discussed above, the CmCyt *c*–CO recombination rate at ~1 mM CO was 1.3×10^3 s^{-1}, which is about the same as that of electron transfer. Consequently, to reduce the fraction CmCyt *c* in which CO recombines prior to electron transfer, the CO concentration was reduced by partly exchanging the CO atmosphere in the cuvette by nitrogen. However, the CO concentration must not be too low because the apparent redox potential of the CmCyt *c*–CO complex decreases with decreasing CO concentration (cf. Ref. [12]). Therefore, the CO concentration must be adjusted so that the apparent redox potential of the complex is higher than that of the electron acceptor (to avoid electron transfer prior to CO dissociation) yet the CO-recombination rate is much slower than that of electron transfer (to avoid CO recombination before electron transfer).

The redox potential of free plastocyanin is about +380 mV vs. NHE [20]. Since there is almost 100% electron transfer from CmCyt *c* in which CO is dissociated to plastocyanin, the redox potential of CmCyt *c* in the complex with plastocyanin must be significantly lower than +380 mV. On the other hand, electron transfer from the CmCyt *c*–CO complex did not take place prior to CO photolysis, which implies that in the complex with plastocyanin, the redox potential of carboxy CmCyt *c* was significantly higher than +380 mV. The maximum change in the apparent redox potential of CmCyt *c* after CO dissociation is approximately 350 mV at 1 mM CO because the CO binding constant is 10^6 mM^{-1} (at pH 5.9) [16], i.e., at equilibrium, CO stabilises the reduced state by $59 \log^{10}(10^6[mM^{-1}]1[mM])$ [meV] ≅ 350[meV]. At about 0.1 mM CO (as used in these experiments), the change in redox potential is about 300 mV.

Assuming a redox potential of at least 380 mV for the CmCyt *c*–CO complex, the redox potential of CmCyt *c* must be at least 80 mV (but lower than 380 mV, see above), which is similar to that of myoglobin (~50 mV), which confines a high-spin heme in a cavity protected from water exposure [21].

The redox potential of CmCyt *c* in aqueous solution has been reported to be −218 mV [22]. The approximately 0.5 V lower redox potential as compared to the native cytochrome *c* [23] has been attributed to a larger degree of water exposure [22] in the modified protein, which stabilises the ferric form [24].

In the experiments described in this work, electron transfer from CmCyt *c* to plastocyanin takes place within about 1 ms after dissociation of CO. If binding of CO reduces the access of water to the heme, and if the displaced water binds much slower than 1 ms, after dissociation of CO, the electron transfer would take place without this water, which would increase the apparent redox potential of CmCyt *c* on the time scale of the electron transfer. Another possibility is that upon formation of the CmCyt *c*–plastocyanin complex, water is likely to be released from the contact surface. As docking most likely occurs near the heme edge [25], this results in less water around the heme, thus increasing the CmCyt *c* redox potential [24]. The value estimated above (~80 mV) is between those of CmCyt *c* in solution and of the native cytochrome *c*.

The docking of CmCyt *c* with plastocyanin most likely occurs near the heme edge of CmCyt *c* and the negatively-charged patch on the plastocyanin surface. The binding is presumably non-specific but most likely involves lysin residues on the cytochrome *c* surface [25]. During preparation of CmCyt *c*, in addition to methionine, also one or more lysin residues may be modified. However, it has been shown that the second-order rate constant for binding of cytochrome *c* to plastocyanin decreases by at most a

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[1] Difference in absorbance after and before the flash.

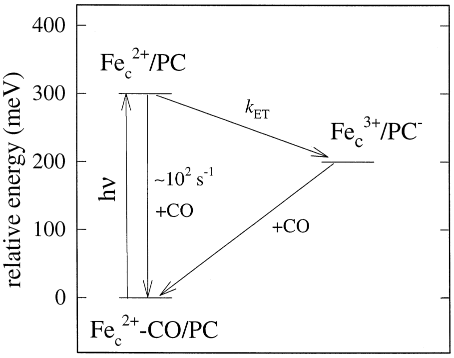

Fig. 4. Energy-level diagram which models the electron transfer after dissociation of CO from the CmCyt *c*–CO complex in the presence of oxidized plastocyanin (PC).

factor of two upon modification of any of the lysins involved in binding [25]. Therefore, the CmCyt *c* preparation procedure is not likely to introduce any significant changes in the binding properties, which is also evident from the fact that a complex is formed upon mixing the two proteins at low ionic strength.

As discussed above, the driving force for electron transfer from CmCyt *c* to plastocyanin is larger than that from cytochrome *c*. However, this is not expected to alter the electron transfer rate as it is most likely preceded by a reorientation within the complex, i.e., the electron transfer is gated (for review see Ref. [1]). The intracomplex electron transfer rate observed in this study (960 s^{-1}) falls in the range of rates observed previously (5×10^2 s^{-1}–2×10^3 s^{-1}) between cytochrome *c* and plastocyanin using other methods ([10,11,26], see Section 1).

Fig. 4 shows an energy-level diagram which models the experiments described in this work. When CO is flashed off from the CmCyt *c*–plastocyanin complex, the apparent redox potential of CmCyt *c* decreases, which results in electron transfer to plastocyanin. If no reductant was present in the solution, eventually, CO would recombine with CmCyt *c* and the system would return to the starting level. However, in our experiments the oxidized CmCyt *c* may be re-reduced by the excess dithionite which leaves both CmCyt *c* and plastocyanin reduced.

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