Ligand-induced monomerization of *Allochromatium vinosum* cytochrome *c'* studied using native mass spectrometry and fluorescence resonance energy transfer

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Abstract Cytochrome *c'* from *Allochromatium vinosum* is an attractive model protein to study ligand-induced conformational changes. This homodimeric protein dissociates into monomers upon binding of NO, CO or CN[−] to the iron of its covalently attached heme group. While ligand binding to the heme has been well characterized using a variety of spectroscopic techniques, direct monitoring of the subsequent monomerization has not been reported previously. Here we have explored two biophysical techniques to simultaneously monitor ligand binding and monomerization. Native mass spectrometry allowed the detection of the dimeric and monomeric forms of cytochrome *c'* and even showed the presence of a CO-bound monomer. The kinetics of the ligand-induced monomerization were found to be significantly enhanced in the gas phase compared with the kinetics in solution, however. Ligand binding to the heme and the dissociation of the dimer in solution were also studied using energy transfer from a fluorescent probe to both heme groups of the protein. Comparison of ligand binding kinetics as observed with UV–vis spectroscopy with changes in fluorescence suggested that binding of one CO molecule per dimer could be sufficient for monomerization.

Keywords Fluorescence resonance energy transfer · Heme protein · Ligand-induced conformational change · Native mass spectrometry · Monomerization

Abbreviations

cyt- <i>c'</i>	Cytochrome <i>c'</i> from <i>Allochromatium vinosum</i>
DTT	Dithiothreitol
ESI	Electrospray ionization
FRET	Fluorescence resonance energy transfer
MBP	Maltose binding protein
MS	Mass spectrometry
OG488	Oregon Green 488 maleimide
sGC	Soluble guanylate cyclase
Tris/HCl	Tris(hydroxymethyl)aminomethane hydrochloride

Introduction

Ligand-induced conformational changes of proteins are crucial for the regulation of protein activity in many biological processes. The binding of diatomic gases such as O₂, CO or NO to gas-sensing heme proteins illustrates that even the binding of small molecules can cause large rearrangements in a protein's secondary, tertiary or quaternary structure [1]. Binding of O₂ to the regulatory heme-PAS domain of FixL is involved in the regulation of gene expression in nitrogen-fixing bacteria by inactivating the FixL kinase domain [2]. CO binding to the heme of the transcription factor CooA activates its DNA binding domain, initiating the transcription of enzymes that allow the photosynthetic bacterium *Rhodospirillum rubrum* to grow on CO as a sole energy source [3]. The generation of the second messenger 3'-5'-cyclic guanosine monophosphate by soluble guanylate cyclase (sGC) in mammals is

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regulated by binding of NO or CO [4]. Via this pathway, NO is involved in various physiological processes such as immune response, vasodilation and neurotransmission [5].

The cytochromes c' are a widespread class of cytochromes found in the periplasm of photosynthetic, denitrifying, nitrogen-fixing and sulfur-oxidizing bacteria [6] and show many similarities to heme-based gas-sensor proteins. They consist of a four-helix bundle with a heme group covalently attached to a CKXCH motif near the C-terminus. Unlike the six-coordinated iron in other cytochromes, the heme iron in cytochromes c' is five-coordinate with a vacant distal coordination site. Similar to sGC, cytochromes c' can bind small ligands like NO, CO and CN^- , but not O_2 . Despite their ubiquity in bacteria, the biological role of cytochromes c' remains unclear. Although a role in electron transfer processes had been assumed for a long time [7], more recently it has been suggested that cytochromes c' are involved in protection against high levels of NO [8, 9]. Scientific interest in cytochromes c' has increased further owing to the similarities in spectral properties and ligand binding properties between cytochrome c' from *Alcaligenes xylosoxidans* and sGC [10, 11]. Its CO and NO binding properties have been applied in electrochemical [12, 13] and fiberoptic fluorescent biosensors [14, 15]. Most cytochromes c' are isolated as stable homodimers, although some occur as monomers or as a mixture of both [16]. Cytochrome c' from the purple photosynthetic bacterium *Allochromatium vinosum* (cyt- c') displays a unique dimer-to-monomer transition upon binding of NO, CO, CN^- or small alkyl isocyanides [9, 17–19]. Like in many gas-sensing proteins [4, 20], the selectivity in ligand binding arises from steric hindrance of the vacant coordination site [21]. In cyt- c' , the vacant site is only accessible to the solvent via a narrow channel in the protein. Tyr16 blocks the distal coordination site and has to be displaced to allow ligand binding to the heme. This displacement probably induces a movement of helix A, which is believed to lead to disruption of the dimer interface, consisting of helices A and B [21]. Monomerization of cyt- c' can be considered as an extreme case of a ligand-induced conformational change and therefore provides an attractive model system to study ligand-induced conformational changes in general and those of the heme-based biosensors in particular.

The heme group is an excellent spectroscopic probe to monitor ligand binding to heme proteins. In contrast, the characterization of the accompanying protein conformational changes has proven to be much more difficult. Although X-ray crystallography and mutational analysis can help to understand how binding of a small ligand can result in a large conformational change [1], additional research tools are needed that give more insight into the nature and kinetics of these conformational changes in solution. Here,

we have explored two techniques that allow the simultaneous monitoring of ligand binding and monomerization of cyt- c' . Native mass spectrometry (MS) was applied to distinguish between monomers and dimers and allowed the detection of CO binding to the protein. In addition, a fluorescent probe was introduced at a specific position in the protein. Fluorescence resonance energy transfer (FRET) between this probe and the two heme groups of the cyt- c' dimer was used to study the kinetics of both ligand binding and the subsequent monomerization.

Materials and methods

General

UV–vis spectra were recorded using a Shimadzu Multispec 1501 photodiode array spectrophotometer. The UV lamp was switched off during kinetic measurements to prevent photochemical reactions. Protein concentrations were determined using a molar extinction coefficient of $87,000 \text{ M}^{-1} \text{ cm}^{-1}$ per oxidized heme at 400 nm [22] and $81,000 \text{ M}^{-1} \text{ cm}^{-1}$ at 490 nm for Oregon Green 488 maleimide (OG488). For all UV–vis and fluorescence spectroscopic measurements performed at 4 °C, the spectrophotometers were equipped with a Peltier-cooled cuvette holder (PerkinElmer) and the measurement chamber was filled with argon gas to prevent condensation.

Mutagenesis of cyt- c'

The construction of the expression vector for cyt- c' (pMAL-CCP) was previously described [23]. This vector encodes for a fusion protein of cyt- c' with a maltose binding protein (MBP) containing an N-terminal periplasmic leader sequence. The T104C mutation was introduced using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) and the following primers: 5'-GCTGAAGTGGCCGCTGCGGTGAGGCCGAGGC-3' and 5'-GCCTCGGCCTACCGCAGGCGGCCACTTCAGC-3'. Correct open reading frames were confirmed by DNA sequencing (BaseClear, Leiden, The Netherlands).

Protein production and purification

Wild-type cyt- c' and cyt- c' -T104C were produced and purified as described before [23]. Briefly, proteins were isolated as MBP fusion proteins from the periplasm of *Escherichia coli* JCB712 cells that were cotransformed with the appropriate expression vector and a pEC86 plasmid that contains eight cytochrome c maturation genes. Fusion proteins were purified using amylose affinity chromatography using amylose resin from New England

Biolabs. MBP was cleaved from the protein by digesting the protein with Factor Xa (Novagen) in the presence of 0.01% (w/v) sodium dodecyl sulfate (SDS) for 8 h at 30 °C. The proteins were subsequently purified from MBP using one or two additional rounds of amylose affinity chromatography and finally by ion-exchange chromatography on a Bio-Rad UNO Q1 column. Protein purity was confirmed by SDS polyacrylamide gel electrophoresis and UV–vis analysis. Pure protein was concentrated to 100–150 μM in 100 mM potassium phosphate, pH 7.0, frozen in liquid nitrogen and stored at $-80\text{ }^{\circ}\text{C}$. Liquid chromatography–electrospray ionization (ESI) MS analysis was used to verify the identity of the proteins. Protein samples were injected on a Prosphere P-HR 4 μ reversed-phase column (Alltech), equilibrated with 20% (v/v) acetonitrile, 0.1% formic acid in water. Proteins were eluted with a 20–70% (v/v) acetonitrile gradient and analyzed with a Thermo Finnigan LCQ Deca ESI ion-trap mass spectrometer. Data deconvolution was performed using the Micromass MaxEnt 1 algorithm.

ESI MS of native cyt-*c'*

ESI mass spectra of native cyt-*c'* were recorded with an Ultima GLOBAL quadrupole time-of-flight mass spectrometer (Micromass) equipped with a Z-spray source. Protein samples were injected in the flow injection analysis mode. Ammonium acetate (50 mM), pH 6.8 in water was pumped with a Shimadzu LC-10ADvp at a flow rate of 300 $\mu\text{L min}^{-1}$. ESI was achieved in the positive ion mode by application of 3.5 kV on the needle. The source block temperature was maintained at 80 °C and the desolvation gas was heated to 60 °C. The pressure in the first vacuum stage was kept at 4.7–4.8 mbar for all experiments. To obtain the spectrum of ferric cyt-*c'*, 2 μL of 20 μM cyt-*c'* in 50 mM ammonium acetate, pH 6.8 was injected into the mass spectrometer. The spectrum of ferrous cyt-*c'* was obtained by incubation of a similar sample with 10 mM dithiothreitol (DTT) prior to injection into the mass spectrometer. CO-ligated ferrous cyt-*c'* was prepared by 1:1 dilution of the ferrous cyt-*c'* with CO-saturated 50 mM ammonium acetate, pH 6.8, after which 2 μL of this solution was injected into the mass spectrometer. After 2 h, a second 2- μL sample of the reaction mixture was injected into the mass spectrometer.

The kinetics of CO binding to reduced cyt-*c'* were monitored by preparing a solution of 4 μM cyt-*c'* in 50 mM ammonium acetate, 10 mM DTT, pH 6.8, purged with helium gas and cooled to approximately 4 °C on ice. Two microliters of this solution was injected into the mass spectrometer to obtain the abundances of each ion prior to reaction with CO. After addition of 100 μM CO from CO-saturated 50 mM ammonium acetate, pH 6.8, 4 °C, the

reaction mixture was rapidly mixed and transferred into a 100- μL precooled gas-tight syringe. The syringe was constantly kept on ice and 2- μL samples from the reaction mixture were injected into the mass spectrometer at different time points. The ion currents for m/z 1,030 $\pm$ 4, 1,110 $\pm$ 4, 1,202 $\pm$ 4, 1,331 $\pm$ 4, 1,442 $\pm$ 4, 1,602 $\pm$ 4, 1,805 $\pm$ 5, 2,063 $\pm$ 5, 2,406 $\pm$ 5, 2,885 $\pm$ 5 and 3,206 $\pm$ 5 were plotted as a function of time and the area under the peak of each injection was calculated for these ions using MassLynx software. The relative abundance for the folded monomer was calculated by dividing the abundances of the m/z 1,805, 2,063 and 2,406 ions by the total abundance of all ions. Similarly, the relative abundance for the dimer was calculated by adding the abundances of the m/z 2,885 and 3,206 ions and dividing this by the total abundance of all ions. The relative abundances for the monomer and the dimer were plotted as a function of time, with the first measurement after addition of CO taken as $t = 0$. The resulting curves were fit with Eq. 1:

$$y = A_0 + A_1[1 - \exp(-k_1t)] + A_2[1 - \exp(-k_2t)]. \quad (1)$$

The same reaction was also monitored using UV–vis spectroscopy at 4 °C. A cuvette was filled with helium gas and sealed with a rubber septum. A solution of 4 μM cyt-*c'* in helium-saturated 50 mM ammonium acetate, 10 mM DTT, pH 6.8, 4 °C was transferred to the cuvette. While spectra were being taken every 6 s 100 μM CO was added from a CO-saturated solution of 50 mM ammonium acetate, pH 6.8, 4 °C. The absorbance at 418 nm, characteristic for the CO adduct, was plotted as a function of time, using the first measurement after addition of CO as $t = 0$. The resulting curve was fit using Eq. 2:

$$y = A_0 + A_1[1 - \exp(-k_1t)]. \quad (2)$$

Fluorescent labeling of cyt-*c'*-T104C

Cyt-*c'*-T104C was incubated with 5 mM DTT (Sigma) for 2 h at room temperature to disrupt the formation of disulfide-bridged oligomers and to remove glutathione adducts. Using a Zeba desalt spin column (Pierce), the DTT was removed from the solution and the buffer was exchanged for argon-purged 100 mM potassium phosphate, 1 mM tris(2-carboxyethyl)phosphine, pH 7.0. The protein was then reacted overnight with a 20 times molar excess of OG488 (Invitrogen) at 4 °C under argon. Unreacted OG488 was removed using either 200- μL dialysis buttons (Hampton Research) fitted with a 6–8-kDa molecular mass cutoff membrane (Spectropore), a Zeba desalt spin column or an Amicon Ultrafree centrifugal filter (Millipore, 10-kDa molecular mass cutoff). None of these methods were able to completely remove the unreacted label (typically

5–10% of the remaining OG488). OG488-labeled glutathione, obtained by reaction of OG488 with an excess of reduced glutathione (Sigma) was used as a control for fluorescence experiments.

Fluorescence spectroscopy

Fluorescence emission spectra were recorded using a PerkinElmer LS 50B photoluminescence spectrometer fitted with a red-sensitive R928 detector and were corrected for the wavelength-dependent efficiency of the instrument. Time-resolved fluorescence decays were recorded using an Edinburgh Instruments time-correlated single photon counting Lifespec-PS spectrometer, consisting of a 400-nm picosecond laser (PicoQuant PDL 800B) operated at 2.5 MHz, an emission monochromator and a Peltier-cooled Hamamatsu microchannel plate photomultiplier (R3809U-50). Decays were recorded at an emission wavelength of 516 nm in 4,096 channels, divided over a 50-ns window with a 4-ns delay until 10,000 counts in the peak channel were obtained.

For glutathione–OG488, the decay was recorded of a 1 μ M solution in 100 mM tris(hydroxymethyl)amino-methane hydrochloride (Tris/HCl), 50 mM NaCl, pH 8.0, 4 °C. The fluorescence decay of 1 μ M cyt-*c'*-T104C-OG488 in 20 mM Tris/HCl, 20 mM NaCl, pH 7.0, 4 °C was recorded using the same instrumental setup. For the fluorescence decay of CO-ligated cyt-*c'*-T104C-OG488, a quartz-glass microcuvette (Hellma) was sealed with a rubber septum and filled with 1 atm CO gas (Hoekloos) by repeated vacuum–CO cycles and cooled to 4 °C. 150 μ l of CO-saturated 20 mM Tris/HCl, 20 mM NaCl, pH 7.0, 4 °C was transferred to the cuvette with a gas-tight syringe and the pressure in the headspace of the cuvette was adjusted with CO gas to 1 atm. By extrapolation of CO solubility data as a function of temperature [24], the CO concentration in solution was determined as 1.44 mM. Cyt-*c'*-T104C-OG488 (1 μ M) and 5 mM DTT were added to the solution and the fluorescence decay was recorded. Decays were fit with PicoQuant FluoFit Pro version 4.1.1 using a monoexponential decay model to describe the decay of glutathione–OG488 and a biexponential decay model to fit the decay of cyt-*c'*-C132-OG488. The goodness of fit was judged from a value of reduced χ^2 near 1 and random distributions of the residuals and autocorrelation traces around 0. From the lifetimes obtained from these fits, energy transfer efficiencies and distances were calculated, using values of 0.9 for the quantum yield Q_D , 2/3 for the orientation factor κ^2 and 1.4 for the refraction index n . Values for the overlap integral $J(\lambda)$ were calculated using the normalized emission spectrum of glutathione–OG488 and the absorption spectra of ferric cyt-*c'* and CO-ligated ferrous cyt-*c'* that were published previously [23].

Kinetics of CO binding to cyt-*c'*-T104C-OG488

The kinetics of the reaction of CO with cyt-*c'*-T104C-OG488 were monitored using UV–vis and fluorescence spectroscopy. A 1 μ M solution of cyt-*c'*-T104C-OG488 in CO-saturated 20 mM Tris/HCl, 20 mM NaCl, pH 7.0, 4 °C was prepared as described in the previous section and the reaction was initiated by addition of 5 mM DTT. The reaction was monitored by recording a UV–vis spectrum every 6 s. The absorbance at 418 nm was plotted as a function of time and fit with Eq. 1. The same reaction was also monitored by fluorescence spectroscopy using a PerkinElmer LS50B using the same setup as described earlier and recording the emission at 516 nm after 490-nm excitation during the entire reaction. A plot of the emission intensity as a function of time was fit with Eq. 1.

Results

Monitoring ligand-induced monomerization using native MS

Recent developments in MS instrumentation have enabled the characterization of native, folded proteins and large protein complexes that are held together by weak noncovalent interactions [25, 26]. Several studies have reported the use of this so-called native MS to study protein folding and cofactor binding [26, 27]. Since MS, unlike many spectroscopic techniques, can detect all species present in solution, we explored its suitability for monitoring the CO-induced conformational changes in cyt-*c'*.

Figure 1a shows the spectrum of oxidized cyt-*c'* in 50 mM ammonium acetate, pH 6.8. The narrow distribution of peaks with a high mass-to-charge (m/z) value is characteristic for a folded protein, which can accumulate relatively few charges at its surface. Deconvolution of the spectrum identifies the observed peaks as the 9+, 10+ and 11+ species of the dimer with a mass of 28,816 Da, which is exactly the calculated theoretical mass. Unfolded cyt-*c'* was previously shown to give a much broader m/z distribution at higher charges (8+ to 21+) [23]. To study CO binding to cyt-*c'*, the protein was first reduced with 10 mM dithiothreitol (DTT), as CO can readily bind to ferrous cyt-*c'*, whereas it is unreactive with ferric cyt-*c'* [28]. Reduction of cyt-*c'* did not significantly alter the mass spectrum, except for the appearance of small peaks at lower m/z values (Fig. 1b). Although these m/z values are consistent with the 12+, 14+ and 16+ peaks of the dimer, a more likely explanation is that they correspond to the 6+, 7+ and 8+ peaks of the cyt-*c'* monomer. Proteins with a lower molecular mass generally accumulate relatively more charges and the absence of peaks (13+ and 15+) from a

Fig. 1 Electrospray ionization mass spectrometry of native cytochrome *c'* from *Allochromatium vinosum* (cyt-*c'*) in 50 mM ammonium acetate, pH 6.8. Sets of peaks corresponding to the dimeric, monomeric or unfolded monomeric species are indicated. **a** Mass spectrum of ferric cyt-*c'*, showing only dimer peaks. **b** Mass spectrum of ferrous cyt-*c'* obtained after reduction with 10 mM

dithiothreitol (DTT). **c** Mass spectrum taken immediately after 1:1 dilution of the ferrous cyt-*c'* with CO-saturated 50 mM ammonium acetate, pH 6.8 at room temperature. **d** Mass spectrum of the fully CO-ligated ferrous cyt-*c'*. The inset is a zoom of the 7+ peak of the monomer, clearly showing the CO adduct

charge envelope is highly unusual. The appearance of monomer after reduction of cyt-*c'* by DTT could reflect the situation in solution, but might also indicate that the reduced form is less stable under the ionization conditions. Immediately after addition of an excess of CO to the reduced protein, the abundance of the 9+, 10+ and 11+ peaks of the dimer decreased and the intensity of the 6+, 7+, 8+ and 9+ peaks of the monomer increased (Fig. 1c). In addition, a third set of small peaks appeared between *m/z* 1,000 and 1,500, corresponding to the 10+ to 14+ ions that probably correspond to denatured monomer. After 1 h, the peaks of the dimer had almost disappeared, while the monomer peaks had further increased in absolute intensity (Fig. 1d). Closer inspection of, e.g., the 7+ peak at *m/z* 2,060 revealed an additional adduct at *m/z* 2,064 (Fig. 1d, inset). This difference in *m/z* corresponds to a mass difference of 28 Da, which is exactly the mass of one CO molecule. Similar adducts were also observed for the 6+, 8+ and 9+ peaks. For the 10+ to 14+ ions of the monomer these adducts were not detected, confirming that these peaks represented denatured protein.

In order to test whether native MS could also be used to study the kinetics of the monomerization reaction, we compared the kinetics of the decrease of the dimer peaks and the increase of the monomer peaks in the mass spectrum with the kinetics of CO binding to cyt-*c'* as observed using

UV–vis spectroscopy (Fig. 2). Successive injections of 2- μ l samples from the reaction mixture were performed at various times after CO addition. By plotting the ion current for each ion species in the mixture, we obtained the abundance of each ion by integrating the area under the curve for each injection. The kinetics observed by MS appeared biphasic and were dominated by a fast component with a rate constant of approximately $6 \times 10^{-3} \text{ s}^{-1}$, followed by a slower process with a rate constant of $4 \times 10^{-4} \text{ s}^{-1}$. The same reaction was also monitored with UV–vis spectroscopy by detecting the formation of the CO adduct at 418 nm. The optical absorbance at 418 nm could be fit satisfactorily using just a single rate constant of $9.1 \times 10^{-4} \text{ s}^{-1}$. The discrepancy between the UV–vis and MS data suggests that the relative abundances of the separate species are affected by the ionization and transmission through the mass spectrometer.

Monitoring ligand-induced monomerization using FRET

Because the monomerization kinetics as observed with native MS might have been affected by the ionization conditions, an independent method was sought to study the ligand-induced dissociation of the dimer in solution. FRET, the nonradiative transfer of excitation energy from a donor fluorophore to an acceptor chromophore, can be used to

Fig. 2 Kinetic measurements of the reaction of 4 μM ferrous cyt- c' with 100 μM CO in 50 mM ammonium acetate, pH 6.8 at 4 $^{\circ}\text{C}$. **a** Relative abundance of the 6+, 7+ and 8+ peaks of monomeric cyt- c' . The solid line is a fit of the data using Eq. 1 and $A_1 = 75\%$, $k_1 = 5.7 \times 10^{-3} \text{ s}^{-1}$, $A_2 = 25\%$ and $k_2 = 4 \times 10^{-4} \text{ s}^{-1}$. **b** Relative abundance of the 9+ and 10+ peaks of the cyt- c' dimer. The solid line is a fit of the data using Eq. 1 and the same values as used for the monomeric species. **c** UV-vis absorbance at 418 nm. The data were fit with Eq. 2 and $k_1 = 9.1 \times 10^{-4} \text{ s}^{-1}$ (solid line)

probe changes within or between proteins with nanometer or even angstrom resolution. The energy transfer efficiency E is dependent on the distance between the two chromophores r and the Förster distance R_0 of the two chromophores [29]:

$$E = \frac{R_0^6}{R_0^6 + r^6}. \quad (3)$$

The Förster distance is dependent on the relative orientation of the donor and acceptor transition dipole moments κ^2 , the overlap of the donor emission and the acceptor absorption spectra $J(\lambda)$, the quantum yield of the donor Q_D and the refractive index of the medium n according to

$$R_0 = 0.211 [\kappa^2 n^{-4} Q_D J(\lambda)]^{1/6} \quad (4)$$

Although FRET from a fluorescent donor is most commonly used in combination with a fluorescent acceptor, nonfluorescent chromophores can also function as an acceptor. For example, energy transfer from a fluorophore to a prosthetic heme group has been used to study protein interactions [30, 31], folding dynamics [32, 33], protein redox state [34] and ligand binding to the heme [14, 31]. In the last two cases, changes in FRET are not due to a change in the distance between donor and acceptor, but are based on a change in spectral overlap. Cyt- c' provides a rare opportunity to use FRET to simultaneously study ligand binding to the heme and the subsequent monomerization, as disruption of the dimer will remove one of the two quenching heme groups from the fluorescent donor.

In order to allow site-specific introduction of fluorophores, we recently developed an efficient expression system for recombinant cyt- c' [23]. Thr104, located in the short loop between helices C and D, was replaced by a cysteine residue, resulting in cyt- c' -T104C (Fig. 3a). This position was chosen such that both hemes would be at equal distances to an attached fluorescent probe. The UV-vis spectrum of cyt- c' -T104C (Fig. 3b) was similar to that of cyt- c' , indicating that the heme environment is identical for both proteins. The circular dichroism spectrum of cyt- c' -T104C was also identical to that of cyt- c' (Fig. S1), providing further evidence that the mutant protein was folded correctly. Cyt- c' -T104C was less stable than cyt- c' at higher temperatures, showing a melting point of 40 $^{\circ}\text{C}$ [23]. To incorporate a fluorescent probe at position 104, cyt- c' -T104C was reduced and subsequently reacted with the fluorescent dye OG488. After removal of unreacted dye, UV-vis analysis of the labeled protein cyt- c' -T104C-OG488 showed an additional strong absorption peak at 490 nm (Fig. 3b) and MS of the protein showed a mass increase of 465 Da, consistent with the addition of one fluorescent label per monomer (Table S1).

Comparison of the fluorescence emission intensity of cyt- c' -T104C-OG488 with OG488 coupled to reduced glutathione (glutathione-OG488) indicated that the fluorescence of cyt- c' -T104C-OG488 was quenched to a large extent by the nearby heme groups (Fig. 3c). The high energy transfer efficiency was confirmed by the decrease in fluorescence lifetime from 4.1 ns for unquenched OG488 to 0.85 ns for cyt- c' -T104C-OG488

Fig. 3 Fluorescent labeling of cyt-*c'*-T104C with Oregon Green 488 maleimide (OG488). **a** 3D model of cyt-*c'*-T104C-OG488, showing the calculated distances between the fluorophore and the heme on the same monomer (r_1) and the opposite monomer (r_2), based on Protein Data Bank entry 1BBH [21]. **b** UV-vis spectra of cyt-*c'*-T104C (solid line) and cyt-*c'*-T104C-OG488 (dashed line) in 20 mM Tris(hydroxymethyl)aminomethane hydrochloride (Tris/HCl), 20 mM NaCl, pH

7.0. **c** Fluorescence emission spectra of 1 μ M glutathione-OG488 (solid line) and cyt-*c'*-T104C-OG488 (dashed line) in 20 mM Tris/HCl, 20 mM NaCl, pH 7.0, excited at 450 nm. **d** Fluorescence decays of glutathione-OG488 (blue) and cyt-*c'*-T104C-OG488 in the absence (green) and presence (red) of 1.44 mM CO in 20 mM Tris/HCl, 20 mM NaCl, 5 mM DTT, pH 8.0. Fits of the data are represented with solid lines

(Fig. 3d). Note that a 4.1-ns lifetime contribution was observed in the fluorescence decay of all samples, which is due to a small amount (approximately 5%) of unreacted dye that remained even after dialysis, size-exclusion chromatography or ultrafiltration. Upon incubation of reduced cyt-*c'*-T104C-OG488 with an excess of CO, the short-lifetime component increased to 1.11 ns. This increase in lifetime can be explained by monomerization of the protein which removes one of the hemes as energy transfer acceptors. Binding of CO to the heme iron itself is also expected to influence the energy transfer efficiency, however. Binding of CO causes a change in the heme absorption spectrum (Fig. S2), resulting in an increase in spectral overlap with the emission spectrum of OG488, leading to more quenching and a shorter lifetime. Apparently the effect of monomerization is stronger, because a decrease in fluorescence due to a larger overlap integral was not observed.

To investigate whether both processes could be distinguished in the kinetics of CO binding to cyt-*c'*-T104C-OG488, the reaction of an excess of CO with this protein was monitored with UV-vis absorption and steady-state fluorescence spectroscopy (Fig. 4). Because CO can only bind to ferrous heme, the reaction with CO was initiated by adding 5 mM DTT to a 1 μ M solution of cyt-*c'*-T104C-

OG488, saturated with CO.¹ CO binding to cyt-*c'*-T104C-OG488 was characterized by a large increase in the absorbance at 418 nm with a rate constant of $1.4 \times 10^{-2} \text{ s}^{-1}$, and a second process with a rate constant of $6 \times 10^{-4} \text{ s}^{-1}$.² Assuming pseudo first-order kinetics [28], the rate constant of the first, major process corresponds to a bimolecular rate constant of $9.8 \text{ M}^{-1} \text{ s}^{-1}$. This is similar to the value obtained for the reaction of CO with wild-type cyt-*c'* (Fig. 2c; $9.1 \text{ M}^{-1} \text{ s}^{-1}$), confirming that introduction of the label did not significantly affect its ligand binding properties. Monitoring the same reaction using fluorescence spectroscopy showed an increase in emission at 516 nm with a rate constant that was twofold smaller ($6.9 \times 10^{-3} \text{ s}^{-1}$). In

¹ Addition of 5 mM DTT results in complete and instantaneous reduction of the ferric heme. However, the absorption spectrum of cyt-*c'* reduced by DTT is slightly different than when reduced by $\text{Na}_2\text{S}_2\text{O}_4$, indicating that DTT alters the heme environment. Nevertheless, initiation of CO binding by addition of 2 mM $\text{Na}_2\text{S}_2\text{O}_4$ or 5 mM DTT resulted in the same CO binding kinetics, showing that the kinetics are not influenced by DTT.

² Some processes described in the text also displayed a second process with only a minor contribution on the timescale of the experiment, possibly related to the diminished stability of the mutant protein. For simplicity, only the rate constants of the primary process are discussed in the text. Both rate constants and their contributions are given in the figure legends.

Fig. 4 **a** Kinetics of CO binding to cyt-*c'*-T104C-OG488 in CO-saturated 20 mM Tris/HCl, 20 mM NaCl, pH 7.0 at 4 °C. The reaction was initiated by addition of 5 mM DTT to the protein and the UV-vis absorbance was monitored at 418 nm. The data were fit with Eq. 1 using $A_1 = 70\%$, $k_1 = 1.4 \times 10^{-2} \text{ s}^{-1}$, $A_2 = 30\%$ and $k_2 = 6 \times 10^{-4} \text{ s}^{-1}$ (solid line). **b** The same reaction was monitored using the fluorescence emission intensity at 516 nm after 490-nm excitation. The solid line is a fit of the data using Eq. 1 with $A_1 = 56\%$, $k_1 = 6.9 \times 10^{-3} \text{ s}^{-1}$, $A_2 = 44\%$ and $k_2 = 5 \times 10^{-4} \text{ s}^{-1}$

addition to steady-state fluorescence, the same process was also monitored using fluorescence lifetime and anisotropy measurements, which showed similar rate constants of $8.5 \times 10^{-3} \text{ s}^{-1}$ and $8.0 \times 10^{-3} \text{ s}^{-1}$, respectively (Fig. S3).

Discussion

In this study we explored two new techniques to directly monitor the unique ligand-induced monomerization of cyt-*c'*. Native MS allowed simultaneous detection of monomeric, dimeric and unfolded forms of cyt-*c'* and was therefore able to monitor the CO-induced monomerization of cyt-*c'*. MS also allowed the detection of the CO-bound form of cyt-*c'*, which to our knowledge is the first time that the binding of a gaseous ligand to a protein has been observed with MS. An explanation for the partial preservation of the CO-bound complex is that the bound CO molecule is located deep inside the channel that gives access to the distal coordination site [26].

The kinetics of the monomerization process as studied using MS were found to be significantly enhanced compared with the kinetics studied in solution using UV-vis spectroscopy. This discrepancy suggests that the relative abundances of the separate species are distorted by the ionization and transmission through the mass spectrometer. For example, reaction intermediates could be sufficiently destabilized to dissociate during ionization. It is also possible that the local temperature or CO concentration is increased during the ionization, resulting in faster kinetics. The detection of both CO-free and CO-bound forms of the monomer, even after completion of the reaction of cyt-*c'* with an excess of CO, provides another indication that ionization conditions can affect the relative abundances of species. We observed that the relative intensities of the CO-free and CO-bound species could be influenced by altering the pressure in the first vacuum stage. A high pressure generally favors the preservation of weak and large macromolecular complexes through multiple collisions with gas molecules (collisional cooling) [35–38], but even at the maximum pressure of our mass spectrometer some disruption of the CO-bound species was observed. A more quantitative study of CO binding and the subsequent monomerization process therefore requires even gentler ionization using, e.g., nanoflow ESI, which will be the focus of future work.

The incorporation of a fluorescent probe in cyt-*c'* provided a rare opportunity to use FRET to simultaneously monitor ligand binding to the heme and the subsequent conformational changes. To establish whether the overall increase in fluorescence that is observed after addition of CO to reduced cyt-*c'*-T104C-OG488 is consistent with the structure of cyt-*c'* and the position of the fluorescent probe, we calculated the distance between the probe and both heme groups from the observed fluorescence lifetimes. First, we calculated the energy transfer efficiency for the CO-bound monomeric cyt-*c'* according to

$$E = 1 - \frac{\tau_{\text{DA}}}{\tau_{\text{D}}} \quad (5)$$

in which τ_{DA} and τ_{D} are the lifetimes of the donor in the presence and absence of the acceptor, respectively. With this calculated energy transfer efficiency ($E^{\text{CO}} = 73\%$), the distance to the heme on the same monomer was calculated to be approximately 40 Å using Eqs. 3 and 4. With this distance and the energy transfer efficiency of unligated dimeric protein ($E^{\text{ox}} = 79\%$), the distance between OG488 and the heme on the opposite dimer (r_2) can be calculated. The efficiency of energy transfer from one donor to two acceptors is given by [29]

Fig. 5 Relative fluorescence intensities of ligand-free cyt-*c'*-T104C-OG488 dimer, after binding of two CO molecules and after the subsequent monomerization. Relative fluorescence intensities of different protein species were calculated using the energy transfer efficiencies (E) mentioned in the text according to $F_{DA} = F_D(1 - E)$, where the fluorescence intensity of unquenched OG488 (F_D) is set to 100%

$$E = \frac{k_{T1} + k_{T2}}{\tau_D^{-1} + k_{T1} + k_{T2}}, \quad (6)$$

where k_{T1} is the rate of energy transfer to the heme on the same monomer and k_{T2} is the transfer rate for the heme on the opposite monomer. As k_{T1} and k_{T2} are given by $k_{T1} = \tau_D^{-1} (R_0^{\text{ox}}/r_1)^6$ and $k_{T2} = \tau_D^{-1} (R_0^{\text{ox}}/r_2)^6$ [29], respectively, Eq. 6 can be rewritten as

$$r_2 = \left(\frac{r_1^6 (R_0^{\text{ox}})^6 (1 - E^{\text{ox}})}{E^{\text{ox}} [(R_0^{\text{ox}})^6 + r_1^6] - (R_0^{\text{ox}})^6} \right)^{1/6}. \quad (7)$$

Using this equation, we obtain $r_2 \sim 40$ Å. Both calculated distances are consistent with the crystal structure of cyt-*c'* that shows that a fluorescent probe connected at position 104 would be at an equal distance of approximately 40 Å from both heme groups.

Knowing the distances between the fluorophores and the heme groups allows us to calculate the relative fluorescence intensity of the CO-ligated dimeric intermediate. Figure 5 shows that the net increase in fluorescence intensity and lifetime that is observed for CO binding to cyt-*c'*-T104C-OG488 is the result of two counteracting processes. If the monomerization after ligand binding was slow compared to ligand binding, the fluorescence intensity would show an initial decrease, followed by a slow increase. The absence of this initial decrease suggests that monomerization is faster and occurs immediately after ligand binding to cyt-*c'*. Another interesting issue that has not been addressed in the literature is whether ligand binding to both monomers is required for monomerization to occur. The kinetics as observed with fluorescence spectroscopy were slightly slower than the ligand binding

observed with UV-vis spectroscopy. Preliminary kinetic simulations (supplementary material) suggest that these slightly slower kinetics are more consistent with a model in which monomerization occurs after binding of the first ligand. Since the largest differences between the various models are expected in first few seconds after mixing, a more thorough interpretation of various models will require measurements using a stopped-flow setup.

In conclusion, we have shown that FRET is a useful tool to study the intriguing ligand-induced monomerization of cyt-*c'*. This monomer-dimer equilibrium had been studied previously using a variety of techniques, but the application of fluorescence spectroscopy for the first time allowed the ligand binding and monomerization to be monitored simultaneously in real-time. In addition, the use of fluorescent probes should make it possible to study this process on a single-molecule level. Native MS enabled us to discriminate between monomeric and dimeric forms of cyt-*c'* in solution and even allowed the detection of CO-bound forms. The monomerization kinetics appeared to be significantly enhanced in the gas phase compared with the kinetics in solution, however. An advantage of MS is that it does not require mutagenesis or introduction of an artificial label. Together, fluorescence spectroscopy and MS form an attractive combination to study the ligand-induced conformational changes in cyt-*c'* that may be applicable to other heme-based sensor proteins as well.

References

- Gilles-Gonzalez M-A, Gonzalez G (2005) J Inorg Biochem 99:1–22
- Rodgers KR, Lukat-Rodgers GS (2005) J Inorg Biochem 99:963–977
- Roberts GP, Kerby RL, Youn H, Conrad M (2005) J Inorg Biochem 99:280–292
- Boon EM, Marletta MA (2005) J Inorg Biochem 99:892–902
- Denninger JW, Marletta MA (1999) Biochim Biophys Acta Bioenerg 1411:334–350
- Meyer TE, Kamen MD (1982) Adv Protein Chem 35:105–212
- Meyer TE, Cheddar G, Bartsch RG, Getzoff ED, Cusanovich MA, Tollin G (1986) Biochemistry 25:1383–1390
- Cross R, Aish J, Paston SJ, Poole RK, Moir JW (2000) J Bacteriol 182:1442–1447
- Mayburd AL, Kassner RJ (2002) Biochemistry 41:11582–91
- Lawson DM, Stevenson CE, Andrew CR, Eady RR (2000) EMBO J 19:5661–71
- Andrew CR, Green EL, Lawson DM, Eady RR (2001) Biochemistry 40:4115–4122
- Ge B, Meyer T, Schoning MJ, Wollenberger U, Lisdat F (2000) Electrochem Commun 2:557–561
- de Groot MT, Evers TH, Merckx M, Koper MTM (2007) Langmuir 23:729–736
- Barker SL, Kopelman R, Meyer TE, Cusanovich MA (1998) Anal Chem 70:971–976
- Barker SL, Clark HA, Swallen SF, Kopelman R, Tsang AW, Swanson JA (1999) Anal Chem 71:1767–1772

16. Weiss R, Gold A, Turner J (2006) *Chem Rev* 106:2550–2579
17. Doyle ML, Gill SJ, Cusanovich MA (1986) *Biochemistry* 25:2509–16
18. Motie M, Kassner RJ, Meyer TE, Cusanovich MA (1991) *Biochim Biophys Acta Protein Struct Mol Enzymol* 1076:97–102
19. Kassner RJ (1991) *Biochim Biophys Acta* 1058:8–12
20. Boon EM, Marletta MA (2005) *Curr Opin Chem Biol* 9:441–446
21. Ren Z, Meyer T, McRee DE (1993) *J Mol Biol* 234:433–45
22. Bartsch RG (1963) In: Gest H, San Pietro A, Vernon LP (eds) *Bacterial photosynthesis*. Antioch, Yellow Springs
23. Evers TH, Merckx M (2005) *Biochem Biophys Res Commun* 327:668–674
24. Lide DR (ed) (1994) *CRC handbook of chemistry and physics*, 75th edn. CRC, Boca Raton
25. Van Den Heuvel RHH, Heck AJR (2004) *Curr Opin Chem Biol* 8:519–526
26. Heck AJR, Van Den Heuvel RHH (2004) *Mass Spectrom Rev* 23:368–389
27. Potier N, Rogniaux H, Chevreux G, Van Dorsselaer A (2005) *Methods Enzymol* 402:361–389
28. Cusanovich MA, Gibson QH (1973) *J Biol Chem* 248:822–834
29. Lakowicz JR (1999) *Principles of fluorescence spectroscopy*, 2nd edn. Kluwer Academic/Plenum, New York
30. Mustonen P, Virtanen JA, Somerharju PJ, Kinnunen PKJ (1987) *Biochemistry* 26:2991–2997
31. Lepesheva GI, Strushkevich NV, Usanov SA (1999) *Biochim Biophys Acta* 1434:31–43
32. Rischel C, Jørgensen LE, Földes-Papp Z (2003) *J Phys Condens Mater* 15:S1725–S1735
33. Sarkar R, Shaw AK, Narayanan SS, Dias F, Monkman A, Pal SK (2006) *Biophys Chem* 123:40–48
34. Kuznetsova S, Zauner G, Schmauder R, Mayboroda OA, Deelder AM, Aartsma TJ, Canters GW (2006) *Anal Biochem* 350:52–60
35. Krutchinsky AN, Chernushevich IV, Spicer VL, Ens W, Standing KG (1998) *J Am Soc Mass Spectrom* 9:569–579
36. Tahallah N, Pinkse M, Maier CS, Heck AJR (2001) *Rapid Commun Mass Spectrom* 15:596–601
37. Schmidt A, Bahr U, Karas M (2001) *Anal Chem* 73:6040–6046
38. Chernushevich IV, Thomson BA (2004) *Anal Chem* 76:1754–1760