# Geminate carbon monoxide rebinding to a c-type haem

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# Geminate carbon monoxide rebinding to a *c*-type haem†

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A chemically modified form of cytochrome *c* (cyt. *c*), termed carboxymethyl cytochrome *c* (cm cyt. *c*), possesses a vacant sixth coordination site to the haem iron that is available to bind external ligands. We present data on the rapid flash photolysis of CO from the ferrous haem iron of cm cyt. *c* and describe the kinetics and spectral transitions that accompany the recombination. This was achieved using 30-femtosecond laser pulses and a white light continuum to monitor spectral transitions. Whereas the photo-dissociation quantum yield is close to 1, the yield of CO escape from the protein (the apparent quantum yield, $\varphi$) relative to myoglobin ($\varphi = 1$) is small due to rapid geminate recombination of CO. On ligand photo-dissociation the haem undergoes a spin-state transition from low-spin ferrous CO bound to penta-coordinate high-spin. Subsequently the system reverts to the CO bound form. The data were fitted with a minimum number of exponentials using global analysis. Recombination of CO with the haem iron of cm cyt. *c* is multiphasic ($\tau = 16$ ps, 120 ps and 1 ns), involving three spectrally distinct components. The fraction of haem (0.11) not recombining with CO within 4 ns is similar to the value of $\varphi$ (0.12) measured on the same preparation by the "pulse method" (M. Brunori, G. Giacometti, E. Antonini and J. Wyman, *Proc. Natl. Acad. Sci. USA*, 1973, **70**, 3141–3144, ref. 1). This implies that no further geminate recombination occurs at $t > 4$ ns. This unusually efficient CO–haem geminate recombination indicates the sterically hindered ("caged") nature of the distal haem pocket in cm cyt. *c* from which it is difficult for CO to escape. The large geminate phase may be contrasted with the behaviour of myoglobin in which geminate recombination is small. This is in general agreement with the well-documented extensive structural dynamics in myoglobin that allow ligand passage, and a higher structural rigidity in cyt. *c* imposed by the restraints of minimising reorganisation energy for electron transfer (M. Brunori, D. Bourgeois and D. Vallone, *J. Struct. Biol.*, 2004, **147**, 223–234, ref. 2). The high pH ferrous form of cm cyt. *c* is a low-spin species having a lysine bound to the central iron atom of the haem (M. Brunori, M. Wilson and E. Antonini, *J. Biol. Chem.*, 1972, **247**, 6076–6081; G. Silkstone, G. Stanway, P. Brzezinski and M. Wilson, *Biophys. Chem.*, 2002, **98**, 65–77, refs. 3 and 4). This high pH (pH $\sim$ 8) form of deoxy cm cyt. *c* undergoes photo-dissociation of lysine (although the proximal histidine is possible) after photo-excitation. Recombination occurs with a time constant ($\tau$) of $\sim$7 ps. This is similar to that observed for the geminate rebinding of the Met80 residue in native ferrous cyt. *c* ($\tau \sim 6$ ps) following its photo-dissociation (S. Cianetti, M. Negrerie, M. Vos, J.-L. Martin and S. Kruglik, *J. Am. Chem. Soc.*, 2004, **126**, 13 932–13 933; W. Wang, X. Ye, A. Demidov, F. Rosca, T. Sjodin, W. Cao, M. Sheeran and P. Champion, *J. Phys. Chem.*, 2000, **104**, 10 789–10 801, refs. 5 and 6).

## Introduction

Redox reactions are at the heart of numerous processes of biological importance.[7] The theoretical framework provided by Marcus,[8] and the availability of three-dimensional structures of many redox proteins have placed studies of electron transfer on a firm footing allowing predictions to be made and experimentally tested. For non-naturally light-activated systems, such studies often rely on techniques such as stopped-flow spectroscopy or methods in which electron transfer is initiated by a laser pulse. Both stopped-flow and flash methods have advantages but also suffer severe limitations. While flow methods have the advantage that reactions between *in vivo* redox partners may be studied without recourse to protein modification they suffer from the disadvantage that the true electron transfer rate constant ($k_{et}$) may not be measured directly. This is because $k_{et}$ is generally much larger (up to $10^5$ $s^{-1}$) than the pseudo-first order rate for complex formation and, in any case, is often too large to be determined with instruments having a dead time of $\sim$1 ms. Similarly, laser pulse methods, while having sufficient time resolution to determine ket have several important disadvantages. First, they often rely on chemical coupling of a light sensitive electron donor to one of the partners that may perturb the system.[9] Secondly, because the excited state of the donor is generally very short lived, the quantum yield of the process *i.e.* electron delivered to acceptor per pulse, is generally very low ($\sim$0.05).[10,11]

Our objective has been to develop a method to study inter-protein electron transfer that combines the advantages of the standard techniques while largely overcoming the drawbacks. Our method employs proteins in "quasi-native" states and, while not requiring chemical coupling of a light inducible reductant, delivers electrons very rapidly on laser activation. In addition it has the potential for delivering one electron for each CO dissociated, a much higher electron stoichiometry than usually available. The general principle of the method depends upon engineering a CO binding site into a redox haem protein (the electron donor) and relies on the fact that CO has a high affinity for ferrous haem proteins but does not bind to the ferric haem counterpart. Invariably CO complexes of ferrous haem proteins are light sensitive, such that a brief light pulse, *e.g.* from a laser, will dissociate the CO from the haem group, leaving this as a five coordinate high-spin ferrous species. Five coordinate haem proteins generally have a lower redox potential than their hexa-coordinate forms and thus photo-dissociation of CO has the effect of rapidly switching the redox potential of the protein, changing a good electron acceptor (the CO adduct) into a good electron donor (five coordinate ferrous haem). Thus, having mixed the CO adduct of such a haem protein with a redox partner and allowed time for a complex to form one may initiate electron transfer at will with a laser. We have exploited this

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strategy in flow-flash experiments using a modified form of cyt. *c* termed cm cyt. *c*, in which the iron is penta-coordinate due to carboxymethylation of the methionine-80 that normally fills the sixth coordination site in the native protein. Mixing the CO complex of cm cyt. *c* with a suitable redox active-partner produces a protein complex which, however, does not undergo electron transfer as the ferrous form of cm cyt. *c* is stabilised by the bound ligand. Laser photolysis of CO from cm cyt. *c* renders this penta-coordinate high spin, but now with the potential to donate electrons.

Our studies on cyt. *c* oxidase and plastocyanin have provided proof of principle for the method but the use of cm cyt. *c*, although useful, is not ideal and we have now produced a set of genetically engineered Met80X mutants that bind CO and are light sensitive. Unfortunately these proteins have very low quantum yields and are unsuitable for the use described.[4] In order to understand why this is so and thus to help further design of mutants we have undertaken a set of experiments to study the events following laser excitation. In this paper we describe our initial studies using femtosecond laser photolysis of the CO adduct of cm cyt. *c*.

From another perspective, we note that in contrast to the high-affinity haem ligand NO, efficient geminate haem-CO rebinding in proteins is unusual. The present system gives the unique opportunity to study parameters that determine CO binding to haem.

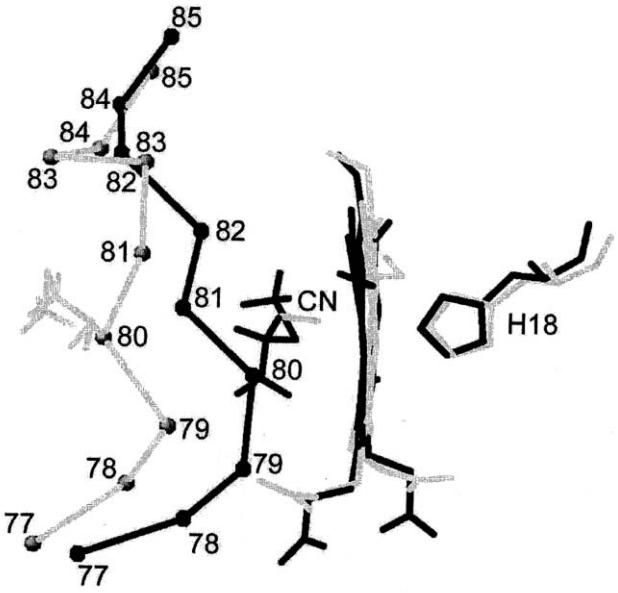
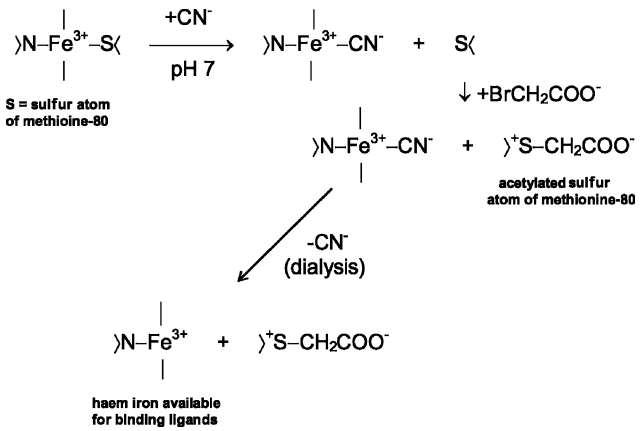



**Fig. 1** The secondary structure (NMR) of the $CN^-$ adduct of horse heart ferri cyt. *c*, the rearrangement of backbone residues 77–85. Native horse heart ferri cyt. *c*, is represented by black and the $CN^-$ adduct by light grey.[13] (Reproduced with permission from *J. Biol. Inorg. Chem.*, © 2002).

## Materials and methods

### Cm cyt. *c* preparation

Cm cyt. *c* was prepared by treatment of native horse heart cyt. *c* (Sigma) with potassium cyanide (KCN) and bromoacetic acid (BrAc) (see Scheme 1). The cm cyt. *c* preparation was adapted from the method of Schejter and George, but with a minimum incubation time of cyt. *c* with the KCN and BrAc.[12] Approximately 10 mg of horse heart cyt. *c* was dissolved into 1 ml of buffer (100 mM phosphate, pH 7.0) with stirring ([protein] ~1 mM). A 0.4 M KCN solution was carefully prepared by dissolving solid KCN in buffer (100 mM borate, pH 9.0) and ensuring the pH did not fall below neutrality by adding small amounts of 1 M NaOH and that the final pH was 7.0. The KCN solution (2 ml) was added to the protein solution. Fig. 1 shows the nmr structure of the cyanide adduct of native horse heart ferri cyt. *c* solved by Yao *et al.* (see Discussion).[13] A 0.4 M BrAc solution was prepared by dissolving solid BrAc in buffer (100 mM phosphate, pH 7.0) and the pH was adjusted to ~7.0 by adding NaOH. This BrAc solution was added to the protein/KCN solution to give a total reaction mixture




**Scheme 1** The reaction of cyt. *c* with $CN^-$ and bromoacetic acid to form cm cyt. *c*. Acetylation of the sulfur atom of methionine-80 renders it unable to bind to the sixth coordination site of the haem iron. The haem iron of cm cyt. *c* is available to bind external (*e.g.* CO) and internal (*e.g.* lysine) ligands.

volume of ~5 ml, equimolar in the modifying reagents KCN and BrAc and ~200 μM in protein. The solution was stirred at room temperature, and at half hour intervals a small aliquot (~10 μl) was removed to monitor the progress of the reaction. To the aliquot was added ~1 ml of buffer (100 mM phosphate, pH 7.0) and a few grains of solid dithionite was added and pure CO bubbled into the solution. Any cyt. *c* that had undergone carboxymethylation of the sulfur atom of Met80 was reduced to the ferrous form (thereby displacing the $CN^-$ from the iron) and combined with CO to give the ferrous CO adduct (CO-cm cyt. *c*), identified by its distinct absorption maximum in the Soret region at 414 nm ($\varepsilon \approx 219\,000\ M^{-1}\ cm^{-1}$).[3] The reaction was stopped once all the cyt. *c* was converted (~4–6 h) by passing the mixture down a column containing Sephadex G25 pre-equilibrated with buffer (100 mM phosphate, pH 7.0), this separated the KCN and BrAc in solution from the cm cyt. *c*. Following passage through the column the cm cyt. *c* was extensively dialysed against buffer (100 mM phosphate, pH 7.0) to remove cyanide bound to the ferric form.

Analysis of the product by mass spectroscopy (MALDI-ToF, Bruker Daltonics/Reflex IV) showed two forms of cm cyt. *c* differing in mass from the native protein by +58 and +113 Da. This closely approximates to the addition of one (+58) or two (+116) acetyl groups. One of these must be to Met80, as all the protein bound CO, the other is most likely to be to Met65 although other residues can be modified. This heterogeneity is in our view not responsible for the kinetics we observe and reasons for this view will be discussed later.

### Sample preparation

For the spectroscopic measurements, cm cyt. *c* was prepared to a haem concentration of ~50 μM in a gas tight optical cell with an optical path length of 1 mm. For the deoxy form of cm cyt. *c*, the de-gassed as-prepared (ferric) sample was reduced with 1 mM sodium dithionite. For the CO form of cm cyt. *c*, the deoxy form was equilibrated with 1 atm (1 atm = 101.3 kPa) CO. The buffer used in all experiments (unless otherwise stated) was Hepes (20 mM, pH 8.0).

### Spectroscopy

Steady-state spectra were recorded using a Shimadzu UV-Vis 1601 or a Cary 5E spectrophotometer. Spectra were unchanged after the femtosecond laser flash experiments. Multicolour femtosecond absorption spectroscopy[14] was performed with a 30fs pump pulse centered at 565 nm and a <30 fs white light continuum probe pulse, at a repetition rate of 30 Hz. Full spectra of the test and reference beams were recorded using a combination of a polychromator and a CCD camera. All experiments were carried out at 21 °C. The sample was continuously moved perpendicular to the beams to ensure sample renewal between shots.

Basic data matrix manipulations and presentation were performed using Matlab (The Mathworks, South Natick, MA). The absorbance changes were treated using the SPLYMOD algorithm,[15] with a Matlab interface.[16] The geminate CO recombination data was also analysed using the maximum entropy method (MEM).[17] The MEM algorithm describes the data by a distribution of rate constants with a minimal number of peaks as justified by the data's signal-to-noise.

The quantum yield of CO-cm cyt. *c* was also measured by the "pulsed" method developed by Brunori and co-workers,[1] and described previously.[4] Lamp flash photolysis was performed using an Applied Photophysics instrument equipped with a xenon flash (instrument dead time 0.2–1 ms depending on flash intensity). The flash intensity was attenuated using a set of neutral density filters. The monitoring beam was attenuated using neutral density filters so as to minimise CO photolysis prior to flash, and myoglobin ($\varphi = 1$) was used as a calibrant protein. The difference spectra, CO-cm cyt. *c* minus ferrous cm cyt. *c* was measured by steady state. The same sample was then flash photolysed in the lamp flash instrument at many values of single wavelength in order to reconstruct another difference spectra (flash constructed. The CO recombination following flash was monitored at 414 nm (the Soret maxima for CO-cm cyt. *c*). The quantum yield was determined by taking the absorbance change following flash as a fraction of the total absorbance change observed for the steady state difference spectra at a given wavelength (in most cases 414 nm). The CO concentration was kept quite low (commonly ~50 μM) so that the rate of CO recombination from bulk was not "lost" in the dead time of the instrument.

## Results

### Cm cyt. *c*-CO

The CO adduct of ferrous cm cyt. *c* was found to be light sensitive, as expected from the behaviour of other ferrous haem proteins, and photo-excitation led to dissociation of CO from the haem. In Fig. 2 (panel A), we present transient absorption spectra that represent the difference spectra ($Fe^{2+}$ minus $Fe^{2+}$–CO) recorded at selected delay times after photo-excitation. The spectra reflect a substantial red shift of the Soret band as expected for a five-coordinate minus six-coordinate ferrous haem spectrum, and are qualitatively similar to the spectra associated with CO dissociation from other haem proteins, and in particular also to those associated with methionine dissociation from native cyt. *c*.[6] Therefore, the spectra are provisionally assigned to formation of five-coordinate haem. The difference spectrum diminishes as CO recombines and the time scale for recombination indicates that the majority of this reaction occurs in a very rapid geminate process. A multi-exponential decay model yields three relaxation processes ($\tau$ = 16, 120 and 1000 ps), see Fig. 2 (panel B). Analysis of geminate CO recombination using MEM, also suggests that CO rebinding is not a gradual distribution of phases but consists of three distinctive exponential processes ($\tau$ = 3, 68 and 600 ps, see Fig. 2, panel C). The values found for the time constants are comparable given that the MEM analyses kinetics at a single wavelength

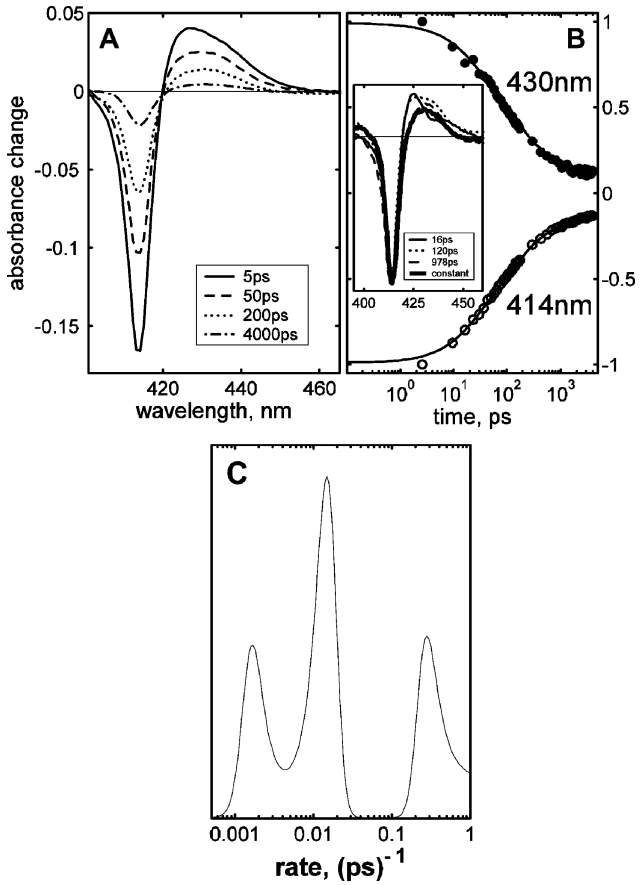



**Fig. 2** Kinetics of CO recombination to cm cyt. *c* following photo-dissociation. Panel A: Transient spectra at selected delay times after photolysis. Panel B: Time courses monitored at 430 and 414 nm (solid line is a theoretical curve according to parameters from the fit); Inset: the spectral components from the multi-exponential global fit (DAS components) and the final spectrum (constant, at 4 ns). The relative amplitude of each component (normalized to the photolysis) is 0.27 (16 ps), 0.45 (120 ps), 0.17 (1 ns) and 0.11 (constant). Panel C: Results from the analysis of the CO rebinding kinetics at 430 nm using the MEM.

while the global fit analyses the whole spectrum. The spectral characteristics of the decay phases are quite distinct (Fig. 2, inset to panel B). The relative amplitude of each component (normalised to 1 at the bleaching at 414 nm) is 0.27 (16 ps), 0.45 (120 ps), 0.17 (120 ps) and 0.11 (constant at 4 ns). The latter relative amplitude of 0.11 represents the percentage (11%) of CO that does not recombine with the haem iron during the first 4 ns following photo-dissociation. This value is consistent with the measured apparent quantum yield of CO release from the protein (see below).

### Cm cyt. *c* (deoxy)

Photolysis of the protein at pH 8 in the absence of added CO led to the appearance of a rapid transient. In Fig. 3 (panel A), we present transient absorption spectra recorded at selected delay times after photo-excitation that we assign to the difference spectrum $Fe^{2+}$ minus $Fe^{2+}$–L, where L is an intrinsic amino acid other than methionine (proximal His18 or distal Lys79). This difference spectrum bleaches as the intrinsic ligand recombines and the time scale indicates that this reaction occurs in a very fast geminate process. Analysis of the transient spectra yields a single picosecond decay phase ($\tau \sim 7$ ps), see Fig. 3 (panel B).

### Measurement of apparent quantum yield ($\varphi$) for cm cyt. *c* by the "pulsed" method

The apparent quantum yield was measured at pH 7.0. In Fig. 4 we present the spectra for the ferrous and ferrous-CO forms

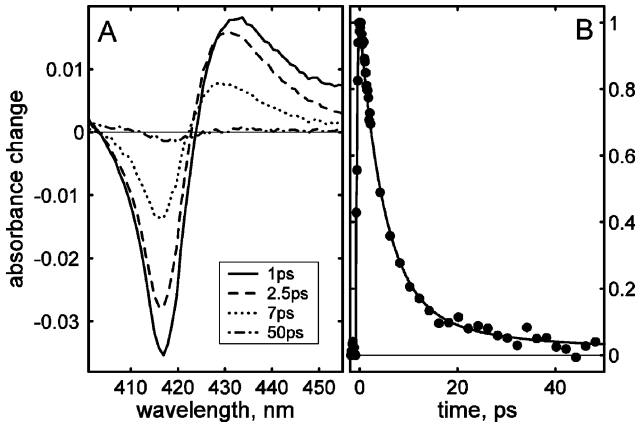



**Fig. 3** Spectral evolution of the high pH (pH 8.0) deoxy form of cm cyt. *c*. following laser excitation. Panel A: transient absorption spectra at the selected delay times after photolysis. Panel B: the kinetics at 430 nm (normalised to the maximum).

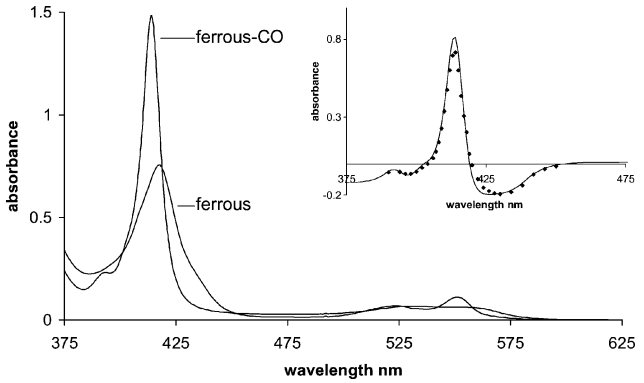



**Fig. 4** The steady-state spectra of ferrous cm cyt. *c* and the CO derivative (sodium phosphate, 20 mM, pH 7.0). Inset: comparison between the difference spectra (carbonmonoxy minus deoxy) obtained statically (solid line) and by lamp flash photolysis (solid symbols)). The protein concentration was ~7 μM. The protein was reduced with a slight excess of sodium dithionite. The temperature was 20 °C.

of cm cyt. *c*. The two difference spectra (steady state and flash constructed) were overlaid and normalised to each other, and it was found they were virtually identical (see inset to Fig. 4). Assuming a lysine binds to the 6th coordination position of the haem iron in the absence of CO (see Discussion section), this indicates that at the millisecond time scale of the Xenon flash experiment, a lysine coordinates to the CO position of the haem. This is generally consistent with the earlier attribution of a 150 μs phase to lysine binding.[4] Following photolysis using a xenon lamp (flash duration in the μs range) a fraction of the photo-dissociated CO escapes to bulk solution and recombines in the ms time range at a rate dependent on the CO concentration. The amplitude of the absorbance change accompanying this CO recombination is a measure of the extent of photo-dissociation to bulk solution and has been employed to determine the apparent quantum yield, $\varphi$, using the "pulsed" method.[1] The method determines the value of the $\varphi$ relative to a standard, in most incidences myoglobin, for which $\varphi \cong 1$ (*i.e.* one CO escapes to bulk solution for each photon absorbed by the haem group). The results are shown in Fig. 5 where the logarithm of the ratio of the concentrations of the CO adduct in the dark to that in the light (of relative intensity I) is plotted as a function of I. As predicted by theory the plots are linear passing through the origin. The slope of the plot for cm cyt. *c* relative to myoglobin yields the apparent quantum yield. The value of $\varphi$ for cm cyt. *c* was calculated to be 0.12, very close to that determined by the laser flash method (see Fig. 2). This value is smaller than that previously reported ($\varphi = 0.26$) for cm cyt. *c*.[4] This is the result of differences in the way in which cm cyt. *c* was prepared.

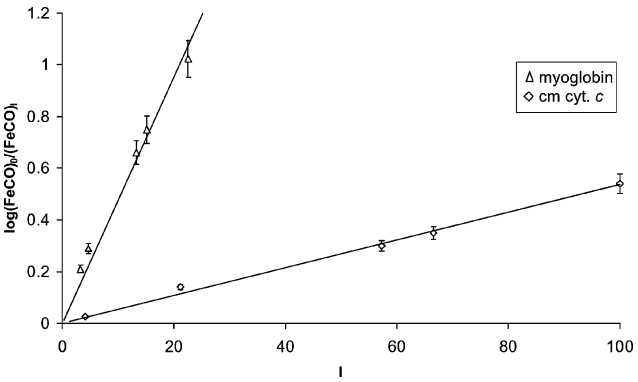



**Fig. 5** Dependence of the extent of photo-dissociation of the CO adducts of myoglobin and cm cyt. *c* on relative light intensity (I) expressed as a percentage of full flash intensity. The protein concentrations were ~5 μM dissolved in 20 mM sodium phosphate buffer, pH 7.0, 50 μM CO and temperature 20 °C.

In particular, the preparation described here employed the minimum incubation time (~4 h) with bromoacetic acid that is needed to fully modify the methionine-80 residue. In earlier work incubation with the haloacid was for at least 12 h. It is known that such lengthy incubation leads to further modification of the protein (lysine and histidine residues being susceptible) and this further disrupts the protein structure allowing easier movement of CO from the haem cavity, resulting in elevated apparent quantum yield.

## Discussion

Following photolysis, geminate recombination of gaseous ligands in the picosecond time scale likely reflects rebinding from the immediate vicinity of the haem iron. This is in contrast to geminate recombination in the nanosecond time scale that is thought to report rebinding from within the protein matrix, and some distance from the iron. For cm cyt. *c*, analysis of the CO geminate process is in the picosecond time scale. This result suggests that following photolysis CO rebinds from within the distal pocket region, very close to the haem iron and is in or can easily adopt an orientation favourable for binding to the haem (*i.e.*, the carbon atom pointing towards the haem iron and the CO axis not far from perpendicular to the haem plane). Analysis by MEM and a global model reveals that three distinct phases contribute to the geminate process. For cm cyt. *c* then, the existence of three phases could be attributed to CO rebinding from three different locations from within the distal pocket region.

The explanation that multiphasic geminate CO recombination results from heterogeneity of the protein sample (see Materials and methods section) can be discounted for two reasons. Firstly, fractionation into separate components of cm cyt. *c* prepared by long incubation with bromoacetic acid has revealed that the CO combination kinetics did not differ between these.[3] Secondly, and crucially, cyt. *c* in which the methionine-80 ligand has been substituted by an alanine residue by site directed mutagenesis displays tri-phasic CO geminate recombination with time constants and spectral contributions very similar to those reported here for cm-cyt. *c*. As the mutant protein is homogeneous we can eliminate protein heterogeneity as the cause of the complexity we observe. The results on this and several other Met80X mutants will be reported elsewhere.

However, this simple explanation needs further elaboration. Examination of Fig. 2(B) (inset) reveals that the kinetic processes exhibit different spectra, implying that CO recombines with a number of distinct spectral forms of the deoxy haem, *i.e.* with vibrationally excited species and/or forms not at structural equilibrium. For vibrationally excited, 'hot', haem species we would expect a red shift of the spectrum compared to

thermalized haem. The finding that the induced absorption lobe of the transient spectrum red shifts in time indicates that, where haem cooling does take place on the timescale of a few ps,[18] this is not the main origin of the spectral evolution. Regarding the possibility of haem structural relaxations, for example, if following photo-dissociation of the CO molecule the haem group does not fully relax from its planar form characteristic of the liganded species, for some ps before relaxing to the fully domed configuration typical of the deoxy form, then the fastest geminate CO binding process may effectively compete with this molecular motion. Thus this kinetic process reports rebinding to a non-equilibrium species and displays a spectrum different from that observed to be associated with the slower geminate phases and that recorded from static spectroscopy. More generally the haem cavity of cm cyt. *c* may allow little mobility for the dissociated CO, which therefore initially strongly interacts with the haem, giving rise to both efficient (possibly barrierless) recombination and distortion of the haem. Competing rebinding pathways for CO rebinding and motion into distinct other positions can then lead to both haem relaxation and less efficient rebinding.

On rapid laser photolysis of CO from cm cyt. *c* the fraction of CO that recombines by a geminate process (intramolecular) in the picosecond time scale was measured at ~89%, and the fraction that does not return after 4 ns was measured at ~11% (see Fig. 2). The quantum yield measured by lamp flash yielded a value for $\varphi = \sim 0.12$ or, in other words, the fraction of CO escaping to bulk solution and recombining with the haem in a bimolecular reaction was ~12%. These two values are almost identical, and this would suggest that there exists no further geminate process after 4 ns. Therefore, it appears that CO geminate recombination is only from within the distal pocket region close to the haem and not from further distances away located in the protein matrix itself. The close correspondence also implies that the photo-dissociation quantum yield is as high as for MbCO, *i.e.* close to 1. The CO geminate recombination efficiency is very high for a haem protein. In many haem proteins like myoglobin the geminate recombination amounts only to a few percent and occurs on a timescale of nanoseconds or longer.[19] In other haem proteins where CO replaces an internal ligand substantial haem–CO rebinding has been observed, in particular in the presumed oxygen sensor *Ec*Dos[20] and in the CO-sensor CooA.[21]

Geminate rebinding of gaseous molecules to haem, particularly where this is multiphasic, has been used to probe the internal structure of the protein. Probably the best example of a protein where this strategy has been employed is myoglobin, with NO as a ligand.[14]

In 1965, Kendrew and co-workers discovered that Xe gas binds to myoglobin,[22] and 1984 Tilton and co-workers solved the crystallographic structure of ferri myoglobin equilibrated with Xe revealing it contained four Xe atoms trapped in four separate cavities ($X^1$–$X^4$) with radii >5 Å and lined with hydrophobic side chains.[23] More recently, several groups have obtained the crystal structures of "intermediate states" of myoglobin that show the location CO following photo-dissociation.[24,25] This crystallographic data shows that photo-dissociated CO can be seen at more than one site inside the protein matrix. The primary docking site is within the distal haem pocket, CO that lies parallel and ~3.6 Å from the haem plane. This site is occupied a few ps after photo-dissociation.[26] Despite their proximity, virtually no, fast geminate CO–haem rebinding occurs from this site, presumably at least in part because the orientation and mobility of, CO is unfavourable. There are two secondary sites at a greater distance from the iron, and these overlap with the Xe binding sites (namely $X^1$ and $X^4$). Brunori *et al.* in 2004 showed that following CO photo-dissociation in myoglobin, the CO that migrated to these cavities could then exit the protein and equilibrate with the bulk solution.[2] As we do not observe any CO geminate process for cm cyt. *c* other than in the picosecond time range that we attribute to re-binding from within the distal pocket, it would seem that cm cyt. *c* does not possess such CO sequestering cavities that facilitate ligand escape to the exterior of the protein. It cannot be excluded, however, that the rebinding from such cavities would be as low as in MbCO, and, given the already low probability of escape from the haem vicinity, too low to detect.

The haem iron in native cyt. *c* is axially coordinated by two endogenous ligands, namely His18 and Met80. It has been shown that one of these axial ligands can be photo-dissociated from the haem iron of ferrous cyt. *c* with high yield.[14] The majority of the photophysical studies on ferrous cyt. *c* have been based on femtosecond transient absorption spectroscopy with high photon energy excitation in the Soret or UV absorption region of the haem.[6,27] The main objective has been to elucidate whether the Fe–His proximal bond or the Fe–Met distal bond is broken on photo-excitation and conclusions have been drawn from comparison of the reconstructed photoproduct absorption spectrum with the stationary absorption spectrum. For example, Wang *et al.* in 2000 found a high similarity of the photoproduct absorption spectrum with the stationary absorption spectrum of a model five-coordinate His-ligated microperoxidase concluding, therefore, that the Met80 was photo-dissociated with a time constant of ~6 ps for geminate re-binding.[6] However, Jongeward *et al.* in 1987 conducted ligand photolysis studies on cytochromes $b_5$ and *c* with picosecond time resolution and concluded that the Fe–His bond is broken with a time constant of ~11 ps for geminate recombination.[27] More recently, Cianetti *et al.* in 2004, working on ferrous cyt. *c* using a novel subpicosecond Raman spectrometer/laser instrument, assigned the photoproduct as being a five-coordinate His-ligated species.[5] The distinctive feature in their 1 ps spectrum of the cyt. *c* photoproduct was the appearance of a strong 216 $cm^{-1}$ band, assigned to the Fe–His stretching. This band is not Raman active in six coordinate cyt. *c*, but is present in five coordinate ferrous high-spin domed haem structures.[28] Therefore, the appearance of the Fe–His stretch indicated the photo-dissociation of an axial ligand, namely the Met80. Geminate recombination of the Met80 axial ligand was found to be with a time constant ($\tau$) of ~6 ps, very similar to that we obtain for the geminate recombination of the photo-dissociated axial ligand in cm cyt. *c*. We believe that the process that we observe is the recombination with the haem of the lysine residue that, at high pH, we know fills the sixth coordination position in cm cyt. *c*, the homologous position that Met80 occupies in the native molecule. The proximity of the amino group of lysine 79 to the haem group (Fig. 1) supports the view. We have previously shown for cm cyt. *c* that, at elevated values of pH, an intrinsic lysine residue binds to the sixth coordination site of the haem iron, and this is the case for both the ferrous (as studied here) and ferric forms of the protein.[4,29] The apparent p*K* values for this transition were found to be ~7 and ~6 for the ferrous and ferric forms respectively, the spectra of the latter being analogous to the spectra of the alkaline form of native ferri cyt. *c*.[3,4,29] The close correspondence of the time constant for recombination of the internal ligand, whether it is methionine or lysine indicates that this recombination may be essentially barrierless. We note further that the fastest time constants observed for internal ligand rebinding in other six-coordinate CO-binding haem proteins like EcDos (methionine) and the CO-sensor CooA (proline) are very similar.[20,21] However, the binding of internal residue ligands after CO dissociation in such protein occurs on the time scale of ~100 μs.[20,30,31] (see below)

An earlier estimate of the rate at which the intrinsic lysine binds to the haem of cm cyt. *c* has been provided by temperature (*T*) jump experiments. In such experiments the "on" rate constant has been evaluated at ~15 000 $s^{-1}$ (giving $\tau$ ~60 μs). A similar value has been reported for Met80Asp ($\tau$ ~ 150 μs) using a nanosecond laser flash method that follows events in the μs time range following CO photolysis to the bulk phase. These values are clearly very different from the relaxation time

we report here for direct lysine recombination. These differences may be reconciled by proposing that in the *T* jump or CO flash experiments the lysine recombines from a location distant from the haem. This is reasonable given that in the T jump experiments the events are triggered by deprotonation of a lysine that, because of the positive charge on the amino group, is displaced outside the haem cavity. Similarly one would expect CO to displace lysine from the vicinity of the haem given the highly caged and packed nature of the haem cavity (as suggested by the low apparent quantum yield). In contrast photodissociation of a lysine already bound to the ferrous iron leads to rapid (ps) recombination, as reported here.

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