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Production and characterisation of Met80X mutants of yeast iso-1-cytochrome *c*: spectral, photochemical and binding studies on the ferrous derivatives

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Abstract

The iron ligand, Met80, of yeast iso-1-cytochrome *c* has been mutated to residues that are unable to bind to the iron. The resultant proteins, Met80Ala, Ser, Asp, Glu, have been expressed and purified. All mutant proteins exhibit well defined pH dependent spectral transitions that report the binding, at high pH, of an intrinsic ligand (probably the nitrogen of an ε-NH_2 of a lysine) that drives the heme low-spin. The p*K* values are mutant dependent. All the mutant proteins bind extrinsic ligands, such as CO, in their ferrous states and we report the apparent quantum yield (φ) for CO photo-dissociation. The values of φ range from 0.004 for Met80Ala to 0.04 for Met80Asp. We also report values for the rate constant for binding the intrinsic lysine residue. The values for this constant, for φ and for the p*K* values are discussed in terms of the rigidity of the cytochrome structure. We also show that the mutant proteins bind with high affinity to cytochrome *c* oxidase, both in the ferric and ferrous states. The potential of these proteins to act as light activated electron donors for the study of electron transfer is discussed.

Keywords: Yeast cytochrome *c*; Quantum yield; Electron transfer; Flash photolysis

1. Introduction

Photochemically induced electron transfer has found wide application in the study of redox proteins (see [1] for review). The method relies on using a brief flash of light from a laser to excite one of the participants in a redox reaction to a state such that it becomes a powerful reductant that then transfers an electron to the other partner. There are many ways in which this may be achieved. One of these exploits the photosensitivity of the bond linking carbon monoxide (CO) to ferrous heme to initiate light activated electron transfer. In this method a chemically modified form of mammalian cytochrome *c* (carboxymethyl cytochrome *c*; Cm cyt *c*) has been employed. In this molecule one of the intrinsic ligands to the iron in the native protein, namely methionine-80 (horse heart numbering) was chemically modified

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rendering it unable to co-ordinate the central metal atom [2,3]. The resultant protein is now penta co-ordinate (depending on pH) and, in the ferrous state, is able to bind CO [4]. Stable complexes may be formed between this ferrous CO adduct and oxidized redox proteins. Electron transfer does not occur within such complexes because the ferrous form of the cytochrome is strongly stabilised by bound CO. Following laser photolysis of the CO adduct (in ns) the apparent redox potential of the cytochrome falls (by ~300 mV, depending on the CO concentration) and the electron residing on the ferrous iron is transferred to the partner [5,6]. The kinetics of this process may be monitored through the associated absorbance changes thereby yielding the rate constant for electron transfer within the complex of the redox proteins.

This method for studying very rapid intrinsic electron transfer rate constants ($k \geqslant 10^5\ s^{-1}$) offers many advantages, in particular the possibility of delivering one electron for each photon absorbed by the heme group, as has been demonstrated for the Cm cyt *c* system [4]. This is generally more efficient than methods based on photoactivation of ruthenium complexes covalently bound to redox proteins where usually less than 10% of photons absorbed lead to electron transfer [7,8]. Studies of electron transfer between Cm cyt *c* and either cytochrome *c* oxidase or plastocyanin have proven the CO photo-dissociation method effective [5,6]. However, this method may be improved by substituting a Met80 mutant of the native protein for the chemically modified protein. Mutation is far more selective than carboxymethylation as the latter leads to modification of residues in addition to Met80 and thus results in poorer recognition between redox partners. The Met80Ala mutant of *Saccharomyces cerevisiae* cytochrome *c* has been expressed in *Escherichia coli* by Lu et al., and was shown to bind CO in the ferrous form [9].

In this paper we describe the preparation of a set of such Met80X mutants (where X = Ala, Ser, Asp or Glu) and describe their properties. In particular we have studied the ferrous and CO derivatives, especially the latter's response to photolysis, and their ability to bind to the native redox partner of cytochrome *c*, cytochrome *c* oxidase. Here we report the quantum yields for CO photo-dissociation for these mutants and use changes in this parameter on binding to its oxidase to determine the binding constant for complex formation. These binding constants are very similar in magnitude to those reported for the native protein and indicate good recognition between cytochrome *c* oxidase and the mutant cytochromes *c*. However, we have found, in agreement with earlier work on semi-synthetic horse heart cytochrome *c* in which Met80 was replaced by Ala [10], that these mutants have small apparent quantum yields for CO photo-dissociation compared with Cm cyt *c*, i.e. the majority of recombination is geminate. Thus at present these mutants are only partially suitable for the study of electron transfer, offering little advantage over well coupled ruthenium systems.

2. Materials and methods

2.1. The yeast iso-1-cytochrome c expression system

Construction of the expression system for *S. cerevisiae* iso-1-cytochrome *c* in *E. coli* has been reported [9,11]. This work involved the cloning of genes CYC1 and CYC3, which encode the yeast iso-1-cytochrome *c* and yeast cytochrome *c* heme lyase respectively, into the vector pUC18 to give the pBPCYC1(wt)/3 plasmid construct. The two genes were arranged in parallel with CYC1 upstream from CYC3. Transcription is directed by the two promoters, Lac and Trc, that are located upstream from CYC1. pBPCYC1(wt)/3 contains a marker for ampicillin resistance. The pBPCYC1(wt)/3 plasmid construct was kindly donated to us by W. Pollock (Vancouver).

2.2. Mutagenesis of the yeast cytochrome c gene

The original pBPCYC1(wt)/3 plasmid construct has the native Cys102 residue substituted by a threonine residue to prevent formation of disulphide bonds between cytochrome *c* molecules. This was further modified by removal of one of only two Kpn1 restriction sites, the site at position 246 outside the CYC1 gene. The remaining Kpn1 site at position 1086 and within the CYC1 gene,

made insertion of small (~100 bp) pieces of DNA containing the modified position-80 straightforward, following cuts with Kpn1 and BamH1 on both the modified plasmid and PCR fragments. Introduction of the modified methionine-80 codons using PCR was carried out using two primers, a 1×forward and 1×backward of ~25 bp's each. The forward primer contained the modified 80 codon and often incorporated a unique restriction site designed to make initial screening of cloned mutagenic PCR an easier task. The yeast cytochrome *c* methionine-80 mutants synthesised were Asp, Glu, Ala, and Ser. Mutant DNA was identified by carrying out restriction enzyme digests on various transformants utilising the aforementioned unique restriction sites, followed by dideoxy DNA sequencing in the region of the introduced mutations.

2.3. Expression and isolation of mutants from E. coli

Plasmid constructs containing the modified methionine-80 codons were transformed into *E. coli* strains HB2151 or XL1-Blue, and were efficiently expressed. Bacteria were grown up in 2 l Erlenmeyer flasks, containing 1.5 l of a modified Luria Broth enriched with ~30% more tryptone (10 g l^{-1}) and yeast extract (8 g l^{-1}) than standard broth and containing sodium chloride (5 g l^{-1}), ampicillin (100 mg l^{-1}), and sodium nitrate (~2 g l^{-1}) which stabilises the heme environment and increases protein yields (Mauk, personal communication). The inoculated flasks were shaken and maintained at 37 °C for ~48 h. Following growth of bacteria, centrifugation (7000×*g*, 10 min) gave ~5 g l^{-1} of bacterial pellet which was lysed at room temperature for 1 h using a lysis buffer containing egg white lysozyme (3 g l^{-1}), Tris–HCl (50 mM, pH 8.0), RNase, DNase, EDTA (5 mM), and a protease inhibitor PMSF (~2 mM), at ~3 ml g^{-1} of bacterial pellet. Following lysis, further centrifugation (10 000×*g*, 15 min) gave a supernatant containing the mutant cytochromes. To the supernatant, adjusted to pH 7, was added solid CM52 cation exchange resin (10 g 100 ml^{-1}) and this was stirred at 4 °C for ~48 h to allow binding of the basic proteins. The CM52 was allowed to settle and then loaded into a column (45×1.5 cm) and the proteins eluted with phosphate buffer (50 mM, pH 7.0, 0.3 M NaCl). The mutant cytochromes and lysozyme were separated using HIC, by small (1 ml) HiTrap Phenyl Sepharose (high performance or fast flow/high substitution) columns obtained from Pharmacia Biotech. The proteins were loaded onto this column at high salt concentration (2.1 M ammonium sulphate) in a Tris–HCl buffer (50 mM, pH 8.0), and were eluted in a very pure form at between 1.2 and 1.4 M salt. Lysozyme was eluted at salt concentrations $\lesssim$1.0 M.

2.4. Preparation of other proteins

Cytochrome *c* oxidase was prepared from beef heart by the method of Yonetani [12]. Concentrations of were determined by using $E_{605}=42\,000$ $M^{-1}\,cm^{-1}$ (per functional unit, containing two heme *a* groups) for the fully reduced protein. Carboxymethyl cytochrome *c* was prepared by the method of Schejter and George [2]. Horse heart myoglobin was purchased from Sigma Chemical Co.

2.5. Spectrophotometry and photolysis

All spectra were recorded using a Cary 5 (Varian) instrument. Lamp flash photolysis was performed using an Applied Photophysics instrument, equipped with a xenon flash (Hi Tech Instruments). The flash intensity was attenuated using a set of neutral density filters. Laser photolysis was carried out using a Nd-YAG laser (Spectra Physics) as described by Brzezinski and Wilson [5].

2.6. ^{1}H-NMR of ferrous Cm cyt c and the Met80Glu mutant

Samples for NMR were prepared by several cycles of concentration by lyophilisation and dilution in D_2O. The buffer added was deuterated potassium phosphate (20 mM), and pD adjusted using small amounts of either HCl (0.1 M) in D_2O, or NaOH (0.1 M) also in D_2O. The final

concentrations of the proteins in buffer solution were ~400 μM (Met80Glu) and 700 μM (Cm cyt *c*). ^{1}H-NMR spectra were recorded on a Varian Unity Inova 600 spectrometer operating at a ^{1}H frequency of 599.167 MHz.

3. Results

3.1. Determination of extinction coefficients

The extinction coefficients of the carbon monoxy (CO) derivatives were determined from which all other extinction coefficients were found by comparison of spectra. The extinction coefficients were determined by titrating the ferrous (sodium dithionite reduced) proteins (at concentrations of approx. 5–10 μM) with solutions of CO, the concentration of which had previously been determined by titration against known concentrations of ferrous myoglobin. At these protein concentrations, and because the affinity of CO for the ferrous proteins is high ($K \sim 10^7$ M^{-1}, data not shown), the titrations yield precise endpoints. From a knowledge of the concentration of CO required to saturate the protein and the stoichiometry of the reaction (1 CO/heme) the concentration of heme (and hence, cytochrome) present could be determined and from this the extinction coefficient at any wavelength could be calculated. The values of the extinction coefficients of the carbon monoxy derivatives of the mutants at their Soret maxima were found to be; Met80Ala $E_{413.5} = 212\,000$ M^{-1} cm^{-1}, Met80Ser $E_{413.2} = 210\,000$ M^{-1} cm^{-1}, Met80Asp $E_{414} = 216\,000$ M^{-1} cm^{-1}, Met80Glu $E_{414} = 217\,000$ M^{-1} cm^{-1}. These values are the average of three determinations each and the standard error on each mean is ~5000 M^{-1} cm^{-1}. The value found for Cm cyt *c* was $E_{414} = 217\,000$ M^{-1} cm^{-1}, very similar to the value 219 000 M^{-1} cm^{-1} reported by Brunori et al. [3].

3.2. Spectral properties of ferrous Met80X mutants

The spectra of the ferrous state of all the Met80X mutants were pH dependent displaying simple, $n = 1$, titration curves characterised by a single p*K*, the value of which depended on the nature of the residue at position 80 [13]. The p*K* values for the mutants are given in Table 1. At high pH values, two pH units above the p*K*, all the mutants displayed very similar spectra having the typical profile of a low-spin *c*-type cytochrome with α, β and γ bands at 520, 550 and ~417 nm, respectively. Under these conditions the corresponding ferric species were shown by EPR spectroscopy to be low-spin, possessing the signal around $g = 3$ characteristic of low-spin ferric heme (data not shown). These spectral features reflect the fact that in the high pH forms the sixth coordination position, occupied by Met80 in the native protein, is filled by another intrinsic ligand in the mutant proteins that stabilises the low-spin form. This behaviour is identical to that of Cm cyt *c* [3,4]. The ligand binding to the iron at high pH values has been assigned as a lysine residue, possibly lysine 79 (see below).

Table 1
Quantum yields for CO photo-dissociation for mutant and Cm cyt *c* relative to myoglobin and p*K* values for the ferrous pH transition

Protein	Relative quantum yield (φ)	pH Transition p*K* (±S.D., $n = 3$)
Myoglobin	1	
Cm cyt *c*	0.26 ± 0.01	7.1 ± 0.03
Met80Asp	0.04 ± 0.002	6.3 ± 0.12
Met80Glu	0.038 ± 0.002	6.8 ± 0.09
Met80Ser	0.006 ± 0.001	9.7 ± 0.14
Met80Ala	0.004 ± 0.0005	9.4 ± 0.08

On lowering the pH, a single protonation event causes this ligand to dissociate from the heme iron leading to substantial spectral changes as illustrated in Fig. 1 [3]. The particular spectral features displayed by the low pH forms of the ferrous proteins are dependent on the nature of the mutant. If alanine is substituted at position 80 the resultant spectrum is consistent with a pure high-spin ferrous derivative, while if serine occupies this position the spectrum retains some low-spin character. Analysis of these spectral features will be reported elsewhere.

Irrespective of pH, i.e. whether or not a ligand stabilizing the low-spin form is bound to the ferrous iron, the mutants all bind CO. At high pH values CO displaces the bound ligand and the

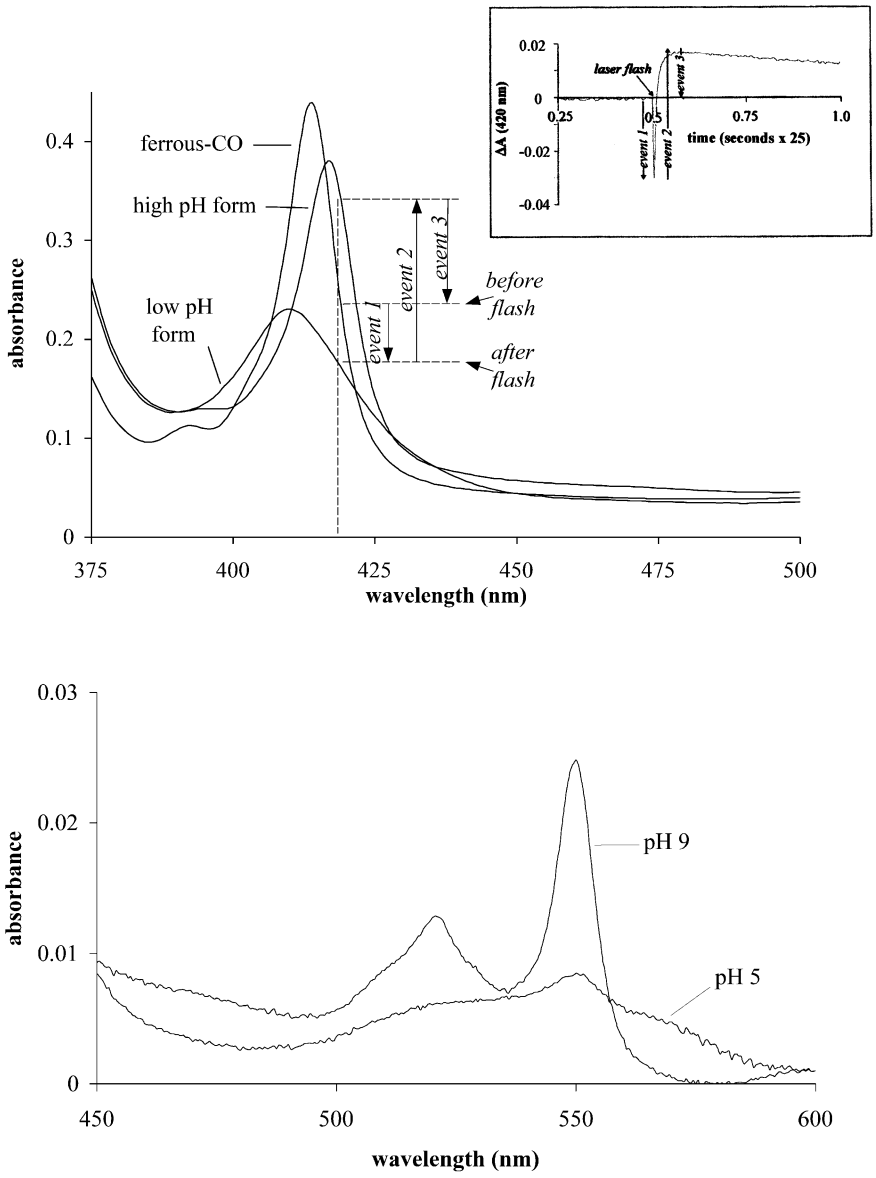

Fig. 1. Spectra of yeast Met80Asp ferrocytochrome *c* at pH 5 (low pH form) and at pH 9 (high pH form), and the CO derivative (in the Soret region). Events 1, 2 and 3 represent the major spectroscopic changes that take place at 420 nm following laser flash photolysis. The inset shows the time course for these events. The protein concentration for the Soret region was 2 μM and for the visible region ~1 μM. The protein was reduced with a slight excess of sodium dithionite. The temperature was 20 °C.

spectra of the carbon monoxy derivatives are all very similar (see extinction coefficients above) and typical of low-spin ferrous CO adducts (see Fig. 1 for example). These spectra are essentially pH independent.

3.3. ^{1}H-NMR of Cm cyt c and Met80Glu

Fig. 2 shows the ^{1}H-NMR spectra of the ferrous Met80Glu mutant and Cm cyt *c* at high and low pH values, and compares these to that of the

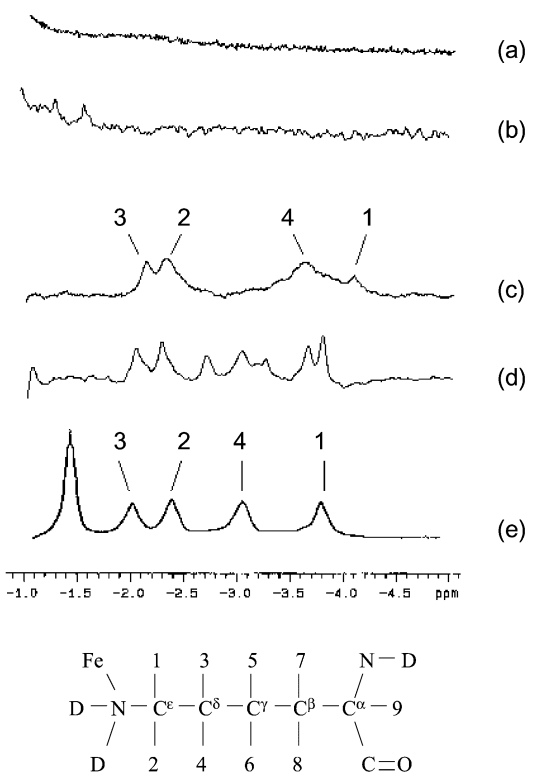

Fig. 2. ^{1}H-NMR spectra of the ferrous forms of the Met80Glu mutant of yeast cytochrome *c*, of Cm cyt *c* (horse), and of the Met100Lys mutant of *Thiobacillus versutus* cytochrome *c*-550 (taken from [14]). (a) The low pH form (pH 5.3) of Cm cyt *c*; (b) the low pH form (pH 5.0) of the Met80Glu mutant; (c) the high pH form (pH 8.5) of Cm cyt *c*; (d) the high pH form (pH 8.5) of the Met80Glu mutant; (e) the Met100Lys mutant (pH 7.0). The numbers indicated are C-H assignments as given in the schematic representation of lysine, 2f.

Met100Lys mutant of *Thiobacillus versutus* cytochrome *c*-550 (pH 7.0) taken from Ubbink et al. [14]. The high pH low-spin form of Cm cyt *c* shows four one-proton peaks in the low-frequency region Fig. 2c, similar to the Met100Lys mutant Fig. 2e. These signals are not present in the spectra at low pH values (~2 pH units below the p*K*), Fig. 2a. The Met80Glu mutant shows seven, possibly eight, one-proton peaks that are not present in the spectra at low pH values (Fig. 2b and d). None of these one-proton peaks are observed in the spectrum of ferrous wild-type cytochromes *c* (horse and yeast) at neutral pH, where the resonances of the side chain of the bound methionine-80 are present.

3.4. Measurement of apparent quantum yield (φ)

The CO adducts of all the mutant cytochromes *c* were light sensitive, as expected from the known behaviour of other ferrous heme proteins, in particular Cm cyt *c* [4]. On illumination CO dissociated, the extent of the dissociation being dependent on the nature of the substitution at position 80 and upon the light intensity. Following the flash the CO that had dissociated and equilibrated with the bulk phase CO recombined in a rapid reaction that was dependent on the CO concentration. The details of the kinetics of CO combination at high pH are made complex by competition between the incoming CO and the intrinsic lysine residue, as reported for Cm Cyt *c* [4], and will not be fully discussed here. The amplitude of the absorbance change accompanying CO recombination is a measure of the extent of photo-dissociation and has been employed to determine the apparent quantum yield, φ, for photo-dissociation using the 'pulsed' method pioneered by Brunori et al. [15]. In this method the extent of photo-dissociation is measured as a function of the intensity of the flash. The method determines the value of φ relative to a standard, generally myoglobin, for which the apparent quantum yield is unity, i.e. one CO molecule is released into bulk solution for each photon absorbed by the heme group. The results are shown in Fig. 3a and b where the logarithm of the ratio of the concentrations of the CO adduct in the dark to that in light (of relative intensity I) is plotted as a function of I. As predicted by theory [15], these plots are linear, passing through the origin. The relative slopes of these plots (the spectral overlap between the lamp and each protein being very similar) yield the apparent quantum yields. Thus from Fig. 3a, in which the data for myoglobin and Cm cyt *c* are given, the value of φ for Cm cyt *c* may be calculated as 0.26, the slope being 0.26 of that for myoglobin. This value for Cm cyt *c* is identical to that reported by Wilson et al. [16]. Table 1 gives the values of φ for four Met80X mutants.

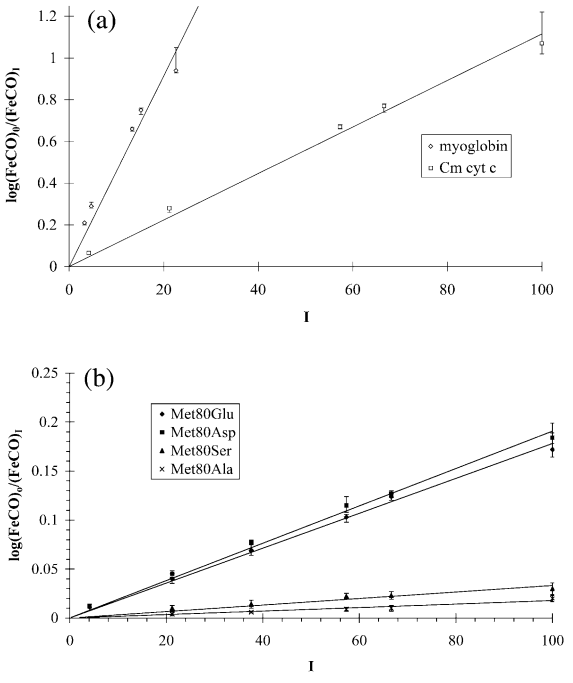

Fig. 3. (a) Dependence of the extent of photo-dissociation of the CO adducts of myoglobin and Cm cyt *c* on relative light intensity (*I*) expressed as a percentage of full flash intensity. (b) Dependence of the extent of photo-dissociation of the CO adducts of the Met80X mutants on light intensity. The protein concentrations were ~5 μM dissolved in 20 mM sodium phosphate buffer, pH 7.0, and the temperature 25 °C.

3.5. Interactions between Met80X mutants and cytochrome c oxidase

3.5.1. [17]*The Hummel and Dryer method*

On passing oxidised cytochrome *c* oxidase through a gel filtration column (Sephadex G100 26 cm long by 0.75 cm diameter) equilibrated with ferric cytochrome *c* yielded the elution profile typical of that seen for protein/protein association [17]. Fig. 4a and b show these profiles for Cm cyt *c* and, as an illustration, for the Met80Glu mutant. It may be seen that the cytochrome *c* concentration is greater than basal concentration in those fractions in which the high molecular weight cytochrome *c* oxidase elutes and there is a corresponding 'trough' in cytochrome *c* concentration following this peak. Even at very low protein concentrations it is apparent that there is a strong association between the mutant cytochromes *c* and cytochrome *c* oxidase. The concentration of cytochrome oxidase in the peak fraction together with the concentrations of bound and free cytochrome *c* yield the association constant for the proteins. For Cm cyt *c* this value is 1.5×10^5 M^{-1} at ionic strength of 0.013 M. For the mutant forms of cytochrome *c* the binding constant, under similar conditions, is much larger. The data in Fig. 4b yield a value of 2.5×10^7 M^{-1}, a value comparable with those reported for the interaction of the native molecule with cytochrome oxidase at this pH and ionic strength [18]. All the mutants gave very similar binding constants and these indicate that the recognition between the mutants and oxidase is largely unperturbed by the mutation at position 80. As expected, at high salt concentrations the electrostatic interactions stabilising the protein/protein complexes were disrupted and the complexes dissociate. This is reported in Fig. 4a and b where it is seen that the 'peak' and 'trough', diagnostic of association, are absent in the presence of high salt concentrations.

3.5.2. Using changes in φ to determine binding constants

On forming a complex with cytochrome *c* oxidase the apparent quantum yield of each mutant was found to decrease. This phenomenon is unhelpful if one wishes to study electron transfer and explains why it proved difficult to monitor electron transfer using these mutants. However, the decrease in φ on binding mutant cytochrome *c* to oxidase proved interesting as it may itself be used to measure binding constants between the *reduced* proteins. Fig. 5 reports the results of an experiment in which Met80Glu mutant was titrated with cytochrome *c* oxidase. On addition of oxidase the amplitude of the signal for CO recombination from the bulk phase, following the flash, decreases. This may result either from a change in the quantum yield or because of significant attenuation of the light intensity in solution due to the increase in absorbance caused by addition of cytochrome *c* oxidase. This latter reason may be excluded by addition of salt to the solution. This will not affect the absorbance (apart from a small dilution effect) but will disrupt electrostatic interactions that sta-

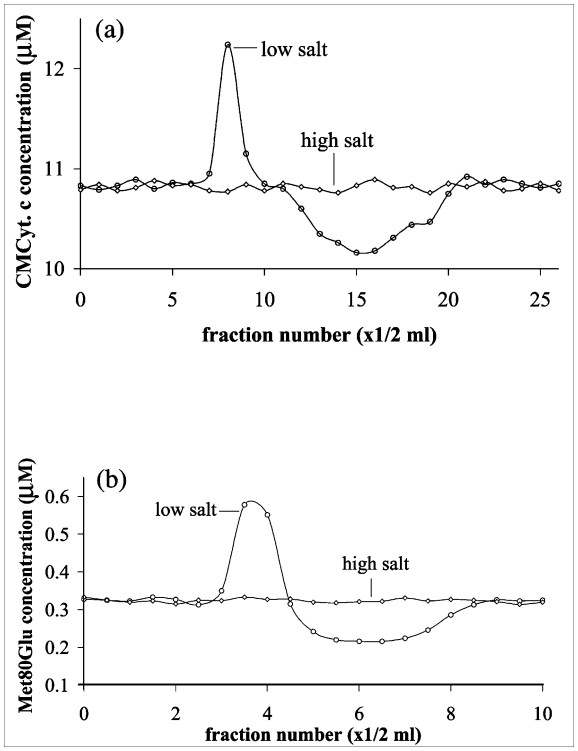

Fig. 4. The interactions between bovine cytochrome *c* oxidase and either Cm cyt *c* or Met80Glu cytochrome *c* monitored through molecular exclusion chromatography. The cytochrome *c* oxidase was loaded onto the column at either 10 μM (a) or 1 μM (b) under conditions of either low or high ionic strength. Low salt conditions were Tris–HCl, 10 mM, pH 7.8 containing 0.1% lauryl maltoside, and high salt conditions were as above plus 250 mM NaCl.

bilise the protein association. From Fig. 5 we see that on progressive addition of salt the absorbance change reporting CO recombination from bulk solution returns to its original value, indicating that the decrease in the absorbance change on titration with oxidase is due to lowering φ, by $\sim$50%, on complex formation. This being so we have fitted the curve to the standard binding equation (see footnote to Table 2) and have obtained the value of the binding constant and the endpoint of the titration. This endpoint corresponds to the condition where all the cytochrome *c* is in the complex, i.e. no free cytochrome *c* is present. Using this endpoint and the initial absorbance change (that corresponds, in the absence of cytochrome *c* oxidase, to all the cytochrome *c* being free), it is possible to calculate the binding constant at each of the ionic strengths shown in Fig. 5.

Table 2 reports these data and the comparable data for Cm cyt *c*.

3.6. Laser flash experiments

On laser illumination of the complex formed between ferrous CO derivatives of Met80Asp or Glu (chosen because of their larger φ values), and oxidised cytochrome oxidase very small, very fast, transients (<100 μs) reporting electron transfer were observed. However, it proved difficult to obtain reproducible and robust results from such signals. In the ms–s time range, however, larger reproducible signals were recorded. These signals were present in the absence of cytochrome oxidase and displayed features that were qualitatively similar for each mutant but which exhibited kinetics that were strongly influenced by the nature of the residue at position 80 and the pH at which the

Table 2
Ionic strength dependence of the binding constant for complex formation between mutant or Cm cyt *c* and cytochrome *c* oxidase

Ionic strength	K M^{-1} ($\times 10^{-4}$)	
	Met80Asp	Cm cyt *c*
0.0115	252	5.0
0.022	25	3.7
0.037	6	2.4
0.062	1.1	1.6
0.072	n.d.	1.4
0.087	n.d.	1.0

Above an ionic strength of 5 mM salt the association between cytochrome *c* and its oxidase is found to conform to a simple equilibrium model, i.e.

Cyt *c* + Oxidase ↔ complex

where K_D = [Cyt *c*][Oxidase]/[complex]. Titrations of cytochrome *c* (at constant concentration, $[\text{cyt } c]_T$) with oxidase (Fig. 5) may be described by the standard quadratic binding equation, namely;

$$Y = (-b - (b^2 - 4ac)^{1/2})/2a \quad (1)$$

where *Y* is the fractional saturation of the cytochrome *c* with oxidase (i.e. [complex]/$[\text{cyt } c]_T$) and $a = [\text{cyt } c]_T$, $b = -([\text{cyt } c]_T + [\text{oxidase}] + K_D)$ and c = [oxidase].

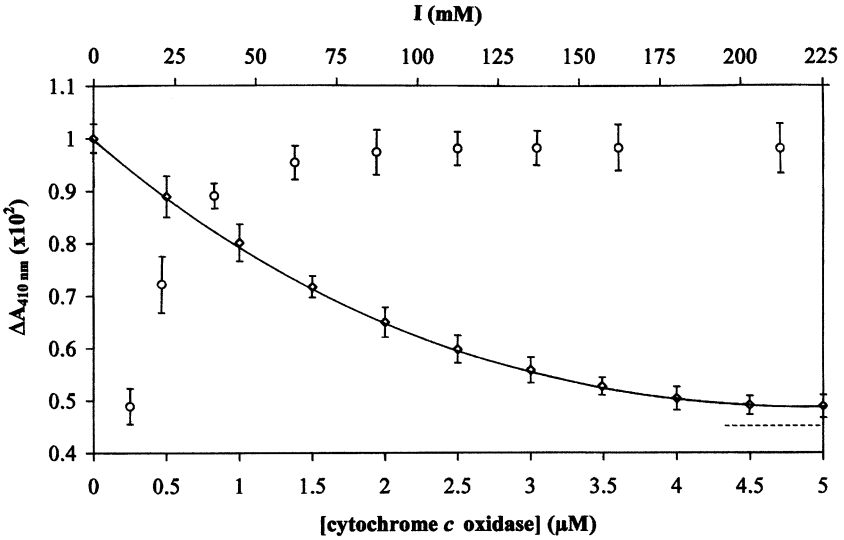

Fig. 5. The extent of photo-dissociation of CO from Met80Asp carbon monoxy cytochrome *c* (2.2 μM) on titration with cytochrome *c* oxidase (open diamonds, lower axis scale) and the subsequent return of photosensitivity on increasing ionic strength by addition of NaCl (open circles, upper axis scale). Recombination of CO to the mutant cytochrome was monitored at 410 nm, a wavelength isosbestic for CO recombination to oxidase. The buffer was 20 mM sodium phosphate, pH 6.3 (ionic strength = 11.5 mM), temperature 20 °C. The solid line is a fit ($R^2 = 0.999$) to Eq. (1) and the predicted end point is indicated (----).

experiment was performed. Fig. 1 (inset) illustrates the transients that follow laser photolysis of CO from Met80Asp cytochrome *c* at high pH (two pH units above the p*K* for the spectral transition). Immediately following the flash the first optical transition, event 1, leads to a decrease in absorbance. This is as expected as CO photolysis leaves the heme penta co-ordinate and thus exhibiting the spectrum associated with the low pH form. The amplitude of this signal is smaller than expected by reference to the static spectrum because of very rapid geminate CO recombination, not resolved on our time scale, that results in the low quantum yields reported above. Following this we observe the kinetics of the transition from the low pH form to the high pH form, that is the binding of the ligand that drives the heme low-spin, event 2. For the Met80Asp mutant this process conforms to a single exponential with rate constant $k = 5750\ s^{-1}$. The final process observed is the slower recombination of the CO from the bulk phase, returning the protein to the original CO adduct, event 3. The profile of this transition depends strongly upon the pH. For example at low pH, below the p*K*, the kinetics of CO recombination for all mutants conform to simple second-order processes (see Fig. 6 for an example), the second-order rate constants of which are 9.5×10^4, 1.5×10^4, 1.7×10^5 and $2.5\times10^5\ M^{-1}\,s^{-1}$ for Met80Ala, Ser, Glu and Asp, respectively. Above the p*K* the rate of CO recombination becomes [CO] independent, and is limited by the rate at which the intrinsic ligand (possibly lysine) dissociates (Fig. 6).

4. Discussion

Mutation of the intrinsic methionine ligand of cytochrome *c* has been reported previously. The Met80Ala mutant of yeast cytochrome *c* was first described by Lu et al. [9] and the structure of the ferric cyanide derivative, determined by NMR methods [19], was found to retain the protein fold and overall conformation of the native protein. This structure, in the vicinity of the heme group, is shown in Fig. 7. The heme group is buried within the hydrophobic core of the protein. This hydrophobic interior is also retained in the ferrous

form as indicated by the great stability of the oxyferrous derivative ([10] and confirmed by us, data not shown). In general, ferrous heme is rapidly oxidised by oxygen unless a protein provides a hydrophobic environment and sterically restrains the bound oxygen. In the case of Met80Ala the oxy complex is very stable (half time for autoxidation 6 h, Silkstone unpublished), though not as stable as that formed by horse heart Met80Ala cytochrome *c* produced by semi-synthetic methods [10]. The structural reasons for this difference are not presently known.

It is apparent from Fig. 7 that lysine 79 retains a position close to that it occupies in the native molecule. It is known from studies of the alkaline transition of native ferric cytochrome *c* that at high pH (~10) lysine 79 (among other lysines) may displace Met80 and occupy the 6th co-ordination position of the heme group [20]. Mutation of *Thiobacillus versutus* cytochrome *c*-550 has shown that when the methionine ligand (Met100) is substituted by a lysine, this residue may bind to the heme iron, confirming that the ε-amino group may act as a ligand for *c*-type heme [14]. This conclusion is strongly reinforced by 2-D NMR spectroscopy (COSY and NOESY), where not only the four one-proton peaks shown in the Fig. 2e are seen, but also five other one-proton peaks (nine in total), therefore assigning lysine as the

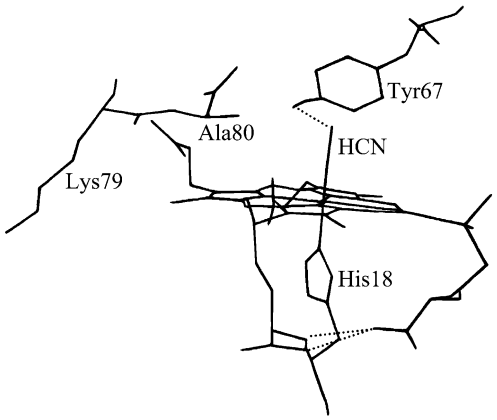

Fig. 7. The structure in the region of the heme pocket of the cyanide adduct of Met80Ala yeast iso-1-ferricytochrome *c*. This structure was determined by ^{1}H-NMR spectroscopy (structure number 17, protein data bank, [14]). Some important amino acid residues lining the distal heme pocket are shown together with the nearby Lys79 residue.

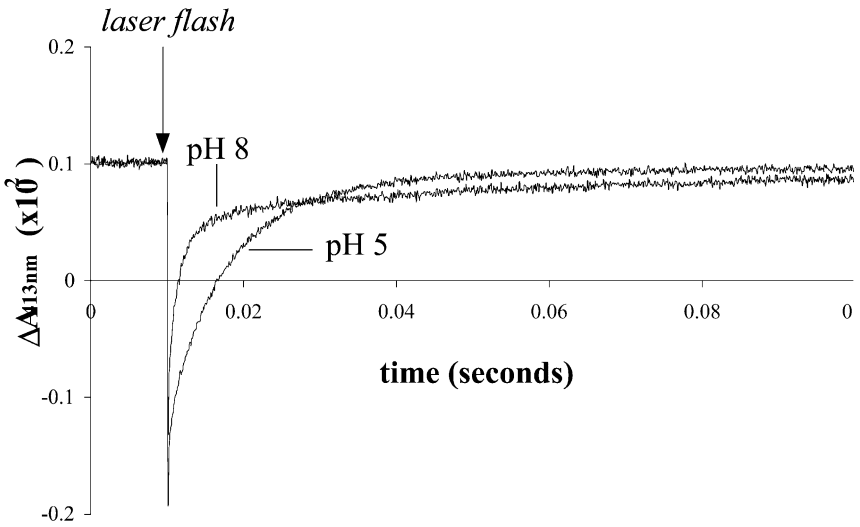

Fig. 6. Laser flash photolysis of the Met80Glu mutant of yeast cytochrome *c* (2 μM), at pH values 5.0 and 8.0. At pH 5.0 the time course reports CO binding from bulk solution, with a k_{obs} of ~175 s^{-1} at 1 mM CO (single exponential fit). At pH 8.0 the time course becomes biphasic. The faster phase reports lysine binding $k_1 = \sim 520\ s^{-1}$, that effectively competes with CO binding from bulk. The slower phase reports replacement of the lysine by CO and is rate limited by the lysine off rate from the ferrous heme iron, $k_2 = \sim 19\ s^{-1}$. The buffer was 50 mM sodium phosphate, temperature 20 °C.

ligand to the ferrous heme iron (very probably the Lys100). Although it is clear from the ^{1}H-NMR spectra presented in Fig. 2c and d, that a ligand binds to the iron of Cm Cyt *c* and the Met80Glu mutant at high pH, rendering the iron low-spin, it is not possible to assign this ligand from this data alone. Assignment of these one-proton peaks can, however, be made with some degree of certainty by comparison with those of the Met100Lys mutant of cyt *c*-550 obtained by Ubbink et al. [14] (Fig. 2e). Although assignment of the protons 1–4 of the proposed lysine ligand appears reasonable for Cm cyt *c*, assignment of the low-frequency resonances for the Met80Glu mutant is more difficult. It is clear, however, from the position of the peaks in comparison with those of Cm cyt *c* and the Met100Lys mutant, that lysine is very likely to be the ligand at pH values above the p*K* of the spin state transition of the ferrous form.

In order that the lysine at position 79 (or indeed another lysine, say Lys 73) may occupy the 6th co-ordination site the protein loop containing this residue must rotate (or otherwise rearrange) to allow the approach of the amino group to the iron. This requires some unfolding and re-packing of the protein in this region. It is evident that the ease with which this takes place is mutant dependent as shown by the p*K* value for the ferrous pH transition (Table 1). In all mutants spectral evidence suggests that lysine is bound in the ferrous alkaline form, the differences in the p*K* values, therefore, reflect the extent to which the free energy of binding of this lysine to the iron is used to remodel the protein. (The addition of a negative charge at position 80 by substitution of Asp or Glu would tend to elevate the p*K* of a neighbouring Lys and hence the apparent p*K*) When hydrophilic residues are substituted the p*K* values fall, indicating that the structure of the protein is disrupted and rearrangement of the loop is easier. This is most clearly seen when a negatively charged carboxyl group occupies position 80. For Met80Asp the transition has p*K* 6.3. Using a simple model [3] in which only the deprotonated amino group can bind to the ferrous iron, this fall in p*K* may be interpreted as the lysine having a 1000-fold greater affinity for the iron in the Asp mutant than in the Ala mutant [13]. This difference in affinity reflects the relative difficulty in distorting the structure of the Ala mutant compared to that of the Asp mutant. From the above we conclude, in agreement with other available information, that the Met80Ala mutant retains the structure of the native molecule in which the heme is embedded in a relatively rigid closed cavity with little access to solvent. This is in keeping with recent NMR studies [21] on *R. capsulatus* ferrocytochrome c_2 in which it was found that the protein structure in the vicinity of the heme is unusually rigid, probably due to the interaction of the protein with the heme prosthetic group. Our results suggest that even in the absence of the strong Met80 to ferrous iron bond these interactions confer rigidity on the protein structure, particularly for the Met80Ala mutant. This structural interpretation explains the very low value of φ for the Ala mutant, as previously pointed out by Bren and Gray [10]. On photolysis all CO/Fe(II) bonds are broken and the dissociated CO possess translational energy, acquired from the excitation of the heme. The CO molecule is now able either to recombine with the iron (in ps, geminate recombination) or migrate away from the iron into the protein and eventually through the protein into the bulk solution. The balance between these alternatives depends on the presence of internal cavities within the protein into which CO may rapidly diffuse. For myoglobin, a molecule evolved to bind and dissociate dioxygen, these cavities are present and define a route to the exterior [22,23]. Thus, for myoglobin, on photolysis essentially all photo-dissociated CO finds its way to the bulk solution and hence the quantum yield is unity. In contrast the Met80Ala mutant provides no such route, the tight structure around the heme group does not permit easy migration of the CO. Thus, a large fraction of the CO recombines with the heme very rapidly, probably within ps, and very little reaches the bulk solution. The apparent quantum yield is therefore very low, Table 1. Geminate recombination of CO is so rapid that it would compete very effectively with even the fastest electron transfer processes and explains why with this mutant we were unable to observe electron transfer to cytochrome *c* oxidase. If the protein backbone loop-bearing position 80 is disrupted by introduction of hydrophilic/charged groups into

the heme cavity the loosened structure now permits some CO migration into the bulk and φ increases 10-fold, but remains very small (Table 1). It is interesting that Cm cyt *c*, that has been modified not only at Met80 but at other (unspecified) lysine residues does provide a more permeable structure with a consequent further 5-fold increase in φ. Once bound to cytochrome *c* oxidase the value of φ falls, indicating that the route from the iron to the exterior becomes partially occluded.

The relative values of the lysine 'on' rate are consistent with this view, the easier it is for the lysine-bearing loop to re-orientate the more rapidly the binding process may take place. Thus, for the relatively rigid Met80Ala mutant the value is $\sim 100\ s^{-1}$ while it increases to $5700\ s^{-1}$ for the Met80Asp mutant which we suggest has the looser structure in the vicinity of the heme. The value reported for the Cm cyt *c*, that has the highest value of φ, is $15\,300\ s^{-1}$ [3].

Although there are clearly structural changes induced by mutation at position 80, as discussed above, these do not influence the recognition of the cytochrome *c* for its redox partner. The interaction between cytochrome *c* and its oxidase, either in their ferric or ferrous forms, is very tight. The data in Table 2 may be used to construct Bronsted plots of $\log K$ vs. $(I)^{\frac{1}{2}}$. Such plots (not shown) are approximately linear and indicate that the binding constant of the mutant cytochromes to the oxidase is $>10^8\ M^{-1}$ at zero ionic strength. This value is in close agreement with to that reported for the interaction with native cytochrome *c* [18]. In addition, the slopes of such plots give an indication of the number of charges involved in docking and stabilising the proteins in the complex. For the mutants the slope is ~ 16 suggesting that up to four charges on each protein interact electrostatically. Although this estimate is crude, being based on an oversimplification of a complicated situation, it does nevertheless agree well with the results of Wang et al. [24] who have identified four carboxyl groups close to the electron entry site on subunit II of cytochrome *c* oxidase that interact with lysine groups surrounding the heme crevice of cytochrome *c*. Similar plots for Cm cyt *c* yield the value of two charge/charge interactions consistent with the fact that carboxymethylation modifies some lysine groups in addition to methionines.

Although the Met80X mutant cytochromes dock with oxidase in what appears to be the native site their low quantum yields make them less than ideal for use as light induced electron donors. In order that they may become of use it is necessary to engineer at least one cavity in the protein that may facilitate the escape of CO once it leaves the iron. It is to that end that we are now working. Clues to how this may be achieved may be found in recent literature. For example, it has been demonstrated that relatively minor alterations to the overall protein structure can have dramatic effects on quantum yield. Sugimoto et al. [25] constructed myoglobin mutants in which geminate yield was increased 10-fold (from 7 to 70%) by mutations in the distal pocket (Val68Thr, little change: Val68Thr/His64Val, 10-fold increase). A recent review [26] discusses the role of packing defects on the reactivity of myoglobin with gaseous ligands and points out the significant effects of single mutations. We are hopeful that the same may apply to cytochrome *c*, such that a further mutation, in addition to Met80, will elevate the value of φ to levels at which these proteins will find a useful role in the study of protein electron transfer reactions.

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