Some reactions of carbon monoxide and oxygen with carbodi-imide-modified cytochrome *c*

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The reactivity of carbodi-imide-modified tuna and horse heart cytochromes *c* with the ferrous ion ligands CO and O₂ has been studied. Both modified cytochromes bind one molecule of CO. Stopped-flow and flash-photolysis experiments indicate the presence of three kinetic processes in the reaction of the cytochromes with CO. The second-order rate constants associated with all three kinetic process are pH-independent being $2.8 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$, $3.8 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $4 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$ under all conditions studied. The concentration-dependence of the contributions made by each of the processes to the overall absorbance change indicates that the fast and slow kinetic phases are associated with two forms of the cytochromes which are in equilibrium, whereas the intermediate phase arises from a separate cytochrome species. The quantum yield for the photodissociation of CO from the ferrous cytochromes is unusually low. Both modified cytochromes are capable of binding and reducing O₂. In the presence of excess reductant, the modified cytochromes can catalytically reduce large molar excesses of O₂. In the absence of excess reducing agent, the oxy complex initially formed undergoes a pH-dependent intramolecular electron-transfer process with half-life approx. 10 min. EDC [1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide]-promoted internal cross-linking is proposed to account for differences between the EDC-modified proteins and carboxymethylated cytochrome *c*.

INTRODUCTION

Although it was originally suggested by Timkovich (1980) that the treatment of cytochrome *c* with the water-soluble 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide (EDC) leads predominantly to modification of the less buried haem propionate group and the carboxylate function of Asp-62, it has been shown by peptide mapping that extensive intramolecular cross-linking and additional covalent attachment of the haem group to the protein occurs (Mathews & Brittain, 1986). The protein produced by chemical modification is interesting not only in that the internal cross-linking should lead to a less flexible structure which might effect the conformational changes normally observed in the ferrous–ferric transition (Simone & Kreishman, 1983), but also because it shows unusual spectral-reactivity correlations (Mathews & Brittain, 1987). In particular, it has been suggested that the loss of the 695 nm band from the visible absorption spectrum of native ferricytochrome *c* corresponds to the formation of five-co-ordinate haem due to the loss of Met-80 ligation (Sreenathan & Taylor, 1971), and that a correlation exists between the property of ascorbate-reducibility and the presence of this absorption band (Greenwood & Palmer, 1965). The carbodi-imide-modified proteins lack the 695 nm band of native ferricytochrome *c*, bind CO and O₂, and yet retain ascorbate-reducibility (Timkovich, 1980; Mathews & Brittain, 1987), suggesting that intermediate structural states exist for ferricytochrome *c* in which the protein structure surrounding the haem is sufficiently disrupted to lead to loss of the 695 nm band while retaining sufficient native structure to allow ascorbate-reducibility (Mathews & Brittain, 1987; Angstrom *et al.*, 1982).

In view of the unusual spectral-reactivity correlations present in the carbodi-imide-modified proteins, we have undertaken an investigation of the reactions of these proteins with both CO and O₂, and compare them with those of carboxymethylated cytochrome *c* (Wilson *et al.*, 1973) and horseradish peroxidase (Phelps *et al.*, 1974).

MATERIALS AND METHODS

Albacore tuna (*Thunnus alalunga*) were caught on long lines off the East Coast of New Zealand's South Island, and the hearts were quickly removed and stored in solid CO₂ until required. Cytochrome *c* was obtained from tuna hearts by a modification of the method of Brautigan *et al.* (1978). In the present preparation, centrifugation replaced filtration in the early steps of the reported procedure. The crude cytochrome was then recovered by batchwise adsorption on CM-cellulose (Whatman CM 52), rather than by adsorption on a bed of Amberlite IRC-50. All other steps were as previously reported. Horse heart cytochrome *c* (type VI) and EDC were obtained from Sigma Chemical Co. Deamidated and polymeric forms of the proteins were removed by chromatography on CM-cellulose (CM 52)

Fig. 1. Effect of CO on the visible absorption spectrum of EDC-modified cytochrome *c*

EDC-modified horse heart cytochrome *c* ($4.8 \mu\text{M}$ for the 370–470 nm region and $24.5 \mu\text{M}$ for the 480–600 nm region) was reduced with dithionite (----) and placed under an atmosphere of CO (—). The buffer employed was 0.05 M-citrate/phosphate, pH 6.5 at 18 °C.

(Brautigan *et al.*, 1978). Chemical modification of both proteins was performed exactly as previously described (Timkovich, 1980). Monomeric protein was carefully separated from the dimeric protein species resulting from EDC-promoted intermolecular cross-linking by passage down a Sephadex G-50 column (83 cm $\times$ 3.2 cm diameter).

All spectra were recorded with an Aminco DW2a spectrophotometer (American Instrument Corp., Silver Springs, MD, U.S.A.). Absorbance coefficients for the proteins were determined by correlation of absorbance measurements with iron content determined by atomic absorption spectroscopy. CO-binding titrations were performed by the procedure of Wilson *et al.* (1973). Stopped-flow and flash-photolysis measurements and subsequent data analysis were performed as previously described (Brittain, 1980, 1984; Light *et al.*, 1987; McKinnie & Olson, 1981). The quantum yield for the photolysis of the CO complexes was determined by the pulse method described previously (Brunori & Giacometti, 1981). O_2 -turnover experiments were performed using a Clark electrode system to monitor dissolved O_2 concentrations.

RESULTS

When the dithionite-reduced modified proteins were exposed to CO, pronounced changes were observed in their visible absorption spectra. The Soret band shifted to shorter wavelength and increased in intensity, whereas the $\alpha\beta$ -spectrum collapsed into two poorly resolved bands at longer wavelengths (Fig. 1). Titrations of the modified proteins with CO at high protein concentrations showed stoichiometric binding, indicative of the formation of a 1:1 protein-CO complex. Titration at low protein concentrations gave hyperbolic binding curves with affinity constants of approx. $2 \times 10^6 \text{ M}^{-1}$ (Fig. 2).

Stopped-flow mixing of the dithionite-reduced proteins with excess CO showed the presence of three kinetic processes. The fastest process was second order with respect to CO concentration, with a second-order rate constant of $2.8 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$. The intermediate and slow processes were also found to be second order, with associated rate constants of $3.8 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $4 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$ respectively. The rates of CO dissociation, as obtained from the intercepts of Fig. 3, were unfortunately too small to be determined with any degree of accuracy. Kinetic difference spectra indicated that all three processes were being monitored as CO-binding reactions, although the contribution from each process at any particular wavelength was not found to be equal. Furthermore the contribution which was made by either the fast or the slow process to the overall absorbance change at 415 nm was found to be CO-concentration-dependent (Fig. 4).

Initial flash-photolysis studies indicated very low quantum yields for the CO complexes of the modified proteins. Using the pulse method (Brunori & Giacometti, 1981), values were obtained for the relative quantum yield for the photolysis process. Making the usual assumptions implicit to this method, a value of 0.003–0.015 was determined for ϕ . The wide range quoted for ϕ , the relative quantum yield, arises from the low levels of photolysis produced even when flash discharges of 25 kV were used. However, even at these low levels of photolysis, measurements of CO binding were made subsequent to photolytic dissociation.

In the case of flash-photolysis studies the results obtained for both cytochromes were completely analogous to those observed in stopped-flow studies (Figs. 3 and 4), although in photolytic studies it was possible to extend measurements up to 1 mM-CO concentration. Even at the highest CO concentration available the pattern of reactivity, initially observed in stopped-flow studies, was maintained.

Fig. 2. CO titration of EDC-modified cytochrome *c*

EDC ($0.5 \mu\text{M}$)-modified cytochrome *c* was titrated with CO in 0.1 M-phosphate buffer at pH 7.0 and 20°C . Experimental data points are shown (●) together with the non-linear least-squares best fit of the data to a hyperbola with $K = 2.3 \times 10^6 \text{ M}^{-1}$ (—).

Fig. 3. CO-concentration-dependence of the rates of reaction of EDC-modified cytochrome *c*

The reaction of dithionite-reduced cytochrome *c* [$3 \mu\text{M}$ for stopped-flow (○, □, △) or $6 \mu\text{M}$ for flash photolysis (●, ■, ▲)] with CO at pH 7.0 in 0.1 M-phosphate buffer at 20°C was monitored at 415 nm. The ligand-concentration-dependences of the pseudo-first-order rate constants for the fast (○, ●; left axis) and the intermediate and slow (□, ■ and △, ▲; right axis) kinetic phases are shown.

Fig. 4. Concentration-dependence of the relative contributions of the three kinetic phases to the overall change for CO binding to EDC-modified cytochrome *c*

The absorbance change observed at 415 nm associated with the fast (○), intermediate (□) and slow (△) kinetic processes observed when dithionite-reduced EDC-modified cytochrome *c* ($5 \mu\text{M}$) is allowed to react with CO in the stopped-flow apparatus in 0.1 M-phosphate buffer, pH 7.0, at 20°C is shown.

Fig. 5. Spectrum of an oxygenated form of EDC-modified horse heart cytochrome *c*

Spectra are shown for a $9\ \mu\text{M}$ solution of the oxygenated form of EDC-modified horse heart cytochrome *c* (separated from dithionite as described in the text), at pH 7.0 in 0.1 M-Tris buffer, at various times after removal of dithionite from the oxygenated protein. The Soret spectra were recorded at 6.5 min (—) and 365 min (---) after preparation. The spectra in the α and β region were recorded at 4.4, 12.3, 22.5 and 360 min after preparation. The spectra represent solutions of increasing age, with decreasing absorbance at 550 nm. Isosbestic points are observed at 525 nm and 578 nm.

Reaction with oxygen

Two different procedures were investigated for the preparation of the oxy complex of the modified cytochromes. Oxygenation of protein reduced with a stoichiometric quantity of dithionite always led to the formation of oxidized protein within a few seconds. However, passage of reduced cytochrome, reduced with an excess of dithionite, down a Sephadex G-25 column (30 cm $\times$ 0.9 cm diameter) led to the formation of a relatively stable oxy species, whose spectra as a function of time after formation are shown in Fig. 5. The ferrous nature of the haem in the oxy species was proved by the formation of the CO complex (identified spectrally), when the oxy species was incubated with CO. By removing samples of a solution of the oxy complex as a function of time after formation and investigating their CO-binding ability, it was possible to follow the conversion of the ferrous oxy complex into ferric protein. If we assume that the EDC-modified cytochrome-CO complex has the same absorbance coefficient as horse myoglobin-CO complex (Antonini & Brunori, 1971), then the spectrum of the cytochrome-CO complex (obtained by incubation of the oxy complex with CO immediately after its formation) indicates that all of the cytochrome is converted into the oxy form by the above procedure. The rate at which CO-binding ability diminished was very close to the rate at which features in the α , β region of the oxy-complex visible spectrum changed with time. Using absorption measurements in this spectral region, half-lives were measured for the stability of the oxy complex as a function of pH.

The oxy complex was found to be maximally stable around pH 6.5, with a half-life of 16 min, and became significantly less stable as the pH was altered, having a half-life of approx. 1 min at pH 5 and 7 min and pH 8.0.

The ability of the modified proteins to reduce O_2 was established using an O_2 electrode. In the presence of excess ascorbate both modified proteins were capable of catalytically reducing O_2 . An investigation of the concentration-dependence of the rate of O_2 reduction showed that the rate was dependent on the concentrations of O_2 , cytochrome and ascorbate. The rate of O_2 reduction under fixed concentration conditions was found

to be pH-dependent, increasing steeply at alkaline pH. Addition of catalase and/or superoxide dismutase had no measurable effect on the rate of O_2 turnover.

Attempts to titrate the reduced protein with O_2 led directly to formation of the oxidized protein. Stopped-flow measurements of the reaction of the reduced protein with O_2 indicated that the reaction was complete within the dead time of the apparatus (4 ms), even at low O_2 concentration. Photolysis of the oxy species indicated a low quantum yield for the photolytic process, and recombination was complete within a few microseconds at low O_2 concentrations. Kinetic measurements employing the ligand-replacement technique (Wilson *et al.*, 1973) were precluded by the instability of the oxy complex over the range pH 5–9.

DISCUSSION

From the results presented above, EDC-modified cytochromes *c* clearly combine with CO to form 1:1 complexes. The equilibrium data are well fitted to a simple binding process with an associated equilibrium constant of $2 \times 10^6\ \text{M}^{-1}$. This apparently simple situation, however, is contradicted by both stopped-flow and flash-photolysis studies of the CO-binding process. In both cases three second-order processes are observed, each of which is associated with a CO-binding reaction, as illustrated by kinetic difference spectroscopy. This apparent contradiction is removed on closer examination of the kinetic data of Fig. 3. Although it is difficult to obtain accurate dissociation rates from Fig. 3 (from the intercepts of the lines on the ordinate), it is clear that the data are consistent with all three binding species having equilibrium constants of approx. $10^6\ \text{M}^{-1}$. This high binding constant necessitates equilibrium measurements at low protein concentrations. The consequent accuracy associated with the titration data is thus such that it would preclude the identification of three similar simultaneous binding processes, and the apparent contradiction between equilibrium and kinetic data is resolved.

A closer examination of the kinetic data obtained for the CO binding to EDC-modified cytochrome *c* can give a better understanding of the origins of the three binding species present in solution. Fig. 4 identifies the species responsible for the fast and slow kinetic phases as being in equilibrium. At high CO concentrations the proportions of each of these phases become limiting. If we assume that under these conditions the first-order reactions linking the two equilibrium forms are too slow to allow any re-equilibration during reaction with ligand, then the ratio of the two phases gives an estimate of the equilibrium constant between the two forms in the absence of ligand. This analysis leads to a value of 3 for the equilibrium constant between these species. The species responsible for the intermediate kinetic process, in contrast, appears to be present as a non-equilibrating component of the mixture of protein species.

Unfortunately all our attempts to separate any of the components from the protein mixture, using ion exchange on CM-cellulose with ionic strength or pH-gradient elution, isoelectric focusing in the pH range 9–11, electrophoresis in polyacrylamide gels at pH 9.1 (Mathews & Brittain, 1986) or reversed-phase h.p.l.c., have failed. Consequently we are led to the conclusion that the three species present in the mixture probably represent structural variants. In the absence of firm experimental data we can but speculate on the nature of the equilibrium existing between two of the protein species. Absorption spectra clearly are consistent with both species being low spin in the ferrous state and hence six-co-ordinate. The equilibrium may thus represent a dynamic exchange of ligands at the sixth-co-ordinate site of the haem iron atom.

It thus appears that the EDC-modified proteins are exhibiting the same fundamental functional characteristics as the carboxy-

methylated protein, but, for the former proteins, the reactivity of all species present is decreased, presumably as a consequence of a more rigid protein structure due to EDC-promoted internal cross-linking (Mathews & Brittain, 1986).

The unusually low quantum yield of the modified protein-CO complexes, compared with myoglobin-CO ($\phi = 1.00$) and the CO complex of carboxymethylated cytochrome *c* ($\phi = 0.25$) (Antonini & Brunori, 1971; Brunori *et al.*, 1973), may arise from geminate recombination of CO after photolysis. Extensive internal cross-linking of the protein, including EDC-promoted cross-linking of the haem group to the protein (Mathews & Brittain, 1986), could readily introduce steric barriers to diffusion of CO away from the haem iron.

Reaction of the reduced modified cytochrome *c* with oxygen leads to the formation of a ferrous oxy complex whose general properties and spectra are similar to those of the oxy complexes of ferrous carboxymethylated cytochrome *c* (Schejter & Aviram, 1970) and horseradish peroxidase (Wittenberg *et al.*, 1967; Phelps *et al.*, 1974). These findings suggest similar electronic environments for the haem iron atoms in each case, including a stronger iron-histidine bond (as might be expected for cytochrome *c* derivatives), for oxy-EDC-modified cytochrome *c* and the oxy complex of carboxymethylated cytochrome *c* than in oxymyoglobin, as has been indicated for oxy-horseradish peroxidase by resonance Raman measurements (Van Wart & Zimmer, 1985). The EDC-modified proteins are capable of both rapid intermolecular electron transfer and slow intramolecular electron transfer. The intermolecular electron-transfer process is observed in the rapid reduction of O₂ when excess reduced protein is mixed with O₂. Slow intramolecular electron transfer is observed in the slow oxidation of the ferrous oxy complex formed when excess O₂ is quickly mixed with dithionite-free reduced protein. In the presence of excess reducing agent the proteins show significant oxidase activity. The formation of free superoxide or peroxide does not appear to be of kinetic significance to the oxidase reactions, as addition of catalase and/or superoxide dismutase does not affect the progress of the reaction.

In conclusion, with respect to their reactions with CO and O₂,

the reduced EDC-modified proteins show reactivity characteristics which are similar to those of carboxymethylated cytochrome *c* (Schejter & Aviram, 1970) and horseradish peroxidase (Wittenberg *et al.*, 1967; Phelps *et al.*, 1974), although the electronic environment of the haem iron in the EDC-modified proteins is modulated by the structural consequences of EDC-promoted internal cross-linking.

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