Kinetics of Carbon Monoxide Binding and Electron Transfer by Cytochrome *c* Polymers

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Studies on horse heart cytochrome *c* polymers were carried out by stopped-flow and photolysis techniques, to investigate the properties of the CO complex and the kinetics of electron transfer, mainly of the dimeric and tetrameric forms. CO binding, which does not occur with native monomers, proceeds at both pH 7.0 and pH 9.6, and appears to follow complex kinetics: an initial phase is observed, which is CO-concentration-dependent, followed by a very slow monomolecular phase ($k \sim 2 \times 10^{-3} \text{ s}^{-1}$ at pH 7) before establishment of equilibrium. Photodissociation of the CO complex has a very low quantum yield, probably less than 0.1. Static titration data of the dimer gave an 'n' value of 0.4. These data strongly suggest heterogeneity of the population of binding sites, and have been interpreted in terms of the existence of different structures, probably owing to the non-unique type of binding of monomers during polymerization. Polymers of cytochrome *c* carboxymethylated on the methionine residue normally ligated to iron show simple CO recombination kinetics after photolytic removal ($k_{\text{on}} = 1.5 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ at pH 6). We therefore suggest that, for native cytochrome *c*, polymerization has an effect on the lability of the haem crevice, rendering the iron available for binding ligands, without, however, forming the structure of a truly open crevice. Electron transfer is, on the other hand, a simple process, and no gross differences are observed between monomer and polymers. A simple model, taking into account all these data, is suggested.

It has been known for many years that preparations of cytochrome *c* made by the classical trichloroacetic acid procedure of Keilin & Hartree (1937) contain variable proportions of polymeric forms of the protein [see Margoliash & Schejter (1966) for a general review].

Margoliash & Lustgarten (1962) have made a detailed study of the interconversion of monomeric and polymeric forms of horse heart cytochrome *c*. It should be pointed out that the type of bonding involved in the stabilization of the polymers is, however, still unknown. Unlike the native monomeric form, the polymers are unreactive in enzyme assays where the cytochrome is fulfilling its redox role. Margoliash & Lustgarten (1962) discuss the possibility that this loss of reactivity arises from a rearrangement of the molecule. Such a rearrangement, leading to inactivation, seems indicated by the fact that the polymers lack the 695 nm band which has been shown to be sensitive to the protein conformational state (Schejter & George, 1964) and associated only with species that are reducible by ascorbate (Wilson & Greenwood, 1971). Although monomeric cytochrome *c* in the reduced form is unable to combine

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with CO or with other ligands of ferrous iron below pH 11, the polymers are able to do so (Margoliash *et al.*, 1959). This fact is again interpretable on the basis of a protein rearrangement influencing the availability and reactivity of the haem iron.

As part of a wider study, seeking to elucidate the role of the protein in determining both the biological specificity and haem reactivity, we have chosen to investigate the electron transfer and the CO-binding reactions of some of the polymers of cytochrome *c* and to compare them with similar reactions involving polymers of carboxymethylated cytochrome *c*, which is modified at the level of methionine-80.

Experimental

Cytochrome *c* (type VI) from horse heart was obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, U.K., and was polymerized by the method of Margoliash & Lustgarten (1962). The polymers were separated by using an upward-flow column (94 cm $\times$ 2.5 cm) of Sephadex G-75 equilibrated with 0.02 M-sodium phosphate buffer, pH 7.0, containing 1 M-NaCl.

Carboxymethylated cytochrome *c* was prepared

from type VI cytochrome *c* by the procedure of Schejter & Aviram (1970). Polymers of carboxymethylated cytochrome *c* were prepared and separated like those of native protein. Native cytochrome *c* concentrations were determined by using the value of ϵ_{550} (reduced) = $27.6 \times 10^3 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$, and those of the dimer by using the value of ϵ_{550} (reduced) = $26.1 \times 10^3 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ given by Schejter *et al.* (1963) as extinction coefficient per haem group. Carboxymethylated cytochrome *c* concentrations were determined by using the value ϵ_{413} (reduced + CO) = $220 \times 10^3 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ (Wilson *et al.*, 1973b). Sodium dithionite was obtained from Hardman and Holden, Miles Platting, Manchester, U.K., and all other chemicals were of AnalaR grade and were obtained from British Drug Houses, Poole, Dorset, U.K.

O₂-free N₂ and CO were obtained from the British Oxygen Company, Deer Park Road, London S.W.19, U.K., and were dispensed from the cylinders and stored over an alkaline solution of dithionite-reduced anthraquinone sulphonate before use.

Stopped-flow experiments were performed in an apparatus identical with that described by Gibson & Milnes (1964) and fitted with 2mm and 2cm light-path cells. Flash-photolytic measurements were made with equipment similar to that described by Greenwood & Gibson (1967).

Spectra and the slow kinetic records were obtained with either a Unicam SP. 700 A recording spectrophotometer or a Cary 14 instrument. Spectrophotometric titrations were performed as described by Wilson *et al.* (1973b).

Results

As indicated in the Experimental section, the polymers of cytochrome *c* and carboxymethylated cytochrome *c* were prepared and separated by the procedure of Margoliash & Lustgarten (1962). A typical elution pattern from the Sephadex G-75 column is presented in Fig. 1.

The elution volumes of peaks A–D correspond to molecular weights of 10500, 22000, 33000 and 44000 respectively (Andrews, 1964) and we therefore conclude that they represent the monomer, dimer, trimer and tetramer of cytochrome *c*.

Carbon monoxide binding

The static titration curve for the dimeric form of ferrocycytochrome *c* is given in Fig. 2. In view of the very slow binding (see below) each point was recorded after at least 1 h to allow sufficient time for the system to reach equilibrium after each addition of CO. A Hill plot of the data, shown as an inset to Fig. 2, yields a value of $n \approx 0.4$. Values of n less than unity can indicate negative site-site interaction

Fig. 1. Elution profile of polymerized horse heart cytochrome *c* from Sephadex G-75

Elution pattern of polymerized cytochrome *c* from a column (2.5cm × 94cm) of Sephadex G-75 equilibrated with 0.02M-sodium phosphate buffer, pH 7.0, containing 1M-NaCl. Peaks A, B, C and D correspond to the monomeric, dimeric, trimeric and tetrameric form of cytochrome *c* respectively. Void volume of column = 151 ml. Total bed volume = 462 ml. Fraction volume = 1.86 ml.

effects or heterogeneity in the population of binding sites. In view of the rather complex kinetics of CO combination, reported below, we consider that the latter explanation is more likely.

On mixing ferrocycytochrome *c* dimers with CO solutions in the stopped-flow apparatus, complex progress curves are observed at both pH 7.0 and 9.6. After a fast phase, whose rate depended on the concentration of CO, there is a very slow reaction which is independent of the ligand concentration (see Table 1). Fig. 3 reports the initial phase of CO binding as seen in stopped-flow experiments and shows that the rate of initial binding depends on the concentration of CO. Analysis of the CO-combination data in terms of a small number of reactions characterized by unique rate constants was found to be fruitless, although the estimate of the combination velocity constant for the initial binding is smaller than $1 \times 10^2 \text{ M}^{-1} \cdot \text{s}^{-1}$.

The CO complex of ferrocycytochrome *c* polymers is light-sensitive, like other CO-haem proteins. Photolytic experiments performed at a number of CO

Fig. 2. Titration of dimer with CO

Static titration of the dimers of horse heart cytochrome *c* ($3.5 \mu\text{M}$ -haem) with CO in 0.1 M-potassium phosphate buffer, pH 7.0, in the presence of sodium dithionite. The inset shows a Hill plot of these data.

Table 1. Slow phase of CO binding to reduced dimers

The overall first-order rate constant for the slow phase in the binding of CO by cytochrome *c* dimers was measured as a function of ligand concentration and pH. The monitoring wavelength used was 413.5 nm and the path length was 1.0 cm.

[Dimer] = $8.2 \mu\text{M}$ (haem) Temp. = 22°C 2% Sodium borate, pH = 9.6		[Dimer] = $3.4 \mu\text{M}$ (haem) Temp. = 35°C 0.1 M-Potassium phosphate, buffer, pH = 7.0	
[CO] (μM)	$10^3 k$ (s^{-1})	[CO] (μM)	$10^3 k$ (s^{-1})
50	0.84	1000	1.5
100	1.0	500	2.2
250	1.2	100	2.5
500	1.0		

concentrations gave results comparable with those of the flow experiment, since they show multiphasic kinetics, and in particular exhibit the slow CO-independent phase of recombination. At no time, even with the highest energies available to us, i.e. approx. 500 J, were we able to achieve complete photolytic breakdown of the CO complex. As shown in Fig. 4, it is possible by virtue of the very low 'on' rate to photodissociate progressively the ligand by repeated flashes. This behaviour would seem to indicate that the quantum yield of photodissociation of CO from cytochrome *c* dimers is very small, probably much smaller than 0.1. The very low

Fig. 3. Stopped-flow kinetics of formation of CO-dimer complex

Stopped-flow progress curves on mixing dithionite-reduced dimers of horse heart cytochrome *c* ($6.8 \mu\text{M}$ -haem) with CO at various concentrations. The buffer was 0.1 M-potassium phosphate, pH 7.0, temperature 20°C , monitoring wave-length = 413.5 nm, path length = 2.0 cm. CO concentrations: $\square$, $50 \mu\text{M}$; $\triangle$, $100 \mu\text{M}$; $\circ$, $500 \mu\text{M}$.

quantum yield of photodissociation illustrates the influence of the protein-haem interaction on this parameter, since carboxymethylated cytochrome *c*, having an identical haem group, has a substantially larger quantum yield (i.e. 0.25) (Wilson *et al.*, 1973a).

Temperature affected the rate of combination of cytochrome *c* dimers with CO. Fig. 5 shows the

Fig. 4. Effect of repeated flashes on photodissociation of the CO complex of cytochrome *c* tetramer

A solution of dithionite-reduced tetramers of horse heart cytochrome *c* ($3.1 \mu\text{M}$ -haem), equilibrated with $500 \mu\text{M}$ -CO, was subjected to repeated flashes at 5 s intervals (at the arrows). The buffer was 0.05 M -potassium phosphate, pH 6.9, the flash energy 150 J and the monitoring wavelength 413.5 nm .

Fig. 5. Temperature-dependence of the reaction between CO and dimers

Stopped-flow progress curves on mixing dithionite-reduced dimers of horse heart cytochrome *c* ($5 \mu\text{M}$ -haem) with 1 mM -CO at various temperatures are shown. The buffer was 0.1 M -potassium phosphate, pH 7.0, and the monitoring wavelength was 413.5 nm , pathlength = 2.0 cm .

effect of temperature on the initial phase of CO combination at a constant CO concentration. Whereas it is possible to assign a value of about $50 \times 10^3 \text{ J} \cdot \text{mol}^{-1}$ to energy of activation of the CO-independent rate, it was not possible to assess the corresponding value for the initial processes on account of the heterogeneity mentioned above.

Kinetics of CO combination with carboxymethylated cytochrome *c*

Comparison of the recombination of CO, after photolytic dissociation, with the monomeric and dimeric forms of carboxymethylated cytochrome *c* showed that they did not differ significantly. The

recombination of the dimer was a simple process with a rate constant of $1.5 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ at pH 6.0 (0.2 M -potassium phosphate), falling to $1 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ at pH 9.8 (2% sodium borate- NaOH), in good agreement with the results found for the monomer (Wilson *et al.*, 1973b).

Electron transfer

The reduction of cytochrome *c* polymers by dithionite follows first-order behaviour. The dependence of the first-order rate constant for the reduction of monomeric, dimeric and trimeric forms of cytochrome *c* is shown in Fig. 6 as a function of dithionite concentration. These experiments were conducted under strictly anaerobic conditions, in view of the observation (Greenwood & Palmer, 1965) that in the presence of O_2 a complex time-course for reduction is observed. It is clear that the pseudo-first-order rate of reduction is not linear with dithionite concentration, in agreement with the observations by Lambeth & Palmer (1973). Fig. 6 also shows that the state of polymerization has a marked effect on the rate of reduction by dithionite, as indeed does the ionic strength.

Discussion

The binding of CO by ferrocycytochrome *c* dimers is a complex process, consisting of several phases. The existence of a fast CO-dependent phase implies that in some molecules the ligand-binding site must be available and the availability must not be dependent on a prior conformational event. In both flow and flash experiments an extremely slow reaction, the rate of which is CO-independent, is observed before equilibrium is established. The presence of this monomolecular phase strongly suggests that a protein-conformational step must precede the ligand-binding step in at least some of the molecules. Extremely slow rates in the reaction between CO and monomeric cytochrome *c* treated with a variety of protein denaturants have been reported by Kaminsky *et al.* (1972). These authors have suggested that under these conditions, CO competes successfully with methionine-80 for the sixth co-ordination site, and presumably the rate of formation of the CO complex is limited by the rate of those processes leading to the dissociation of the methionine-80 from the haem iron.

A formal model to describe the CO-binding behaviour of the system should therefore take into account the following: (a) the time-course consists of two main phases, one of which is CO-dependent and the other is not; (b) the first of these two phases shows a clear heterogeneity, since its course cannot be fitted with a single exponential process. In addition heterogeneity is demanded by the low value of n found in the static titration.

Fig. 6. Reduction rate of polymers: dependence on dithionite concentration

Dependence of the pseudo-first-order rate constant of reduction of monomeric, dimeric and trimeric forms of horse heart cytochrome *c* on dithionite concentration. The protein concentration was $5\mu\text{M}$ in terms of haem, dissolved in 0.1M-potassium phosphate buffer, pH7.0, at a temperature of 20°C . The inset shows a typical stopped-flow record of the reaction between the dimers of cytochrome *c* ($5\mu\text{M}$ -haem) and sodium dithionite (0.05mg/ml). On the vertical scale 1 grid division corresponds to an absorbance change of 0.01 and on the horizontal scale to 20ms. Δ , Native, 0.2M-potassium phosphate (pH7.0); $\circ$, native, 0.02M-potassium phosphate (pH7.0)+1M-NaCl; $\square$, dimer, 0.02M-potassium phosphate (pH7.0)+1M-NaCl; $\bullet$, trimer, 0.02M-potassium phosphate (pH7.0)+1M-NaCl.

In the absence of ligands, the cytochrome *c* dimers may be thought to consist of open-crevice 'a' and closed-crevice 'b' structures in equilibrium. In flow experiments the faster process is represented by structure 'a' reacting with CO, whereas the slow phase arises because of the transition of structure 'b' from closed to open crevice. This model would make the photolytic results compatible with those obtained by flow.

Two points deserve comment. First, the attribution of the faster phase to an open-crevice structure needs qualification. In fact no 'free' co-ordination position is necessary to explain the data, and probably the

effect of polymerization is only on the lability of the crevice, which becomes available for binding CO. The absence of truly open structures is shown by the parallel data on dimers of carboxymethylated cytochrome *c*. Presumably the polymers of carboxymethylated cytochrome *c* are produced in a similar fashion; however, the kinetics of CO recombination after photolysis correspond to a very fast and simple process, similarly to that observed for the monomeric form (Wilson *et al.*, 1973b). This must mean that, where the crevice structure is widely open (as a result of the chemical modification of methionine-80), the influence of the protein environment on CO

binding is independent of polymerization. The absence of any monomolecular step with the carboxy-methylated cytochrome *c* dimers supports the idea that the monomolecular processes observed with the native dimers may reflect crevice opening.

Secondly, it may almost be said that heterogeneity of ligand binding seems the dominant feature of the system. Although the type of interactions that hold together the cytochrome *c* molecules is unknown, it may be safely assumed that no preferential interactions among the various monomers are expected. Consistent with the fact that cytochrome *c* is a monomer under normal conditions up to extremely high concentrations, we think that a multiplicity of possible assembled structures is likely and that heterogeneity of CO binding should be related to this multiplicity of assembly.

Although the ligand-binding studies reveal large differences in behaviour between monomeric and polymeric forms of native cytochrome *c*, as well as differences within a single polymeric species, no gross differences are observed in the dithionite reduction of the protein. The results presented in Fig. 6 suggest that the reduction process is simple. Thus the similarity in the electron-transfer kinetics of cytochrome *c* monomers and polymers suggests that some structural features in the vicinity of the haem are maintained in the polymers. Therefore it may be concluded that dimerization of cytochrome *c* molecules has a minor effect on the structures involved in the electron-transfer pathway, but induces a weakening of the structure of the crevice, which becomes labile enough to allow CO binding at neutral pH. The decrease in, or even loss of, reactivity of the polymer with its natural partners (Shur-Perek & Avi-Dor, 1972), the reductase and oxidase systems, should therefore be attributed to

steric effects and hindrance of the normal interactions arising from polymerization.

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References

- Andrews, P. (1964) *Biochem. J.* **91**, 222–233
- Gibson, Q. H. & Milnes, L. (1964) *Biochem. J.* **91**, 161–171
- Greenwood, C. & Gibson, Q. H. (1967) *J. Biol. Chem.* **242**, 1782–1787
- Greenwood, C. & Palmer, G. (1965) *J. Biol. Chem.* **240**, 3660–3663
- Kaminsky, L. S., Burger, P. E., Davison, A. J. & Helfet, D. (1972) *Biochemistry* **11**, 3702–3706
- Keilin, D. & Hartree, E. (1937) *Proc. Roy. Soc. Ser. B* **122**, 298–308
- Lambeth, D. O. & Palmer, G. (1973) *J. Biol. Chem.* **248**, 6093–6103
- Margoliash, E. & Lustgarten, J. (1962) *J. Biol. Chem.* **237**, 3397–3405
- Margoliash, E. & Schejter, A. (1966) *Advan. Protein Chem.* **21**, 113–286
- Margoliash, E., Frohwirt, N. & Weiner, E. (1959) *Biochem. J.* **71**, 559–570
- Schejter, A. & Aviram, J. (1970) *J. Biol. Chem.* **245**, 1552–1557
- Schejter, A. & George, P. (1964) *Biochemistry* **3**, 1045–1049
- Schejter, A., Glauser, S. C., George, P. & Margoliash, E. (1963) *Biochim. Biophys. Acta* **73**, 641–643
- Shur-Perek, T. & Avi-Dor, Y. (1972) *Biochem. J.* **126**, 709–716
- Wilson, M. T. & Greenwood, C. (1971) *Eur. J. Biochem.* **22**, 11–18
- Wilson, M. T., Brunori, M., Bonaventura, C. & Bonaventura, J. (1973a) *Biochem. J.* **131**, 863–865
- Wilson, M. T., Brunori, M., Rotilio, G. & Antonini, E. (1973b) *J. Biol. Chem.* **248**, 8162–8169