Kinetic Studies on [Methionine Sulphoxide]Cytochrome *c*

By THOMAS BRITTAIN and COLIN GREENWOOD

School of Biological Sciences, University of East Anglia, Norwich NR4 7TJ, U.K.

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A cytochrome *c* haem ligand, methionine-80, was photo-oxidized to methionine sulphoxide and the subsequent changes in redox properties and ligand binding were monitored kinetically. Isoelectric focusing of the product showed the presence of a single oxidized species, capable of binding CO when reduced. The binding of CO to the reduced protein was followed in stopped-flow experiments, which revealed the presence of two binding processes, at neutral pH, with rate constants of $k_{+1} = 3.4 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $k_{+2} = 5.80 \times 10^2 \text{ M}^{-1} \cdot \text{s}^{-1}$. When CO was photolytically dissociated from the reduced protein two recombination processes were observed with rates almost identical with those observed in the stopped-flow experiments ($k_{+1} = 3.3 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $k_{+2} = 6.0 \times 10^2 \text{ M}^{-1} \cdot \text{s}^{-1}$). These findings provide evidence of two reduced forms of the protein. The reduction of [methionine sulphoxide]cytochrome *c* by Cr^{2+} at neutral pH in stopped-flow experiments showed the presence of a single second-order reduction process ($k = 7.2 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$, activation energy = 44 kJ/mol) and one first-order process. This protein was compared with some other chemically modified cytochromes.

Cytochrome *c* transfers electrons in the respiratory chain by a redox cycling of its covalently bound haem group. The structure of the protein has been determined (Takano *et al.*, 1973), but the mode of electron transfer remains something of a mystery. A number of mechanisms have been proposed, which range from attack at an exposed haem edge (Suttin & Yandell, 1972) to transfer via the amino acids of the protein (Winfield, 1965; Dickerson *et al.*, 1972) and to electron tunneling (De Vault & Chance, 1966). In a number of proposed schemes a role has been envisaged for the haem ligand methionine-80. To test these schemes, the ligand has been chemically modified and the subsequent alterations in properties monitored (Schejter & George, 1964; Stelwagen, 1968; Margoliash *et al.*, 1973; Brittain *et al.*, 1974). However, the disruption of the haem ligand is usually accompanied by gross structural changes.

Folin *et al.* (1972) reported a modification that apparently did not grossly alter the electron-transfer properties of cytochrome *c* in the mitochondrion. However, difficulty in reproducing this preparation of [methionine sulphoxide]cytochrome *c* reported by Jori *et al.* (1970) restricted further study until Ivanetich *et al.* (1976) introduced a preparation of the protein by using Methylene Blue-mediated photo-oxidation.

The [methionine sulphoxide]cytochrome *c* so produced seems an interesting species, which lies between the fully bound methionine-80 found in native cytochrome *c* and the fully unbound dicarboxymethylated cytochrome *c*. Thus we investigated its kinetics to

gain information about the role that methionine-80 may play in electron transfer. Also by comparison with other chemically modified cytochromes it may be possible to probe the necessity for the integrity of the electronic structure of the haem to electron transfer.

Experimental

[Methionine sulphoxide]cytochrome *c* was prepared essentially by the procedure of Ivanetich *et al.* (1976). Cytochrome *c* [Sigma type VII; Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K.] was photo-oxidized by illumination with three 375W Philips Photolita type PF 215E/49 bulbs in the presence of Methylene Blue while pure O_2 was bubbled through the solution for 90 min. The solution was then passed through a column (35 cm $\times$ 5 cm) of Sephadex G-25 equilibrated and eluted with 0.05 M-ammonium acetate buffer, pH 7.0. The protein was then dialysed against water at 4°C and applied to a column (10 cm $\times$ 1 cm) of CM-cellulose equilibrated with 0.01 M-Tris/HCl buffer, pH 6.5. The protein was eluted with either 0.2 M-Tris/HCl, pH 6.5, or 0.1 M-cacodylate/HCl, pH 6.5, as required.

Solutions of Cr^{2+} were prepared as described by Brittain *et al.* (1974) and the concentration of stock solutions was found by using the methods described by Dawson *et al.* (1972).

Stopped-flow experiments were carried out by using the apparatus described by Gibson & Milnes (1964), equipped with a 2 cm path-length cell having a dead

Fig. 1. Isoelectric focusing of [methionine sulfoxide]-cytochrome *c*

Protein (20mg) was applied to the LKB 8101 column and focused for 3 days in the pH range 8.0–11.0 (Δ). Fractions (approx. 1.5ml) were collected and protein concentration measured at 280nm (●).

time of 3 ms. Flash-photolysis experiments were performed by using the instrumentation and methods described by Greenwood & Gibson (1967). A Bausch and Lomb monochromator (500mm grating, 1200 grooves/mm, $f=4.4$) was used in conjunction with the flash apparatus. Isoelectric focusing was performed in an LKB 8101 110ml column.

CO buffers were prepared by equilibration of the appropriate degassed buffer under 101 kPa pressure of CO at room temperature (20°C) and the concentration was found by reference to standard solubility tables. The concentration of [methionine sulfoxide]-cytochrome *c* samples was calculated from the E_{530} of the oxidized protein at neutral pH, where $E_m = 1.12 \times 10^4 \text{ cm}^{-1}$ (Margoliash & Frohwirt, 1959; Ivanetich *et al.*, 1976).

Results

When subjected to isoelectric focusing in the range pH 8.0–11.0, oxidized [methionine sulfoxide]-

Fig. 2. Visible spectra of [methionine sulfoxide]cytochrome *c*

The oxidized (—), reduced (----) and CO-complex forms of [methionine sulfoxide]cytochrome *c* (— · —) are shown in the visible region. The protein concentration used was $13 \mu\text{M}$ in the Soret region (a) (380–500 nm) and $40 \mu\text{M}$ in the α/β region (b) (500–600 nm). Spectra were recorded at 20°C in 0.1 M-Tris/HCl buffer, pH 6.9, in 1 cm-path-length cells.

Fig. 3. Typical progress curves for the reaction of reduced [methionine sulfoxide]cytochrome *c* with CO

(a) Typical reaction trace observed when 2 μM -reduced protein was mixed in the stopped-flow apparatus with 0.5 mM-CO at 20°C and pH 7.0. The reaction was followed at 414 nm in a 2 cm path-length cell. (b) Typical reaction trace observed when 1.5 μM -reduced CO complex was photolysed in the presence of 0.47 mM-CO at 20°C. The reaction was followed at 414 nm in a 4 cm-path-length cell.

cytochrome *c* shows a single band with pI 10.1 (Fig. 1).

The spectrum of [methionine sulfoxide]cytochrome *c* in the visible region is shown in Fig. 2. The oxidized material in the Soret region shows a maximum at the same wavelength as the native protein, but has a higher extinction (Ivanetich *et al.*, 1976), whereas in the α/β region the spectrum is identical with that of the native protein (Fig. 2b). As the pH of the solution is lowered below 6.0, however, a band at 620 nm appears, indicating the formation of a high-spin species. The reduced protein is again similar to the native cytochrome in the Soret region, but in the α/β region the spectrum is much altered. The β band has approximately the same extinction as the native protein, but the α band is greatly decreased in extinction. Fig. 2(a) also shows that the reduced protein is capable of forming a CO complex; this complex was found to be photodissociable.

The protein could be reduced by ascorbate even though it does not possess a 695 nm band. But even in the presence of over 100 mM-ascorbate the reduction

requires over 15 min to reach completion, when the spectrum is identical with that of the dithionite-reduced protein.

Kinetics of CO complex formation

When reduced [methionine sulfoxide]cytochrome *c* was mixed with CO-equilibrated buffer in the stopped-flow apparatus, two kinetic processes were observed, both leading to an increase in E_{414} (Fig. 3a). Analysis of the concentration-dependence of the two phases shows that both are CO-concentration-dependent (Figs. 4a and 4b). The slopes of these plots yield the second-order rate constants for the two processes as $k_{+2} = 5.8 \times 10^2 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $k_{+1} = 3.4 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$. A comparison of the static and kinetic difference spectra (Fig. 5) shows that no process is being overlooked in the dead-time of the apparatus.

Fig. 4. CO-concentration-dependence of the rate of combination of [methionine sulfoxide]cytochrome *c* observed by stopped flow and flash photolysis

The concentration-dependence of the two kinetic processes (a) and (b) observed by stopped-flow (●) and flash-photolysis (□) techniques is shown. In the flow experiments 2 μM -protein was mixed with CO in a 2 cm cell at pH 7.0 at 20°C. In the flash experiments 1.5 μM -protein was photolysed in the presence of CO in a 4 cm cell at pH 7.0 at 20°C. In both cases the reaction was monitored at 414 nm.

Fig. 5. Static and kinetic difference spectra of CO binding to [methionine sulfoxide]cytochrome *c*

The static (●) and kinetic (○) difference spectra were obtained between the reduced protein-CO complex and the reduced protein at a cytochrome concentration of $13.5 \mu\text{M}$ in 0.1M-Tris/HCl buffer, pH7.0, in a 1 cm-path-length cell.

When the CO complex of the reduced [methionine sulfoxide]cytochrome *c* was subjected to photolytic dissociation, two recombination processes were observed (Fig. 3b). An analysis of the CO-concentration-dependence of these processes is shown in Figs. 4(a) and 4(b), the slopes of which yield second-order rate constants of 6.0×10^2 and $3.3 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$, that is, rates identical with those observed in the stopped-flow experiments.

Reduction by Cr^{2+}

When mixed anaerobically with a solution of Cr^{2+} in the stopped-flow apparatus, [methionine sulfoxide]cytochrome *c* showed the presence of two kinetic phases, each leading to an increase in E_{420} (Fig. 6a). The faster phase was dependent on the concentration of Cr^{2+} , with second-order rate constant $7.2 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$ (Fig. 6b), whereas the slower phase was independent of Cr^{2+} concentration and had a first-order rate constant of 3.0 s^{-1} . A comparison of

the static and kinetic difference spectra shows that no process is being overlooked in the dead-time of the apparatus.

The dependence of the rate of reduction on tem-

Fig. 6. Progress curve and concentration dependence of Cr^{2+} reduction of [methionine sulfoxide]cytochrome *c*

(a) Typical progress curve observed when $1.5 \mu\text{M}$ -protein was mixed with 3.125 mM - Cr^{2+} in a 2 cm-path-length cell of the stopped-flow apparatus at 20°C and pH6.5. The absorbance change was measured at 420 nm. (b) Concentration-dependence of the faster of the two phases observed.

Fig. 7. Effect of temperature on the rate of Cr^{2+} reduction of [methionine sulfoxide]cytochrome *c*

Protein ($1.5 \mu\text{M}$) was reduced at pH6.5 in 0.1M-sodium cacodylate buffer with 0.39 mM - Cr^{2+} . The reaction was followed at 420 nm in a 2 cm-path-length cell in the stopped-flow apparatus.

perature was measured and the data were used to determine the activation energy of the process by means of the Arrhenius equation. Fig. 7 shows the nature of the temperature-dependence of the reaction and yields an activation energy of 44 kJ/mol.

Discussion

The modification process used to produce [methionine sulphoxide]cytochrome *c* has been shown to lead only to the oxidation of the haem ligand methionine-80 to methionine sulfoxide (Ivanetich *et al.*, 1976). This modification thus does not drastically alter the structure of the molecule, but would be predicted to affect the electronic environment of the haem. That this is the case is illustrated by the spectral observations reported. Perhaps the most striking spectral feature of [methionine sulphoxide]cytochrome *c* is the much lowered extinction of the α band of the reduced protein. This lowering of extinction of the α band is also observed in [N-formyltryptophyl]-cytochrome *c* (Brittain & Greenwood, 1975) and may well be due to the introduction of an oxygen function close to the haem iron atom, so producing a change in its electronic environment.

The oxidation of methionine-80 should remove the possibility of a lone-pair interaction with the haem iron. This electronic interaction has long been thought to be responsible for the presence of the 695 nm band associated with the native cytochrome (Sreenathan & Taylor, 1971). It is found that on photo-oxidation this band is eliminated. The presence of this ligand has also been proposed to be a prerequisite for reducibility by ascorbate (Greenwood & Palmer, 1965), but this proposition has been challenged (Skov & Williams, 1968). In [methionine sulphoxide]cytochrome *c* we find that the 695 nm band is absent, but the protein is still reducible by ascorbate, although a large excess of the reagent is necessary. Whether this requirement is thermodynamic in nature (the redox potential of [methionine sulphoxide]cytochrome *c* is only 184 mV (Ivanetich *et al.*, 1976) compared with 260 mV for the native protein (Henderson & Rawlinson, 1961)), or kinetic, is not yet clear.

From the kinetic studies reported above it seems that oxidized [methionine sulphoxide]cytochrome *c* exists in one form, as indicated by isoelectric focusing and also by the observation of a single reduction process in the studies with Cr^{2+} . The reduced protein appears to exist in two forms, as illustrated by the CO-binding studies. In both stopped-flow and flash-photolysis experiments, the same two binding processes are observed with the same spectral characteristics. This may well arise from the structural changes accompanying modification, which would be more apparent in the reduced state, as it is known that the reduced form of cytochrome *c* is a more compact conformation (George & Schejter, 1964).

Table 1. Comparison of some kinetic parameters of chemically modified cytochrome *c*

Kinetic parameters	<i>k</i> , Second-order rate constant; <i>K</i> , $k_{\text{CO binding}}/k_{\text{CO release}}$. References: (a) Brittain & Greenwood (1975); (b) Wilson <i>et al.</i> (1973); (c) Brittain <i>et al.</i> (1974).			
	Native cytochrome <i>c</i>	[N-Formyltryptophyl]-cytochrome <i>c</i> (a)	[Methionine sulphoxide]-cytochrome <i>c</i>	Carboxymethylated cytochrome <i>c</i>
Rate of CO binding (stopped flow at pH 7.0)	—	$k = 1.8 \times 10^2$, $1.6 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ $K = 1.2 \times 10^4$, $8 \times 10^4 \text{ M}^{-1}$	$k = 3.4 \times 10^3$, $5.8 \times 10^2 \text{ M}^{-1} \text{ s}^{-1}$ $K = 3.4 \times 10^3$, $6 \times 10^4 \text{ M}^{-1}$	$k = 1.5 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ (b) $K = 1.2 \times 10^9 \text{ M}^{-1}$ (b)
Rate of CO binding (flash photolysis at pH 7.0)	—	$k = 1.9 \times 10^2 \text{ M}^{-1} \text{ s}^{-1}$	$k = 3.3 \times 10^3$, $6 \times 10^2 \text{ M}^{-1} \text{ s}^{-1}$	$k = 1.5 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ (b)
Rate of Cr^{2+} reduction (stopped flow at pH 6.5)	$k = 1.2 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$	$k = 4 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$	$k = 7.2 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$	$k_{+1} = 1.1 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ (c) $k_{+2} = 1.0 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ (c)
Activation energy of Cr^{2+} reduction at pH 6.5 (kJ/mol)	38.6 (c)	38.6	44	42 (c) 23 (c)

It is interesting to compare some kinetic parameters of [methionine sulfoxide]cytochrome *c* with some other cytochromes (see Table 1). In native cytochrome *c* it is known that methionine-80 provides one of the haem ligands, whereas in [methionine sulfoxide]cytochrome *c*, Ivanetich *et al.* (1976) suggest that methionine sulfoxide binds to the haem iron, but more weakly so than methionine does in the native protein. In [N-formyltryptophyl]cytochrome *c* it is not clear whether methionine or lysine-79 ligates the haem iron, but in dicarboxymethylated cytochrome *c* lysine-79 is known to ligate the haem (Brunori *et al.*, 1972). Thus the proteins represented in Table 1 show a gradation of the strength of methionine ligation from native cytochrome to carboxymethylated cytochrome *c*. It is noteworthy that the affinity constants of the reduced proteins for CO (*K*) follow the order of strength of methionine ligation, as do the rates of CO combination. For Cr²⁺ reduction, even though the activation energies of the reactions are very similar, the rates of reduction also follow this pattern; whether these results are a consequence of methionine binding itself or some related property is not proved. Another interesting point seen in Table 1 is the close similarity in properties of [N-formyltryptophyl]cytochrome *c* and [methionine sulfoxide]cytochrome *c*. In both cases an oxygen function has been introduced in close proximity to the haem group and the resultant similarity in both spectral and kinetic properties is marked. This seems strong evidence that in both proteins it is the modification of the local haem environment, which is the prime cause of the alterations in properties.

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