The Reduction of Carboxymethyl-Cytochrome *c* by Chromous Ions

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The reduction of ferricytochrome *c* and ferricytochrome *c* carboxymethylated at the haem-linked methionine (residue 80) by Cr^{2+} ions was studied by stopped-flow techniques. At pH 6.2 the kinetics of reduction of ferricytochrome *c* are simple and correspond to a second-order rate constant of $1.21 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$. Under identical conditions the kinetics of reduction of the carboxymethyl derivative, carboxymethyl-cytochrome *c*, are complex; two Cr^{2+} -concentration-dependent processes ($1.5 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $1.3 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$) lead to the formation of an intermediate which decays in monomolecular fashion (0.15 s^{-1}) to form the normal fully reduced material. The kinetic difference spectrum for the overall process corresponds to that found statically, whereas the kinetic difference spectrum of the intermediate minus the oxidized form resembles that of the low-spin ferrous form of carboxymethyl-cytochrome *c* minus oxidized carboxymethyl-cytochrome *c*. A model is proposed in which the reduction of low-spin ferric carboxymethyl-cytochrome *c* to high-spin ferrous carboxymethyl-cytochrome *c* involves a low-spin ferrous intermediate. The monomolecular step involving the decay of this low-spin ferrous intermediate is associated with an activation energy of approx. $126 \text{ kJ} \cdot \text{mol}^{-1}$ and is thought to involve both a change of spin state and a protein-conformational event. Although carboxymethyl-cytochrome *c* represents a mixture of species separable on a charge basis, the above observations were independent of which species was chosen for study.

Since the elucidation of the three-dimensional structure of horse heart ferricytochrome *c* by Dickerson *et al.* (1967), there have been a number of attempts to link the known structural features of the molecule with the mechanism and possible pathways of reduction. Following the proposals of Winfield (1965), Dickerson *et al.* (1972) suggested that electrons can migrate through the protein via the aromatic residues of the 'left-hand channel' and eventually reach the haem group via methionine-80. This scheme has, however, recently been modified to take account of the work of Myer (1972) and Myer & Pal (1972) on bromosuccinimide-modified cytochrome *c*. Myer (1972) now suggests that although the sequence:

reductase $\rightarrow$ some structure of the protein
 $\rightarrow$ Trp-59 $\rightarrow$ Tyr-67 $\rightarrow$ Met-80
 $\rightarrow$ haem Fe

may be attractive, electrons may pass from the exterior of the protein to the haem group via the aromatic residue tryptophan-59 and terminate with the direct donation of the electron to the porphyrin without the intervention of the methionine-80 residue.

An alternative scheme has been proposed by Yandell *et al.* (1973) and Creutz & Sutin (1973),

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who report that the kinetics of reduction of ferricytochrome *c* by Cr^{2+} and dithionite is compatible with a mechanism in which the reducing agent reduces the iron directly (or through a bridging anion). The rate of reduction in this case is limited by the rate of crevice opening (approx. 60 s^{-1} ; Sutin & Yandell, 1972) which must precede the approach of the reducing agent to the iron.

In order to learn something more about the possible role of specific amino acids in the reduction process we commenced a study of the effects of chemical modification on this process.

The present paper deals with the kinetics of reduction of native and carboxymethylated cytochrome *c* by Cr^{2+} . Cr^{2+} was the reductant of choice, as there is evidence (Kowalsky, 1969) to suggest, at least in native cytochrome *c*, that very tight binding accompanies electron donation, and this property is being exploited by Dickerson in further X-ray studies (R. E. Dickerson, personal communication). In carboxymethylated cytochrome *c* the iron-methionine-80 bond is ruptured and direct electron transfer between this methionine residue and iron, if present in the native molecule, is now unlikely. Moreover, owing to the rupture of this bond the whole crevice structure on the methionine-80 side of the haem group is weakened (Kaminsky *et al.*, 1972) and, presumably, to some extent disrupted. The kinetics of reduction of carboxymethyl-cyto-

chrome *c* may therefore throw some light on the importance of the integrity of this section of the cytochrome molecule to electron transfer and reduction of the haem iron.

Experimental

Native cytochrome *c* (type III) was purchased from Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K., and was used without further purification. The concentrations of cytochrome *c* solutions were determined by using the extinction coefficient at 550nm of $27.6 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for the reduced protein (Schejter *et al.*, 1963).

Carboxymethyl-cytochrome *c* was prepared by the method of Schejter & George (1965) by reaction of bromoacetic acid and NaCN with cytochrome *c* at pH 7.0 at room temperature, followed by exhaustive dialysis against water.

Most experiments were carried out with whole modified material as prepared, except for experiments after isoelectric focusing, where specific components could be used. The concentration of carboxymethyl-cytochrome *c* was determined by using the extinction coefficient of the reduced CO complex at 414nm of $219 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Brunori *et al.*, 1972).

Cr^{2+} was produced by dissolving electrolytically pure chromium metal (approx. 99% pure) in O_2 -free HCl essentially by the method of Dawson *et al.* (1972) and was stored under O_2 -free N_2 . The concentration of Cr^{2+} was determined spectroscopically by titrating the Cr^{2+} solution against excess of KMnO_4 in an acid solution under anaerobic conditions and following the disappearance of MnO_4^- at 545nm by using an extinction coefficient at 545nm of $2.34 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$. Determinations of the concentration of Cr^{2+} found by this method were in good agreement with those found by the procedure of back titration with ferrous ammonium sulphate.

Sodium cacodylate buffers were used in the region pH 5–8 and glycine–NaOH buffers were used for values greater than pH 8; these buffers were chosen as they were found to be two of the common buffer systems that did not cause Cr^{2+} to precipitate on mixing.

During these experiments it was found that there was a pH-dependence of the colour of Cr^{2+} ion in the sodium cacodylate solutions, probably owing to the presence of a Cr^{2+} –cacodylate–buffer complex; a distinct change in the colour occurs in the region of the pK of cacodylate ($pK = 6.27$).

Stopped-flow measurements were carried out in an apparatus identical with that described by Gibson & Milnes (1964) equipped with 2cm or 2mm light-path cells, having dead times of 3ms and 1ms respectively. Isoelectric focusing was performed in an LKB 8101 110ml column. Approx. 25mg of carboxymethyl-

cytochrome *c* was placed on the column and focused in the range pH 7–10. The material was removed from the column and collected in 1.5ml fractions. Both protein and Cr^{2+} solutions were made up in the required O_2 -free buffer and stored under N_2 . The stock solutions thus produced were diluted to the required concentration by using deoxygenated buffer.

The pH values of solutions were measured with a Radiometer 51 pH-meter fitted with a micro-electrode unit E5021a.

Results

Stopped-flow experiments in which native ferri-cytochrome *c* was mixed with Cr^{2+} in sodium cacodylate buffer revealed a single exponential process at all wavelengths which corresponded spectrally to the production of ferrocytochrome *c* (see Fig. 1*a*). The rate of this process was linearly dependent on the Cr^{2+} concentration over the concentration range explored (up to $12 \text{ mM} \cdot \text{Cr}^{2+}$). The second-order rate constant was $1.21 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$, in reasonable agreement with Yandell *et al.* (1973), who report a value of about $3 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$ under somewhat different conditions from those used here.

In distinct contrast, the reduction of carboxymethyl-cytochrome *c* under identical conditions was complex. Fig. 1(*b*) shows a typical oscilloscope trace of the time-course of the reaction carried out at pH 6.2 and monitored at 550nm. At this wavelength two kinetic processes leading to an increase in absorbance are clearly discernible in the millisecond time-range and a third process, in the second time-range, leads to a decrease in absorbance.

The dependence of the rates of the two faster processes (phases 3 and 2) on Cr^{2+} concentration are shown in Fig. 2, from which the second-order rate constants may be evaluated as $1.5 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $1.3 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$ respectively, at pH 6.2. The slow rate (phase 1) was independent of the Cr^{2+} concentration (Table 1), and its activation energy was approx. $126 \text{ kJ} \cdot \text{mol}^{-1}$.

In order to ascertain whether the kinetic complexity was due to chemical heterogeneity, a sample of the carboxymethyl-cytochrome *c* was subjected to isoelectric focusing. In agreement with earlier reports (Wilson *et al.*, 1973) five distinct species were observed after equilibrium had been attained (Fig. 3). However, it was found that each of these components, when reduced by Cr^{2+} in the stopped-flow apparatus, gave the same result as was obtained for the whole material. Fig. 2 shows that the two faster processes (phases 3 and 2) have identical rate constants for each species isolated after isoelectric focusing. The kinetics of the reduction by Cr^{2+} is therefore seen to be independent of whatever may be the differences between the fractions obtained by isoelectric focusing. This finding is in agreement

Fig. 1. Progress curves for the reduction of native and carboxymethylated ferricytochrome *c* by Cr^{2+}

(a) Typical oscilloscope trace (above), and log plot of data (below), for reduction of native ferricytochrome *c* by Cr^{2+} . The vertical scale on the oscilloscope corresponds to a change in extinction of 0.05/division and the horizontal scale to 100 ms/division. (b) Oscilloscope traces (above) for the reduction of carboxymethyl-cytochrome *c*. Traces at sweeps of 50 ms/division (i) and 1 s/division (ii) are shown. The vertical scale corresponds to a change in extinction of 0.01 s/division. Log plots of these data are shown below. The two faster processes ($\circ$, $\bullet$) are plotted on the time-scale shown on the lower abscissa, and the slow phase (Δ) is plotted with regard to the upper abscissa. The experiments were carried out at 20°C in 0.2M-sodium cacodylate buffer, pH 6.2. The Cr^{2+} concentration was 2.75 mM and the reactions were followed at 550 nm (3.3 nm bandwidth) in a 2 cm path-length cell.

with that of Wilson *et al.* (1973), who have observed that the binding of CO by ferrous carboxymethyl-cytochrome *c*, although complex, was also independent of which isoelectric fraction was chosen for study.

Both the rates and the proportions of the three kinetic phases were pH-dependent. Fig. 4 shows the dependence of the two faster rates on the pH at which the reaction was carried out and contrasts their behaviour with that of native cytochrome *c*.

At lower pH values the rate of reduction of native cytochrome *c* is similar to that of the slower of the two Cr^{2+} -concentration-dependent phases found for carboxymethyl-cytochrome *c*. However, whereas the former was pH-independent, the latter rate fell sharply above pH 6.0 (see Fig. 4), as did the faster Cr^{2+} -concentration-dependent phase found for carboxymethyl-cytochrome *c*. This difference between the pH behaviour of native cytochrome *c* must certainly reflect differences between the proteins,

Fig. 2. Cr^{2+} -dependence of rate of reduction of carboxymethyl-cytochrome *c*

The fast phase (k_3) (b) and intermediate (k_2) (a) are shown. Whole carboxymethyl-cytochrome *c* (Δ) and isoelectric fractions I ($\bullet$) and IV ($\circ$) (see Fig. 3) were used at $17 \mu\text{M}$ in 0.2M-sodium cacodylate-HCl, pH 6.0. The reduction was followed at 550 nm in a 2 cm path-length cell at 20°C .

as such results cannot be explained on the basis of pH-induced changes in any Cr^{2+} -cacodylate complex that may exist in solution. In contrast with the two fast processes occurring during the reduction of carboxymethyl-cytochrome *c* by Cr^{2+} , the slow phase showed a steady increase in rate and amplitude over the pH range studied. The effect of pH on the proportions of each of the three phases observed in the reduction of carboxymethyl-cytochrome *c* is shown in Table 2.

Fig. 5(a) shows a comparison between the overall difference spectrum of reduced minus oxidized carboxymethyl-cytochrome *c* obtained kinetically and statically. Over the spectral range explored these two difference spectra are identical, indicating that no kinetic process is being overlooked in the dead-time of the flow apparatus. Fig. 5(a) also

Table 1. Cr^{2+} -concentration-independence of slow phase

k_1 is the pseudo-first-order rate constant for the slow phase of carboxymethyl-cytochrome *c* reduction measured at 550 nm in a 2 cm light-path cell at 20°C . The protein concentration was $17 \mu\text{M}$, dissolved in 0.2M-sodium cacodylate-HCl, pH 6.

$k_1 \text{ (s}^{-1}\text{)}$	$[\text{Cr}^{2+}] \text{ (mM)}$
0.154	15
0.115	10.3
0.152	5.1
0.110	2.6
0.130	1.3

Fig. 3. Isoelectric focusing of carboxymethyl-cytochrome *c*

Approx. 25 mg of carboxymethyl-cytochrome *c* was focused at 4°C on an LKB 8101 column in the pH 7–10 gradient (Δ). Fractions (1.5 ml) were collected and protein concentrations measured at 280 nm ($\bullet$).

Fig. 4. pH-dependence of the rates of reduction of, carboxymethyl-cytochrome *c* and native cytochrome *c* by Cr^{2+}

The pH-dependence of the rates of the fast phase, k_3 (Δ), and the intermediate phase, k_2 ($\circ$), seen during the reduction of carboxymethyl-cytochrome *c* are shown along with the rate of reduction of native cytochrome *c*, k ($\bullet$). The reactions were followed at 415nm in a 2cm path-length cell at 20°C. The proteins were at a concentration of 1.5 μM and were reduced with 1.85mM- Cr^{2+} .

Table 2. Effect of pH on the relative proportion of each phase seen during the reduction of carboxymethyl-cytochrome *c*

The percentage of each phase is shown for the reduction of 1.5 μM -carboxymethyl-cytochrome *c* by 1.8mM- Cr^{2+} in 0.2M-sodium cacodylate-HCl. The reaction was measured at 415nm in a 2cm light-path cell at 20°C. All proportions are expressed as percentages of the maximum extinction at this wavelength, i.e. from the fully oxidized condition to the reduced intermediate.

pH	Proportion (%)		
	Slow (phase 1)	Medium (phase 2)	Fast (phase 3)
5.1	5	85	15
5.5	15	80	20
5.9	19	77	23
6.4	26	54	46

shows the difference spectrum between the intermediate formed in the reductive process (i.e. that species formed before the slow Cr^{2+} -independent step), and the fully oxidized protein. It is clear that this intermediate has very different spectral properties from those of the fully reduced material.

Discussion

Since Kowalsky (1969) introduced Cr^{2+} to investigate problems of ion-cytochrome *c* interaction its application as a probe of redox mechanisms and structure has become widespread (e.g. Dawson *et al.*, 1972; Yandell *et al.*, 1973). Kinetic experiments indicate that the reduction of native ferricytochrome *c* is monophasic and there are indications that the site of Cr^{2+} interaction with the protein may also be the physiologically significant site for reaction with the cytochrome *c* reductase (R. E. Dickerson, personal communication).

The work of Schejter & Aviram (1970) has demonstrated that at pH 6.2 carboxymethyl-cytochrome *c* in the ferric form exists largely as a low-spin species, whereas the ferrous form is predominantly high-spin under similar conditions. N.m.r. studies by Keller *et al.* (1972) have confirmed this and also indicated that the ferrous iron is penta-co-ordinated under these conditions. When carboxymethyl-cytochrome *c* is reduced at this pH it may be expected that two processes might result, the change of valence of the iron and the coupled alteration in spin state and co-ordination. One simple model that could be written for the reduction of ferric carboxymethyl-cytochrome *c* at pH 6.2 is as follows:

Experimentally we find fast Cr^{2+} -dependent steps which correspond to the reduction of the haem iron. The Cr^{2+} -concentration-dependent processes lead to the formation of an intermediate which decays in a first-order manner to produce the final reduced species, which is in general accord with eqn. (1). The kinetic difference spectrum between the oxidized and the final reduced material is in good agreement with the static difference spectrum between low-spin ferric and high-spin ferrous at this pH (see Fig. 5a). The difference spectrum between the kinetic intermediate and oxidized carboxymethyl-cytochrome *c* in the region 500–600nm (Fig. 5b) corresponds well to the static difference spectrum between hexa-co-ordinated low-spin ferrous and low-spin ferric haem. We were able to make this comparison by obtaining the difference spectrum between oxidized and reduced carboxymethyl-cytochrome *c* at pH 9.0, where it is known that both valence states exist as low-spin species (Schejter & Aviram, 1970). These experimental findings therefore are in general accord with the simple model proposed in eqn. (1), in which a low-spin ferrous species precedes the formation of the high-spin fully reduced protein. However, although the spectral changes in the visible region

Fig. 5. Static and kinetic difference spectra

(a) Difference spectra between fully reduced and fully oxidized carboxymethyl-cytochrome *c* obtained statically ($\circ$) and kinetically in stopped-flow experiments ($\bullet$). Also shown is the difference spectrum between the intermediate formed and the oxidized protein ($\square$). (b) Kinetic difference spectrum between the intermediate and the fully oxidized protein ($\square$) and static difference spectrum between fully reduced and fully oxidized carboxymethyl-cytochrome *c* (Δ) obtained at pH9 in a 0.2M-glycine-NaOH buffer. The protein concentrations were $7.142\mu\text{M}$ in the Soret region (390–460nm) and $71.4\mu\text{M}$ in the α and β (500–570nm) region. The buffer used was 0.2M-sodium cacodylate, pH6.0, unless stated otherwise.

accompanying the slow Cr^{2+} -independent step are in agreement with the change from hexa-co-ordinated low-spin ferrous carboxymethyl-cytochrome *c* to high-spin penta-co-ordinated ferrous carboxymethyl-

cytochrome *c*, the rate is at variance with that of 2.5s^{-1} measured at this pH for this step by Brunori *et al.* (1972). Evidence that some coupled process occurs which is not shown in the simple

model can also be found in Fig. 5(b), which reveals clear differences between the static difference spectrum at pH 9.0 and the kinetic difference spectrum of the intermediate observed at pH 6.0 during Cr^{2+} reduction. These observations in the Soret region clearly indicate that the intermediate produced does not have the full spectral characteristics of the hexa-co-ordinate low-spin intermediate alluded to above, and therefore the first-order step cannot reflect a simple co-ordination change. We therefore conclude that the co-ordination change is in fact only signalling some other, slower, process to which it is coupled and which makes a spectral contribution in the Soret region. The Cr^{2+} independence and high activation energy of this step seem to lend support to the idea that this represents a protein conformational change. The most obvious assignment for this would be the change in protein conformation between the oxidized and reduced states, greatly decreased in rate as a result of the loss of structural integrity in the chemically modified protein.

In view of our finding that there are two Cr^{2+} -concentration-dependent steps it seems necessary to propose that two forms of ferric carboxymethyl-cytochrome *c* exist in solution. The observations in Table 2 indicate that the proportions of the two Cr^{2+} -dependent processes (phases 2 and 3) are influenced by the conditions. Over the pH range 5–6.5 the contribution made by phase 3 increases at the expense of phase 2, suggesting that the two forms of carboxymethyl-cytochrome *c* which are responsible for these processes are in pH-dependent equilibrium. Our kinetic observations indicate that these two forms are characterized by second-order rate constants which differ by an order of magnitude, that for phase 2, however, being very similar to the second-order rate constant for the reduction of native cytochrome *c* (see the text and Fig. 2a). This may indicate that the normal reduction pathway is operational in this form of carboxymethyl-cytochrome *c*; however, unlike the native material its rate of reduction was found to be pH-dependent above pH 6.0.

The fact that we observe a Cr^{2+} -concentration-dependent reduction (phase 3), which is an order of magnitude faster than that found for the native molecule, may indicate that in this form of carboxymethyl-cytochrome *c* the haem group is reduced via a different pathway. This situation may represent a more direct donation of the electron from Cr^{2+} to the haem group, made possible by a loosening of the crevice structure. The direct reduction of the haem is then followed by a slow monomolecular event

(phase 1 of Table 2), thus making the reduction of this form of carboxymethyl-cytochrome *c* analogous to the direct reduction of native cytochrome *c* by hydrated electrons (Pecht & Farragi, 1971). If this is correct it means that the hexa-co-ordinated low-spin intermediate is produced as the product of that form of carboxymethyl-cytochrome *c* responsible for phase 3. Some support for this conclusion is given in Table 2, which shows that the amount of intermediate formed, as indicated by the amplitude of phase 1, increases in step with the amount of quickly reacting material measured by the amplitude of phase 3.

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