Kinetic Studies on Mammalian Cytochrome *c* Modified with 2-Hydroxy-5-nitrobenzyl Bromide

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The reduction of 2-hydroxy-5-nitrobenzyl tryptophyl cytochrome *c* by the chromous ion was studied by stopped-flow techniques. At pH 6.5 the reduction of 2-hydroxy-5-nitrobenzyl tryptophyl cytochrome *c* is complex, showing the presence of three distinct phases. Two chromium concentration-dependent phases are observed ($1.1 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$, phase 1; $1.25 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$, phase 2) and one slow first-order process (0.25 s^{-1} , phase 3). A comparison of the static and kinetic difference spectra, along with the data from the reduction of the reoxidized reduced protein, suggests that the slow chromium concentration-independent phase is due to a slow conformational event after fast reduction of the NO_2 group. The rates of the chromium concentration-dependent phases show a marked variation with pH above 7.5. The activation energies for the three processes were also measured at 33.2, 38.6 and $69.7 \text{ kJ} \cdot \text{mol}^{-1}$ for phases 1, 2 and 3 respectively. The reaction of reduced 2-hydroxy-5-nitrobenzyl tryptophyl cytochrome *c* with CO was followed by means of both stopped-flow and flash photolysis. The combination with CO at pH 6.8 as measured in stopped-flow experiments showed two phases, one CO-dependent phase (phase 2, $2.4 \times 10^2 \text{ M}^{-1} \cdot \text{s}^{-1}$) and one CO-independent phase (phase 1, 0.015 s^{-1}). Investigation of the pH-dependence of the phases showed both the rates and amounts of each phase to be pH-invariant. CO recombination, after photolytic removal, was found to be biphasic; a CO-dependent phase (phase 2, $2.4 \times 10^2 \text{ M}^{-1} \cdot \text{s}^{-1}$) and a CO-independent phase (phase 1, 1.0 s^{-1}) were observed. A tentative model which can accommodate these observations is proposed.

Since Winfield (1965) first proposed, on theoretical grounds, a mechanism for intramolecular electron transfer in cytochrome *c*, many workers (Dickerson *et al.*, 1972; Myer & Pal, 1972; Myer, 1972) have sought to extend this by incorporating new structural and spectroscopic evidence. All these proposals invoke a significant functional role for the species-invariant tryptophan residue at position 59.

Myer (1972) and Myer & Pal (1972), working on bromosuccinimide-modified cytochrome *c*, suggest its function to be accomplished by π overlap with the haem group which thus facilitates electron transfer to the haem iron. On the other hand, Aviram & Schejter (1971) have suggested a significant structural role for this residue, on the basis of the properties of the molecule when formylated on the ring nitrogen atom of tryptophan-59. Weight has been added to this proposal of structure-function properties of this residue by the solid-state synthesis of a tryptophan-deficient cytochrome *c* which has marked changes in its properties (Sano & Kurihara, 1969).

To learn more about the role of this amino acid in

the redox and other processes a study of the effect of specific chemical modification of the tryptophan residue has been made. The present communication deals with the kinetics of the reduction of 2-hydroxy-5-nitrobenzyl tryptophyl cytochrome *c* by the chromous ion and compares this with that of the native molecule. The chromous ion was chosen as reductant partly because of its high redox potential and ease of production and storage, and also because of an ever-growing corpus of information on its use and effects on cytochrome *c* molecules in general (Kowalsky, 1969; Yandell *et al.*, 1973; Grimes *et al.*, 1974).

The work of Wilson *et al.* (1973) on carboxymethylated cytochrome *c* and the unpublished work of A. J. Nascimento in this laboratory on nitro cytochrome *c* indicate that the haem iron becomes more susceptible to combination with ligands of ferrous iron on protein modification. 2-Hydroxy-5-nitrobenzyl tryptophyl cytochrome *c* has proved to be no exception to this emerging pattern and this paper reports investigations of the CO-binding kinetics either by mixing procedures or else by flash photolysis. The relative ease of reaction between the haem iron

and CO may give some indication of the disruption of the structure subsequent to the chemical modification.

Experimental

2-Hydroxy-5-nitrobenzyl tryptophyl cytochrome *c* was prepared as described in Brittain & Greenwood (1975).

Chromous chloride was produced by dissolving electrolytically pure chromium metal in O₂-free HCl by the method of Dawson *et al.* (1972) and was stored under O₂-free N₂. The concentration of chromous ion was determined by spectrometric titration against KMnO₄, after the disappearance of the band at 545 nm, by using an extinction coefficient of $2.34 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$; for more details the reader is referred to Brittain *et al.* (1974).

Stopped-flow measurements were carried out in an apparatus identical with that described by Gibson & Milnes (1964) equipped with 2 cm- and 2 mm-light-path cells, having dead times of 3 and 1 ms respectively.

Sodium cacodylate was used as the buffering system and the pH of solutions were measured by using a Radiometer 51 pH-meter fitted with a micro-electrode unit E 5021a.

2-Hydroxy-5-aminobenzyl tryptophyl cytochrome

c was prepared by reducing a sample of 2-hydroxy-5-nitrobenzyl tryptophyl cytochrome *c* with a small excess of sodium dithionite and passing the resulting mixture through a small column of Sephadex G-25 which had previously been equilibrated with the required sodium cacodylate buffer.

CO buffers were prepared by equilibrating the appropriate degassed buffer under 101 kPa pressure of CO gas at room temperature (20°C). The concentration of CO was then found by reference to standard solubility tables.

Flash-photolytic experiments were conducted by using the instrumentation and methods described by Greenwood & Gibson (1967). A Bausch and Lomb monochromator (500 mm grating, 1200 grooves/mm, $f=4.4$) was used in conjunction with the flash-photolysis apparatus.

Results

Chromous ion reduction

Figs. 1(a) and 1(b) show the complex nature of the reduction process occurring at pH 6.5 when 2-hydroxy-5-nitrobenzyl tryptophyl cytochrome *c* was mixed with the chromous ion in the stopped-flow apparatus. An analysis of the chromium concentration-dependence of these phases is shown in Fig. 2,

Fig. 1. Progress curve for the reduction of 2-hydroxy-5-nitrobenzylated cytochrome *c* by Cr^{2+}

(a) A typical oscilloscope trace and (b) log plot of the data for the reduction of 2-hydroxy-5-nitrobenzyl tryptophyl cytochrome *c* by Cr^{2+} . The protein at $3 \mu\text{M}$ in 0.2 M-sodium cacodylate buffer, pH 6.4, was mixed with 1 mM- Cr^{2+} in a 2 cm-path-length cell at 20°C. The vertical scale on the oscilloscope trace represents a change of 0.02 *E* unit per division and the horizontal scale 20 ms per division (○) (●) (lower trace) and 1 s per division (Δ) (upper trace).

Fig. 2. Cr^{2+} concentration-dependence of reduction of modified cytochrome *c*

The fast phase (k_1) (a) and intermediate phase (k_2) (b) are shown. The protein was used at a concentration of $6 \mu\text{M}$ in 0.2 M-sodium cacodylate buffer, pH 6.4. The reduction was followed at 420 nm in a 2 mm-path-length cell at 20°C. The dependences for both the nitro (○) and amino (Δ) forms of the protein are shown.

Table 1. Effect of $[\text{Cr}^{\text{II}}]$ on the rate of the slow phase seen in the reduction of 2-hydroxy-5-nitrobenzyl tryptophyl cytochrome *c*

Protein ($6 \mu\text{M}$) was mixed with Cr^{II} in a 2 cm-path-length cell in the stopped-flow apparatus at 20°C. The buffer used was 0.1 M-sodium cacodylate-HCl, pH 6.5. The reaction was observed at 420 nm.

$10^4 \times [\text{Cr}^{\text{II}}]$ (M)	k_3 (s ⁻¹)
21.8	0.23
10.9	0.24
7.3	0.29
4.4	0.34
3.6	0.28
2.2	0.22
1.1	0.22

Table 2. Activation parameters of the phases observed in the reduction of 2-hydroxy-5-nitrobenzyl tryptophyl cytochrome *c* by Cr^{II}

Protein ($6 \mu\text{M}$) in 0.2 M-sodium cacodylate-HCl buffer, pH 6.5, was treated with 0.5 mM- Cr^{II} in a 2 mm-path-length cell in the stopped-flow apparatus. The progress of the reaction was observed at 420 nm.

Phase	E_a (kJ·mol ⁻¹)
1	33.2
2	38.6
3	69.7

from which it is clear that the rates of two of the processes are linearly dependent on chromium concentration, whereas the slowest rate is chromium concentration-independent (Table 1). The gradients of Fig. 2 give the second-order rate constants for the two faster processes at $1.1 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $1.25 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$, and the first-order process has a rate constant of 0.25 s^{-1} .

The temperature-dependence of the rates of the processes allows a calculation of the activation energy by use of the logarithmic form of the Arrhenius equation. The activation energies so calculated are shown in Table 2.

As the spectrum of 2-hydroxy-5-nitrobenzyl tryptophyl cytochrome *c* is known to show marked variation with pH (Brittain & Greenwood, 1974) a study of the effect of pH on the reduction of 2-hydroxy-5-nitrobenzyl tryptophyl cytochrome *c* by the chromous ion was carried out (Fig. 3). The two rates observed undergo a drastic decrease as the pH is raised, whereas the rate of reduction of native cyto-

Fig. 3. pH-dependence of the rates of reduction of 2-hydroxy-5-nitrobenzylated cytochrome *c* by Cr^{2+}

The pH-dependence of the fast phase, k_1 (●), and the intermediate phase, k_2 (○), seen during the reduction of 2-hydroxy-5-nitrobenzyl tryptophyl cytochrome *c* are shown along with the rate of reduction of native cytochrome *c*, k (Δ), in the same range. The reactions were followed at 420 nm in a 2 cm-path-length cell at 20°C. The proteins were used at concentrations of $3 \mu\text{M}$ and were reduced by mixing with 1 mM- Cr^{2+} .

chrome *c* over this pH range is totally invariant. It is noteworthy that a high spin/low spin transition has been found for this protein with a pK of 7.5 (Brittain & Greenwood, 1975). Table 3 shows, however, that the relative amounts of phases 1 and 2 are pH-independent.

A comparison of the static and kinetic difference spectra in the Soret region (Fig. 4) shows that a process that contributes to the absorption band at 420 nm is being missed in the dead time of the stopped-flow apparatus. As the reaction of the reagent group with the protein introduces a reducible NO_2 group, a sample of the protein was reduced with a small excess of sodium dithionite and then reoxidized to yield 2-hydroxy-5-aminobenzyl tryptophyl cytochrome *c*. This protein was then used to determine the effect, if any, of the presence of the NO_2 group on the observed complexity of the reduction process. Only two processes are now observed. The chromous ion concentration-dependence of these processes was identical with those of phases 1 and 2 in the reduction of 2-hydroxy-5-nitrobenzyl tryptophyl cytochrome *c* (see Fig. 2).

CO flow

The reaction of reduced 2-hydroxy-5-nitrobenzyl tryptophyl cytochrome *c* with CO at pH 6.8 as followed in the stopped-flow apparatus was found to be biphasic. The analysis of the CO concentration-dependence of the two phases shows that the rate of one process (phase 2) is CO concentration-dependent and Fig. 5 yields a second-order rate constant for this process of $2.4 \times 10^2 \text{ M}^{-1} \cdot \text{s}^{-1}$. The other process is found to be CO concentration-independent with a first-order rate constant of approx. 0.012 s^{-1} .

A comparison of the difference spectra obtained statically and kinetically, shown in Fig. 6, clearly reveals that no process is being overlooked in the dead time of the stopped-flow apparatus. Table 4 indicates that both the rates and amounts of each of the two phases observed are pH-independent within the range pH 5.0–10.0.

Table 3. Effect of pH on the amount of each of the reductive phases observed in the reaction between Cr^{II} and 2-hydroxy-5-nitrobenzyl tryptophyl cytochrome *c*

Protein ($4 \mu\text{M}$) in 0.5 M-sodium cacodylate-HCl buffer was treated with 0.4 mM- Cr^{II} in a 2 cm path-length cell in the stopped-flow apparatus. The amount of each phase is expressed as a percentage of the total excursion observed.

pH	Amount of phase 1 (%)	Amount of phase 2 (%)
6.0	25	40
6.4	30	50
6.8	25	60
7.3	38	50

Fig. 4. Static and kinetic difference spectra of the reduction of 2-hydroxy-5-nitrobenzyl tryptophyl cytochrome *c* by Cr^{2+}

The static ($\circ$) and kinetic ($\square$) difference spectra were obtained between reduced and oxidized 2-hydroxy-5-nitrobenzyl tryptophyl cytochrome *c* by using a protein concentration of $8 \mu\text{M}$ in 0.2 M-sodium cacodylate buffer, pH 6.4, at 20°C . The kinetic difference spectrum was obtained by mixing the protein with 1 mM- Cr^{2+} in the stopped-flow apparatus in a 2 cm-path-length cell.

Fig. 5. CO concentration-dependence of the rate of combination with 2-hydroxy-5-nitrobenzyl tryptophyl cytochrome *c* observed by stopped-flow and flash photolysis

The dependence of the rate of combination of 2-hydroxy-5-nitrobenzyl tryptophyl cytochrome *c* with CO was followed by mixing the protein at $20 \mu\text{M}$ ($\bullet$) with CO-equilibrated buffer. The dependence of the rate of recombination of the protein with CO was followed by applying a photolytic flash ($\approx 300 \text{ J}$) to $4 \mu\text{M}$ -protein in the presence of various concentrations of CO ($\square$). In both cases the buffer system was 0.02 M-potassium phosphate, pH 6.8, with the reaction monitored at 415 nm.

Fig. 6. Static (○) and kinetic (Δ) difference spectra of CO binding to 2-hydroxy-5-nitrobenzyl tryptophyl cytochrome *c*

The static (○) and kinetic (Δ) difference spectra were obtained between the reduced protein-CO complex and the reduced protein by using a protein concentration of $6\mu\text{M}$ in 0.02M-potassium phosphate, pH 6.8, at 20°C . The kinetic difference spectrum was obtained by mixing the protein with the CO-equilibrated buffer in the stopped-flow apparatus in a 2cm-path-length-cell.

Table 4. Effect of pH on the rates and amounts of the two phases observed in the reaction between 2-hydroxy-5-nitrobenzyl tryptophyl cytochrome *c* and CO

Dithionite-reduced protein ($4\mu\text{M}$) in 0.02M-buffer was treated with 0.5mM-CO in a 2cm-path-length cell in the stopped-flow apparatus at 20°C . The reaction was monitored at 415nm. Sodium acetate-acetic acid buffer was used for pH 5–6, potassium phosphate for pH 6–7 and glycine-NaOH for pH 8–10. The amount of each phase is expressed as a percentage of the total excursion observed.

pH	k_1 (s^{-1})	k_2 (s^{-1})	Amount of phase 1 (%)	Amount of phase 2 (%)
5.0	0.011	0.1	69	31
5.8	0.01	0.08	75	25
6.8	0.012	0.08	78	22
8.0	0.012	0.09	71	29
9.0	0.012	0.09	74	26
10.0	0.013	0.08	80	20

CO flash

The complex nature of the reaction of CO and 2-hydroxy-5-nitrobenzyl tryptophyl cytochrome *c* observed in rapid-mixing experiments was also seen when the reaction was investigated by using the

technique of flash photolysis. Analysis of the CO concentration-dependence of the rates shows one phase to be CO concentration-dependent and the other CO concentration-independent.

It is noteworthy that the CO concentration-dependent process has the same second-order rate constant ($2.4 \times 10^2 \text{M}^{-1} \cdot \text{s}^{-1}$) as the CO concentration-dependent step in the stopped-flow experiments (Fig. 5). However, the rate of the CO concentration-independent process observed by photolytic dissociation is two orders of magnitude faster than for the flow experiments.

Fig. 7 shows that the two processes seen in photolysis experiments contribute equally to the overall, kinetically determined, difference spectrum.

Discussion

Since the *p*-nitrophenyl group introduces a new chromophore absorbing at 420nm (Koshland *et al.*, 1964) it is clearly important to establish its contribution, if any, to the kinetic processes we are studying.

Fig. 7. Kinetic difference spectra for the recombination of reduced 2-hydroxy-5-nitrobenzyl tryptophyl cytochrome *c* with CO after photolytic dissociation

The kinetic difference spectrum of initial minus final absorbance (□) is compared with initial minus intermediate absorbance (○). The protein concentration was $4\mu\text{M}$ in 0.02 M-potassium phosphate, pH 6.8, and the spectra were obtained by applying a photolytic flash of approx. 300 J to the protein contained in a 1 cm-path-length cell at 20°C .

The reduction of *p*-nitrophenyl groups by chromous ions is extremely rapid (A. J. Nascimento, personal communication), and too fast to be studied in the stopped-flow apparatus. The differences observed in the Soret region between the static and kinetic difference spectra (Fig. 4) probably represent the very rapid reduction of the NO₂ group. The nitrophenyl group also appears to be responsible in some way for the slow first-order process seen in Fig. 1, since, when this group is pre-reduced as described in the text, the slow phase is abolished. A possible explanation for this observation is that the reduction of the nitrophenyl group, by chromous ions in kinetic experiments, triggers a protein-conformational event which is responsible for the slow concentration-independent step. Whatever is the nature of this step it does not, however, appear to influence the reduction of the haem iron, since we can observe identical rates of reduction in both forms of the modified cytochrome *c*. If we now consider the nature of the two chromium concentration-dependent processes, Table 3 clearly indicates their pH invariance. This seems to suggest that the two species responsible for these two phases are not in pH-dependent equilibrium or else the equilibrium has a *pK* far removed from neutrality. The homogeneity of the sample, considered along with this finding, indicates only a small difference in the two species, and it is suggested that the two rates may, in fact, be due to the presence of the two structural isomeric products of the modification. The position of the introduced group facilitates electron transfer to differing degrees in each of the two species.

It is thus clear that the modification of the tryptophan residue, which has been carried out as described above, markedly alters the redox kinetics of the protein. The complexity of the processes observed, however, appears, in part at least, to arise from the nature of the modifying agent used. As with most chemical-modification procedures it is difficult to separate the contributions of the effect of the change in the amino acid modified and any subsequent conformational changes, which result in a changed reactivity of the protein.

We have observed the same reduction rates in both the nitro and amino forms of the modified protein, which in themselves must have different hydrophobic characteristics. With this in mind it seems that gross conformational changes are not responsible for the alteration in properties, and, in fact, the alterations are indeed highlighting the functional role of the tryptophan residue in the redox properties of the cytochrome.

CO flow

The results of the reaction of CO with 2-hydroxy-5-nitrobenzyl tryptophyl cytochrome *c*, as followed in the stopped-flow apparatus (Fig. 6), show the

presence of a slow CO concentration-independent step and a relatively fast CO concentration-independent step. This situation could arise in a number of ways, two of which are shown:

or

in which Fe* and Fe represent two different conformational states of 2-hydroxy-5-nitrobenzyl tryptophyl cytochrome *c* only one of which, Fe*, binds CO. In case (a) the Fe* form reacts with CO in a rapid second-order process which is then followed by a first-order step as Fe is converted into Fe* with a rate *k*₁. In the second situation, (b), all the protein is considered to be able to bind CO and does so in a second-order process, which leads to an unstable product which then decays in a first-order fashion. The stopped-flow data so far obtained do not permit an unambiguous conclusion to be reached as to which of these possibilities applies. In an attempt to resolve this problem we resorted to flash photolysis, and we observed biphasic recombination kinetics similar to those obtained in flow kinetics. However, analysis of the CO concentration-dependence of the two phases reveals a relatively slow second-order step and a fast first-order process. The rate of this first-order process exceeds at all times the pseudo-first-order rate of the concentration-dependent step, a situation which arises because of our inability to produce CO concentrations in solution in excess of 1 mM.

It is noteworthy that the second-order process observed in the flash experiments has the same rate constant as that seen in the flow experiments. If it is assumed that these two processes are, in fact, one and the same, then it would appear that model (b) is not operative, since in this system, CO binding, of necessity, requires the observation of the subsequent slow conformational change, which is not seen.

A model which would describe the observed flash kinetics and be consistent with the flow data system (a) is given below.

In this system the process of CO binding (*k*₂) is identical with that of system (a) of the flow experiments; it is, however, necessary to assume that the process designated *k*₄ is spectrally silent.

The product of photolysis, Fe, decays rapidly at a rate *k*₁ to produce Fe*, which combines with CO in a second-order process designated *k*₂, which is

common to the flow kinetics. Since we observe only one CO concentration-dependent rate, then k_3 cannot be significantly larger than k_2 . The equilibrium process between Fe and Fe* is of course inoperative under the conditions of a photolysis experiment.

Although this model is consistent with the observed flow and flash data it must be pointed out that it is only a tentative scheme and further experimental work will be required to establish its validity.

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