Kinetic Studies on [*N*-Formyltryptophyl]Cytochrome *c*

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(Received 24 March 1975)

The formylation of the ring nitrogen atom of the tryptophan residue in cytochrome *c* was carried out and consequent changes in the kinetic properties of the protein were investigated. The reduction of formylated cytochrome *c* by Cr^{2+} was studied by stopped-flow techniques. At pH 6.5 the reduction process shows the presence of two phases. One phase ($k = 4 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$) is dependent on Cr^{2+} concentration and one phase ($k = 5.0 \text{ s}^{-1}$) is not. A study of the temperature dependence of the two phases yields values for their activation energies of $38.6 \text{ kJ} \cdot \text{mol}^{-1}$ and $42.4 \text{ kJ} \cdot \text{mol}^{-1}$ respectively. The reaction of the reduced formylated cytochrome *c* with CO was followed by means of both stopped-flow techniques and flash photolysis. The combination with CO at pH 6.8 measured in stopped-flow experiments shows two phases, both dependent on the concentration of CO ($k_1 = 1.8 \times 10^2 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $k_2 = 1.6 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$). If CO was dissociated from the protein by photolysis and then allowed to recombine with it, it was found to do so in a simple manner, at a rate which depended on the concentration of CO ($k = 1.9 \times 10^2 \text{ M}^{-1} \cdot \text{s}^{-1}$). A tentative model which can accommodate these findings is proposed. The reaction of the oxidized form of formylated cytochrome *c* with NO was followed by means of stopped-flow techniques. The reaction was found to be biphasic with one phase dependent on the concentration of NO ($k = 2.8 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$) and one phase ($k = 0.2 \text{ s}^{-1}$) independent of the concentration of NO. This behaviour is compared with that of the native molecule. A comparison of these kinetic observations with those on other tryptophan-specific modifications leads to the conclusion that the main alteration in kinetic properties is due, not to the nature of the modifying group, but rather to the disruption of the normal environment of the haem.

Horse heart cytochrome *c* contains 104 amino acids, of which the tryptophan residue at position 59 has been found to be invariant over a large number of species (Margoliash & Schejter, 1966), and this finding alone suggests an important role for this amino acid within the cytochrome molecule. A number of workers (Dickerson *et al.*, 1972; Myer, 1972) have suggested its involvement in the electron-transfer properties of the protein, possibly by means of π overlap with the haem group, made possible by its position and close proximity to the porphyrin ring. However, a more intimate effect involved in the maintenance of the integrity of the haem environment has also been proposed (Aviram & Schejter, 1971). This suggestion has been put forward on the basis of chemical modification studies as well as the fact that it has been found that in both oxidation–reduction states, tryptophan-59 is hydrogen-bonded to one of the oxygen atoms of the propionic acid side chains of the haem (Takano *et al.*, 1973).

A reaction allowing the specific modification of tryptophan was introduced by Previero *et al.* (1967) and Coletti-Previero *et al.* (1969), which has been applied by Aviram & Schejter (1971) to

the chemical modification of cytochrome *c*. This procedure has been demonstrated to produce a chromatographically pure fully modified single species by reaction solely at the ring N atom of tryptophan-59 (O'Hern *et al.*, 1975). We have extended this approach to the problem of the role played by this residue in the functionality of cytochrome *c*, by carrying out kinetic studies on both the behaviour on reduction and ligand-binding properties of the modified protein.

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 formylation procedure was essentially that of Aviram & Schejter (1971), in which cytochrome *c* is treated with HCl in 100% formic acid.

CrCl_2 solutions were prepared as described in Brittain *et al.* (1974). Buffers containing CO were prepared by equilibrating the appropriate degassed buffer under 101 kPa of CO gas at room temperature (20°C). The concentration of CO was

then found by reference to standard solubility tables. NO buffers were prepared in an analogous fashion. Spectroscopic measurements were made with an SP.700A instrument (Pye Unicam, Cambridge, U.K.).

Stopped-flow measurements were carried out in an apparatus identical with that described by Gibson & Milnes (1964) equipped with 2 cm or 2 mm

light-path cells, having dead times of 3 ms and 1 ms respectively.

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

Fig. 1. Absorption spectra of the formylated cytochrome *c*

Oxidized (—), reduced (----) and CO-complex (-.-.-) forms in the visible region. The protein was used at $7.5\ \mu\text{M}$ for measurements in the Soret region (390–450 nm) and $33\ \mu\text{M}$ in the α/β 490–600 nm region. Spectra were recorded at 20°C in 0.1 M-potassium phosphate buffer, pH 7.0; reduction was performed by addition of a small excess of solid sodium dithionite.

Sodium cacodylate was used as the buffering system in chromium-reduction studies. The pH values of solutions were measured with a Radiometer 51 pH-meter fitted with a micro-electrode unit E 5021 a.

Results

Fig. 1 shows the absorption spectra of oxidized and reduced formyl-cytochrome *c* as well as that of the complex formed by the addition of CO to the reduced protein. Anaerobic titrations of reduced formyl-cytochrome *c* in the presence of dithionite suggest a relatively low affinity for this ligand,

and Fig. 2(a) shows a typical titration in which the increase in E_{414} is plotted as a function of CO concentration. When replotted by the method of Hill (1910) the data in Fig. 2(b) yield a value of $3.0 \times 10^5 \text{ M}^{-1}$ for the affinity constant and a Hill number (h) of 1.0. Mixing reduced formyl-cytochrome *c* with CO in the stopped-flow apparatus showed the presence of two second-order processes. Analysis of the dependence on CO concentration yielded values of the rate constants as $1.8 \times 10^2 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $1.6 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$ for the two processes. The distribution of the two kinetic events as a function of wavelength was identical but, as Fig. 3 clearly shows,

Fig. 2. CO titration of reduced formylated cytochrome *c*

(b) shows a Hill plot of the titration of (a). Dithionite-reduced protein, at a concentration of $4.4 \mu\text{M}$ in 0.2M-potassium phosphate buffer, pH 7.0, was titrated with portions of CO-equilibrated buffer (1 mM in CO). The additions were made anaerobically and the absorbance increase at 414nm was monitored against the additions made. In (b), $\bar{y}$ represents the fraction of protein that bound ligand and $[\text{CO}]_F$ the concentration of free CO in solution after each addition. The Hill plot yields $K_a = 3.9 \times 10^5 \text{ M}^{-1}$ and Hill number, $h = 1.0$ (----).

Fig. 3. Dependence on CO concentration of the ratio of the amounts of kinetic phases observed in stopped-flow experiments

F and S represent the amounts of the fast and slow phases observed, respectively. In these experiments $3.5 \mu\text{M}$ reduced protein in 0.02M-potassium phosphate buffer, pH 6.8, was treated under a range of CO concentrations at 20°C in a 2cm path-length cell in the stopped-flow apparatus. The reaction was monitored at 414nm.

their relative contributions at a fixed wavelength varied with the concentration of CO. In experiments where the CO recombination was followed after photolytic removal of the ligand a single second-order process was observed with a rate constant of $1.9 \times 10^2 \text{ M}^{-1} \cdot \text{s}^{-1}$.

NO was used as a convenient probe for the oxidized form of the protein. Standard ligand-titration techniques yielded an affinity constant of $3.8 \times 10^4 \text{ M}^{-1}$ and a Hill number of 1.0 for NO. The kinetics of NO binding by formylated ferricytochrome *c* was followed by using rapid-mixing procedures and produced evidence for two processes, one second-order ($k = 2.8 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$) and the other first-order, $k = 0.2 \text{ s}^{-1}$.

Any alteration in the redox properties of the protein, owing to modification, were investigated by rapid-mixing studies by using Cr^{2+} ions as reductant. The reduction was biphasic and analysis of the chromous-ion concentration-dependence showed the presence of one chromium-ion-concentration-dependent phase ($k = 4 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$) and one independent rate, ($k = 5.0 \text{ s}^{-1}$). Analysis of the temperature-dependence of these rates by the Arrhenius equation revealed activation energies of $38.6 \text{ kJ} \cdot \text{mol}^{-1}$ and $42.4 \text{ kJ} \cdot \text{mol}^{-1}$ for the second-order and first-order processes respectively.

Discussion

The present results clearly show the rather drastic alteration in the properties of cytochrome *c* consequent on the introduction of the formyl group on to the ring *N* atom of tryptophan-59. The properties of both the oxidized and reduced protein are affected, as indicated by the binding studies using NO and CO. Native ferrocycytochrome *c* only binds the latter ligand at high pH (Butt & Keilin, 1962), and although ferricytochrome *c* reacts with NO at neutral pH it does so almost two orders of magnitude more slowly than formyl-ferricytochrome *c*.

The data in Fig. 3, together with the presence of two second-order rates for CO combination, strongly suggest that the modified protein exists as two ligand-binding species in equilibrium, although the exact nature of the differences remains a matter for speculation. It is, however, possible to rationalize the data in terms of the single model below:

in which A and B represent the two forms in equilibrium and k_1 and k_2 the two second-order rate constants. In principle it should be possible to assign a value to the equilibrium constant K from Fig. 3(c); however, owing to the constraints placed on us by the limits of solubility of CO, this is not practical. If such a model as that proposed in eqn. (1) is indeed operative then the flash-photolytic data could be explained by supposing that photolysis of XCO leads either to A or B and that the recombination reaction is fast in comparison with the equilibrium. The recombination reaction would thus be identical with one of the two processes observed in flow experiments, as is the case.

The redox properties of the modified protein are also drastically altered, for whereas the native molecule shows a simple exponential rate constant of $1.2 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$, the formylated protein shows biphasic kinetics. The progress of the reduction of formyl-cytochrome *c* indicates a reductive and subsequent conformational change.

From the above, it is clear that modification of this specific residue at the indole ring *N* atom brings about marked functional changes. It is very striking, however, to compare the properties of this modification with those of another tryptophan modification (Brittain & Greenwood, 1975a,b). In all cases the second-order rates of ligand binding and reduction show a remarkable similarity. If one considers the nature of the modifying groups then the formylation at the 3 position and the 2-hydroxy-5-nitrobenzylation at the 3 position clearly are very different in character except for the fact that both groups tend to be hydrophilic. However, another possibility is that both modifications remove the hydrogen atom from the ring nitrogen atom. Aviram & Schejter (1971) have suggested that it is in fact the disruption of hydrogen-bonding by the tryptophan residue that is the cause of the alterations subsequent to formylation. From a comparison of the properties of these two modifications, the actual cause of the alteration in function is not absolutely clear; however, it is clear that it is a product of the disruption of the normal environment of the tryptophan which is the key factor. Whether this is due to the removal of the ability to form hydrogen bonds or the increase in hydrophilic character remains to be clarified.

T. B. thanks the S.R.C. for a graduate studentship. C. G. gratefully acknowledges a grant from the Royal Society for the purchase of the oscilloscope, type 7514.

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