The Preparation and Some Properties of Mammalian Cytochrome *c* Modified with 2-Hydroxy-5-nitrobenzyl Bromide

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2-Hydroxy-5-nitrobenzyl bromide reacts with horse heart cytochrome *c* at acid pH to yield a chemically modified protein. Chromatography of the protein on CM-cellulose allows separation of a single chemically modified species. This species is shown by gel chromatography to be monomeric, and isoelectric focusing shows the pI to be lowered from 10.5 to 9.8 on introduction of the reagent molecule. The changes observed in the u.v. region of the spectrum are consistent with the introduction of a single residue of the reagent, and the normal fluorescence of tryptophan is lost. The chemically modified protein exhibits marked changes in its functional properties as compared with native cytochrome *c*. Unlike the native monomer, the modified cytochrome *c* has a pH-dependent spectrum which is typical of a high-spin species in the α/β region at low pH, changing to a low-spin species with an apparent pK of 7.5. The modified protein is autoxidizable and the ferrous form binds CO at neutral pH with an affinity constant of $2.6 \times 10^5 \text{ M}^{-1}$. The ferrous form of the modified cytochrome *c* binds CN^- at pH 10.0 with an affinity constant of $3.5 \times 10^2 \text{ M}^{-1}$. The modified cytochrome *c* was incapable of restoring the electron-transfer activity to mitochondria depleted of cytochrome *c*.

Horse heart cytochrome *c* contains 104 amino acid residues, and of these a number have been found to be invariant over a large number of species (Margoliash & Schejter, 1966). In particular a tryptophan residue at position 59 has been found to be totally invariant in all eukaryotic cells. The close proximity of its π -system to the haem group has led a number of workers (Winfield, 1965; Dickerson *et al.*, 1972; Myer, 1972; Aviram & Schejter, 1971) to propose a significant structural and functional role for this particular residue in the normal intramolecular electron-transfer process.

One approach to the study of the role of particular residues in structure/function relationships is via chemical modification, ideally by using a modification which is highly specific for the amino acid concerned. Benzyl halides have long been used for protein modifications, but in general suffer from sluggish reactivity and insolubility. However, it has been shown that the introduction of an *o*-hydroxyl group enormously enhances reactivity and specificity (Koshland *et al.*, 1964). Indeed Horton & Koshland (1967) have introduced a reagent, 2-hydroxy-5-nitrobenzyl bromide, which in the absence of free thiol groups and in acid media is specific for tryptophan. In addition to modifying the tryptophan residue, the use of 2-hydroxy-5-nitrobenzyl bromide introduces a new chromophoric group with similar properties to those of *p*-nitrophenol. Knowledge of the extinction co-

efficient of this group permits an accurate measurement of the degree of modification.

Part of this work has already appeared in preliminary form (Brittain & Greenwood, 1974).

Experimental

Native horse-heart cytochrome *c* (type III) and 2-hydroxy-5-nitrobenzyl bromide were obtained from Sigma (London) Chemical Co., Kingston-on-Thames, Surrey, U.K., the cytochrome *c* being used without further purification. All other chemicals were of reagent grade and were obtained from BDH, Poole, Dorset, U.K.

Spectroscopic measurements were made with a SP.700 A instrument (Pye Unicam, Cambridge, U.K.). All pH measurements were made with an EIL23 A pH-meter fitted with a set of micro-combination electrodes, which were calibrated with a buffer standard, Radiometer Type S1001, pH 6.5, before use. Fluorescence measurements were carried out with a Perkin Elmer 204 fluorescence spectrophotometer.

Isoelectric focusing was carried out by applying approx. 30 mg of protein to the LKB 8101 column and focusing in the range pH 8.0–11.0 (Karlsson & Ohman, 1971). The material on the column was removed after 3 days and collected in approx. 1.5 ml fractions.

The molecular weight of the protein was deter-

Fig. 1. Chromatographic analysis of the elution from CM-cellulose of the protein products of reaction of cytochrome *c* with 2-hydroxy-5-nitrobenzyl bromide

The protein was eluted by using a linear ionic-strength gradient (Δ ; 2×400 ml) of 0.02–0.2M potassium phosphate buffer, pH 7.8. Fractions (approx. 1.5 ml) were collected, showing the presence of fraction I (modified protein) and fraction II (unchanged protein).

mined essentially by the gel-filtration method of Andrews (1962). Approx. 40 mg of protein was applied to a column (100 cm $\times$ 2.5 cm) containing Sephadex G-75 and eluted with 0.02M-potassium phosphate buffer, pH 7.0, in 1M-NaCl.

The activity was measured by adding modified cytochrome *c* to mitochondria that had been depleted of cytochrome *c* by the methods of Yacobs & Sanadi (1960). Measurement of O_2 utilization during succinate oxidation was made polarographically with a Rank Brothers (Bottisham, Cambridge, U.K.) oxygen-electrode system, coupled to an Esterline Angus strip chart recorder.

Results

A portion (0.0464 g) of 2-hydroxy-5-nitrobenzyl bromide, dissolved in acetone that had previously been dried over a molecular sieve, was added to a solution containing 200 mg of type III horse heart cytochrome *c* dissolved in 10 ml of 0.17M-acetic acid with consistent stirring in the dark at room temperature (about 21°C).

After 20 min the reaction mixture was applied to a Sephadex G-25 column equilibrated with 0.02M-potassium phosphate buffer, pH 5.8. The mixture was eluted with the same buffer and the red protein band collected.

The protein fraction was absorbed on a column (1 cm $\times$ 50 cm) of CM-cellulose which had been equilibrated at pH 5.8 with 0.02M-phosphate buffer. The protein was then eluted from the column with a linear gradient of 0.02–0.2M-phosphate buffer, pH 7.8, (2×400 ml), after the column had been washed with 200 ml of 0.02M-phosphate buffer, pH 7.8. Fractions

Fig. 2. Isoelectric focusing of modified cytochrome *c*

This was carried out by applying approx. 20 mg of the modified protein to the LKB 8101 column and focusing for 3 days in the pH range 8.0–11.0 (Δ). Fractions (approx. 1.5 ml) were collected and protein concentrations measured at 280 nm ($\bullet$).

(approx. 2 ml) were collected and the E_{410} was measured.

Fig. 1 shows the elution profile of the products of the reaction between ferricytochrome *c* and 2-hydroxy-5-nitrobenzyl bromide. Under the conditions described above, CM-cellulose clearly resolved two components. The component eluted last from the CM-cellulose column behaved identically with native ferricytochrome *c*, and in further experiments was found not to bind CO when reduced at neutral pH, and further, to have the same pI as the native protein. In view of these findings it seems reasonable to suppose that this fraction II represents unchanged ferricytochrome *c*, and therefore its properties have not been further investigated.

Fraction I from the chromatographic step was

subjected to isoelectric focusing in the pH range 8–11 and, as shown in Fig. 2, was found to behave as a single species. The isoelectric point was 9.8, compared with the value of 10.5 found by Tint & Reiss (1950) for the native protein. Molecular-weight determina-

tion by gel filtration on Sephadex G-75 gave a value of 12600 for the fraction I component.

Fig. 3 shows the absorption spectrum of fraction I compared with that of native ferricytochrome *c* over the range 280–320 nm. Clearly significant changes have occurred in this region, and these are consistent with the introduction into the molecule of a chromophore related to *p*-nitrophenol. At pH 4.0, where all the introduced groups are in the un-ionized form, it is possible to estimate the number of groups introduced into the molecule by using the molar extinction coefficient $\epsilon = 9600 \text{ cm}^{-1}$ at 320 nm (Horton & Koshland, 1967). Calculations based on this procedure are consistent with a protein molecule containing 0.98 mol of reagent/mol of haem.

As with most haemoproteins, severe quenching of protein fluorescence occurs as a result of the presence in the molecules of the haem group. Nevertheless, Tsong (1973) has demonstrated for cytochrome *c* that, under suitable conditions, it is possible to observe the tryptophan fluorescence. By this method Fig. 4 shows that the normal fluorescence of tryptophan in ferricytochrome *c* is absent in the chemically modified species, as would be expected if the tryptophan had reacted with the reagent.

The spectrum of the modified protein, unlike that of native cytochrome *c*, shows a marked dependence on pH. In the visible region (Fig. 5a) a typical transition between the low-spin state and the high-spin state in the oxidized material is seen, characterized by the

Fig. 3. *U.v. spectra of native and modified cytochrome c*

The spectrum of native (○) and modified (—) cytochrome *c* are shown; both proteins were $30 \mu\text{M}$ in 0.05 M-ammonium acetate buffer, pH 4.0.

Fig. 4. *Relative fluorescence (F) of native (—) and modified cytochrome c (---)*

Both proteins were $2 \mu\text{M}$ in 0.05 M-Tris-HCl, pH 7.0, containing 6 M-guanidine hydrochloride; (a) excitation, (b) emission.

Fig. 5. Effect of pH on the spectrum of modified protein in the α/β region

(a) A, B, and C represent spectra at pH 10.0, 8.0 and 4.0 in glycine-NaOH, potassium phosphate, and sodium acetate-acetic acid buffers all at 0.2M concentration. The protein was $30\mu\text{M}$ in all cases. (b) Titration curve of the high-spin form of the modified protein at 620nm. The protein was $60\mu\text{M}$, supplemented with NaCl to a final concentration of 0.3M. The spectrum was recorded in a 4cm-path-length cell, titration being carried out with 0.5M-HCl or 0.5M-NaOH as necessary.

appearance of a band at about 610nm. Fig. 5(b) shows a titration of this band which yields an apparent pK of 7.5.

Ligand binding

Apart from the changes noted above in the spectrum of this modified protein, functional changes are also observed, particularly in its reactions towards haem iron ligands. Many chemically modified cytochrome *c* species show a marked increase in their affinity towards such ligands, as noted previously (Schejter & Aviram, 1970; Wilson *et al.*, 1973). Fig. 6(a) shows a titration of the reduced protein with CO at neutral pH. Unlike native cytochrome *c*, which does not bind CO when reduced at neutral pH, the modified cytochrome shows a marked affinity towards this ligand. The Hill plot of these data yields a Hill coefficient of 1 and an affinity constant of $3.5 \times 10^5 \text{M}^{-1}$ (Fig. 6b).

Native ferrocyanochrome *c* has been shown to bind CN^- only at pH greater than 12.0 (Butt & Keilin,

1962), whereas the modified protein, although inactive towards this ligand at neutral pH, binds it at pH 10.0. The data in Fig. 7(a), which represent a titration of the modified protein at pH 10.0 with CN^- , yield a Hill coefficient of 1 and an affinity constant of $2.5 \times 10^2 \text{M}^{-1}$ (Fig. 7b). It is not possible to prepare stable modified ferrocyanochrome *c* in the absence of a reducing agent, e.g. sodium dithionite, and consequently we conclude that the protein is capable of reacting with O_2 , although this reaction has not been investigated in detail.

Enzymic activity

The modified cytochrome, although not reducible by ascorbate at neutral pH, is very slowly reduced at high pH in strictly anaerobic conditions. Fig. 8 shows that in contrast with native cytochrome *c* the modified protein is incapable of restoring respiratory activity to cytochrome *c*-depleted mitochondria.

Fig. 6. CO-binding titration of the reduced form of modified cytochrome *c*

(a) Titration curve obtained by titration of $5.1\mu\text{M}$ -modified protein with 0.1mM-CO. Reduced protein (3.404ml) in 0.2M-potassium phosphate buffer, pH 7.0, was titrated by the addition of small amounts of CO-equilibrated buffer, the reaction being followed by monitoring the increase in absorbance at 414nm. (b) Hill plot of the data obtained from the titration, in which y represents the fractional saturation of the protein and $[\text{CO}]_F$ represents the free concentration (μM) of ligand after reaction.

Fig. 7. CN-binding titration of the reduced form of the modified cytochrome *c*

(a) Titration curve obtained by titration of $3.4 \mu\text{M}$ -modified cytochrome *c* with 0.5M-KCN. Reduced protein (6.616 ml) in 0.3M glycine-NaOH buffer, pH 10.0, was titrated by the addition of small amounts of neutralized KCN. The reaction was followed by monitoring the increase in absorbance at 424 nm. (b) Hill plot of the data obtained from the titration in which y represents the fractional saturation of the protein and $[\text{CN}]_F$ represents the free concentration (μM) of ligand after reaction.

Fig. 8. Comparison of the ability of native and modified cytochrome *c* to restore succinate-oxidation activity to mitochondria depleted of cytochrome *c*

The rates of oxygen utilization were measured polarographically at 37°C for native cytochrome *c* (Δ) and modified cytochrome *c* ($\circ$). Each experiment contained 3.5 ml of O_2 -saturated buffer [10 mM-potassium succinate, 100 mM-KCl, 5 mM-2-(*N*-2-hydroxyethylpiperazin-*N'*-yl)-ethane sulphonic acid, pH 7.6], 10 mg of mitochondrial protein and the added cytochrome *c* (initial concentration $30 \mu\text{M}$).

Discussion

A number of chemical modifications have been introduced in order to probe the structure and reactivity of mammalian cytochrome *c*. In most cases it has proved difficult to avoid the production of multiple forms of the protein, although it has been possible to devise experiments which overcome this difficulty and permit specific conclusions to be drawn (Wilson *et al.*, 1973; Brittain *et al.*, 1974).

In contrast with many modification procedures, the one described in this paper, involving the use of 2-hydroxy-5-nitrobenzyl bromide, results in the specific modification of a single group. Since its introduction by Koshland *et al.* (1964), under appropriate conditions many workers have confirmed the specificity of the reagent towards tryptophan (Barman, 1972; Paetkau *et al.*, 1968). The fact that cytochrome *c* contains only one tryptophan residue and no free thiol groups makes it a particularly favourable protein to investigate with this reagent.

The data presented in Figs. 1 and 2 of this paper indicate that the modified product can be readily separated from unchanged cytochrome *c*, and further, appears homogeneous when subjected to isoelectric focusing. The u.v. absorption of the modified protein is consistent with the introduction of a chromophore related to *p*-nitrophenol, and by using the extinction coefficient found by Horton & Koshland (1967), we calculate that a single molecule of reagent has reacted for each molecule of cytochrome *c*. The fluorescence data strongly suggest that the group modified is tryptophan. The pH-dependence of the spectrum in the visible region is characteristic of a typical transition between high and low spin, with an apparent pK of 7.5. It is noteworthy that the pK of the introduced group is also 7.5 (Kreshkov *et al.*, 1968), and since this group itself does not absorb at 610 nm one conclusion might be that the haem iron transition observed in the above titration is signalling the deprotonation of the introduced group. Several explanations for this are possible, one of which might be that the protein undergoes a conformational change to accommodate the appearance of the charged group formed (in fact this conclusion is borne out by pH-jump experiments, which show a slow spin change comparable in rate with that normally observed for conformational changes).

In contrast with native cytochrome *c*, the modified protein shows a marked affinity for ligands of the ferrous iron, e.g. CO and CN^- . However, the measured affinity constants are much lower than those for CM-cytochrome *c*, in which gross conformation change is known to have occurred. The cyanide-binding data seem to indicate that only limited structural alterations have taken place, as suggested by the lack of reactivity at neutral pH.

By far the most drastic change in properties is seen

in the complete lack of ability of the modified cytochrome *c* to restore electron-transfer activity to the mitochondria depleted of cytochrome *c*. This loss of redox activity, when coupled with the evidence from ligand-binding experiments (which are suggestive of rather limited changes in structure), clearly implies a fundamental role for the tryptophan in electron transfer.

Because the reduced protein autoxidizes, we have been unable to test its ability to transfer electrons to cytochrome *c* oxidase *in vitro*. We are therefore unable to relate this modification to the topography of intramolecular electron transfer proposed by Dickerson (1974). If Dickerson's (1974) ideas are accepted, then the positioning of this residue on the reductive pathway is not inconsistent with the observed redox properties of the modified cytochrome *c*.

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