Properties of Cytochrome *c* Modified by Attachment to a Carbohydrate Polymer

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(Received 28 April 1977)

By enzymic digestion of the polysaccharide part of the covalent complex between cytochrome *c* and Sephadex G-200, a new water-soluble cytochrome *c* derivative is obtained (called cytochrome *c_r*). Measurement of the free amino groups of this derivative indicates that on average the molar ratio between cytochrome *c* and polysaccharide is close to 1. Chemical determination of the sugar content gives a value of approx. 24000 for the molecular weight of cytochrome *c_r*. On these bases the soluble cytochrome *c_r* complex may be thought of as a folded protein to which a long polysaccharide tail is covalently bound. The functional behaviour of cytochrome *c_r* is much more similar to that of the native molecule than to that of the insoluble complex (cytochrome *c_i*). In particular the kinetics of the reaction of cytochrome *c_r* and cytochrome *c_n* (native) with ascorbate, ferrocyanide–ferricyanide, O₂ and cytochrome *c* oxidase were investigated in considerable detail. The results of these experiments, together with the observation that the insoluble complex of cytochrome *c* is a very poor substrate of cytochrome *c* oxidase [Colosimo, Brunori & Antonini (1976) *Biochem. J.* 153, 657–661], indicate that hindrance effects constraining the approach between cytochrome *c_r* and its oxidase are of greater importance than specific chemical modifications in determining the functional behaviour of the protein.

In a paper from this laboratory (Colosimo *et al.*, 1976) it was shown that reduced mammalian cytochrome *c* covalently bound to a Sepharose matrix is oxidized extremely slowly in the presence of O₂ and mammalian cytochrome *c* oxidase unless catalytic amounts of soluble cytochrome *c* are present in the reaction mixture.

In principle two different mechanisms may be put forward to account for this result: (i) the chemical modification of the basic residue(s) of the protein involved in the coupling to the resin may itself be responsible of the effect; or (ii) the stereochemical hindrance due to the presence of the insoluble carrier may impose constraints to the approach between the oxidase and its substrate. Thus, to distinguish between the effect of immobilization and the change in chemical properties associated with the covalent binding to the carbohydrate, we have undertaken a number of experiments, reported here, on a new derivative of cytochrome *c*, namely cytochrome *c_r*, the soluble product obtained by the action of dex-

tranase on the Sephadex–cytochrome *c* complex. The results obtained may have a general relevance to a deeper understanding of the change in structural and functional properties produced by linkage of a protein to a carbohydrate.

Experimental

Materials

Horse heart cytochrome *c* and dextranase (1,6- α -D-glucan 6-glucanohydrolase, EC 3.2.1.11) from a *Penicillium* species (activity 100–200 units/mg) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and used without further purification. Cytochrome *c* oxidase (EC 1.9.3.1), isolated by the method of Yonetani (1960) from ox heart, was kindly given by Dr. Colin Greenwood (School of Biological Sciences, University of East Anglia, Norwich, U.K.). Sephadex G-200 was from Pharmacia Fine Chemicals (Uppsala, Sweden), CNBr from Fluka A.G. (Buchs, Switzerland) and Emasol 4130 from Kao Atlas (Tokyo, Japan). All other reagents were of analytical grade.

Absorption spectra were measured on a Cary 14 or on a Beckman DB-GT spectrophotometer. Protein concentrations were measured spectrophotometric-

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Abbreviations used: cytochrome *c_r*, resolubilized cytochrome *c*; cytochrome *c_n*, native cytochrome *c*; cytochrome *c_i*, the insoluble complex of Sephadex G-200 and cytochrome *c*.

ally by using the following absorption coefficients: reduced horse cytochrome *c*, $\epsilon_{550} = 27\,600 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ (Margoliash & Frohwirt, 1959); reduced cytochrome *c* oxidase, $\epsilon_{605} = 21\,000 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ (Yonetani, 1961).

Methods

Preparation of the insoluble complex Sephadex G-200–cytochrome *c* (cytochrome *c*_i). The use of Sephadex instead of Sepharose is a necessary prerequisite to obtain hydrolysis with dextranase, because of the different chemical constitutions of the parent polymers.

Sephadex G-200 was activated by CNBr by the method of Axen *et al.* (1970). Throughout the activation process the pH was kept constant at 9.8 by adding 0.4M-NaOH. Cytochrome *c* dissolved in 0.2M-sodium phosphate buffer, pH 7.1, was added in the proportion of 1mg/ml of packed resin to the freshly activated Sephadex G-200, the pH adjusted to 9.0 and the mixture left overnight at 4°C under gentle stirring. Under these conditions the yield of coupling is not quantitative, but the resulting complex behaves as a better substrate for dextranase. The unchanged protein was washed off on a glass filter with 0.2M-phosphate buffer, pH 7.1, containing 1.5M-NaCl.

Preparation and purification of the soluble derivative (cytochrome *c*_s). In a typical experiment 1.2ml of dextranase at 0.2mg/ml in 0.05M-Tris/HCl, pH 8.1, was added to the cytochrome *c*_i suspension (20ml) in 0.05M-potassium phosphate buffer, pH 5.8 (Axen *et al.*, 1970), and the mixture left at 37°C for 2h. After overnight dialysis at 4°C against water, which inactivated dextranase, the product of digestion was centrifuged at 15000 rev./min for 30 min (r_{av} , 8cm) and purified from the soluble carbohydrates by chromatography on a column (10cm × 1cm) of CM-Sephadex C-50. The resin was equilibrated with 0.01M-sodium acetate buffer, pH 5.0, and the non-covalently bound carbohydrates were washed off with the same buffer. Finally cytochrome *c*_s was eluted from the column with 0.01M-Tris/HCl buffer, pH 9.0, containing 1M-NaCl. The total sugar content of various samples throughout the purification procedure was tested by the colorimetric method of Dubois *et al.* (1956).

Reduction of various cytochrome *c* species

Before study of their oxidation kinetics, cytochrome *c*_s, cytochrome *c*_n and cytochrome *c*_i were reduced by addition of minute amounts of solid sodium dithionite, the excess of which was removed by filtration on a column (15cm × 1.5cm) of Sephadex G-25 for the soluble proteins (cytochrome *c*_n and cytochrome *c*_s) or by washing on a glass filter for the insoluble one (cytochrome *c*_i), by using in both cases the same buffer.

Direct reduction by sodium ascorbate was done

by adding 50μl from a freshly prepared aqueous 10mM-sodium ascorbate to the cuvette containing 1.2ml of 30μM oxidized protein in 0.1M-sodium phosphate buffer, pH 7.4.

Measurement of amino groups in cytochrome *c*_n and cytochrome *c*_s

The number of free amino groups in cytochrome *c*_n and cytochrome *c*_s was measured by the colorimetric method of Habeeb (1966), based on the reaction with 2,4,6-trinitrobenzenesulphonic acid with only minor modifications. Various amounts of protein (10–80μg) were added to 3ml of a mixture (1:2, v/v) of aqueous 0.1% (w/v) 2,4,6-trinitrobenzenesulphonic acid and 0.5M-Na₂CO₃ buffer, pH 9.5. The reaction was stopped after 2h of incubation at 37°C by adding 0.5ml of 1M-HCl, and the A_{335} read against an appropriate reference solution.

Rapid kinetics measurements

Stopped-flow experiments were performed in a Durrum–Gibson apparatus having an optical path of 2cm and a measured dead time of 4ms. Relaxation kinetics were measured by using an instrument built by Messanlagen (Göttingen, Germany) with a cell of 1cm light-path and 6ml total volume.

Ultracentrifugation

Ultracentrifuge runs were carried out in 0.05M-potassium phosphate buffer, pH 7.4, in a Beckman model E analytical ultracentrifuge with a rotor-temperature-indicating unit.

Results

Insoluble derivative (cytochrome *c*_i)

All the characteristic properties observed by Colosimo *et al.* (1976) for the cytochrome *c* immobilized on Sepharose 4B are retained when Sephadex G-200 is used as the insoluble carrier. This is summarized in Fig. 1, which shows that, in the presence of O₂ (i.e. in air) and of cytochrome *c* oxidase, cytochrome *c*_i is oxidized very slowly; the rate of the process can be enhanced by the addition of a small amount of soluble cytochrome *c* to the reaction mixture [cf. Fig. 1(c) respectively with Fig. 1(b) (no cytochrome *c*_n) and Fig. 1(d) (no cytochrome *c* oxidase)].

Soluble derivative (cytochrome *c*_s)

Preparation. The quantities (i) of soluble, but not diffusible sugars and (ii) of solubilized cytochrome *c* present in the reaction mixture after several additions of freshly prepared dextranase (see under 'Methods') are reported as a function of time in Fig. 2. Fresh enzyme was added at fixed time-intervals to prevent possible inhibition of the digestion with the progress

Fig. 1. Reaction with O_2 of cytochrome c_1 in the presence of cytochrome c oxidase and cytochrome c_n . A suspension of cytochrome c_1 in 0.1M-phosphate buffer, pH7.4, containing 1% Emasol was reduced by sodium dithionite (see under 'Methods') and placed in four identical cuvettes of 1 cm light-path (a-d). Solutions of cytochrome c_n (150 μ M) and cytochrome c oxidase (24 μ M) were prepared in the same buffer. Then 50 μ l of cytochrome c_n was added to (c) and (d) and 100 μ l of oxidase to (b) and (c), and the spectra were observed after 10 min.

of the reaction. It is clear that, under our experimental conditions, the solubilization of cytochrome c and the complete digestion of the polysaccharidic part of the complex occur at very different rates. Thus, although solubilization of cytochrome c was complete in about 5 min (Fig. 2, insert), to decrease the size of polysaccharides in solution the time of digestion was in all cases prolonged to 120 min. Purification of cytochrome c_r from non-covalently bound sugars was achieved by prolonged dialysis followed by column chromatography on CM-Sephadex C-50 (see under 'Methods'). Whereas after prolonged dialysis the average number of sugar monomers per cytochrome c molecule was approx. 2300, after this column purification the value fell drastically to approx. 75 sugar monomers/cytochrome c molecule (see below). Different preparations obtained by this procedure behave all alike.

Structural properties. Absorption spectra in the visible region, as well as shape and position of the shoulder centred at 695 nm, a sensitive test of crevice integrity in cytochrome c (Moore & Greenwood, 1975; Schejter & George, 1964), are practically identical for cytochrome c_n and cytochrome c_r .

From this it may be concluded that differences between the two do not reside in the immediate environment of the haem nor do they involve relevant changes in the folding of the polypeptide chain.

It is known that the coupling between cytochrome c and Sephadex G-200 involves, under our experimental conditions, the formation of covalent linkages between the primary amino groups on the

protein and the CNBr-activated hydroxylic groups on the resin. The most probable structure of the linkage is the following:

Thus they are not affected by the enzymic hydrolysis of the polysaccharide carrier.

The soluble complex obtained by enzymic digestion has a high content of sugars after chromatographic purification also. Analytical determinations carried out for three different preparations of cytochrome c_r give a weight ratio between the protein and the polysaccharide in the complex of approx. 1:1. From this, taking a value of 12000 for the mol.wt. of cytochrome c and of 162 for the mol.wt. of the sugar monomer, the molar ratio of protein to glucose was estimated to be 1:75.

To determine the number of the polysaccharide tails/molecule of cytochrome c_r , determination of the free amino groups present in cytochrome c_n and cytochrome c_r was carried out by the 2,4,6-trinitrobenzenesulphonic acid method of Habeeb (1966). The results of such measurements performed at many different protein concentrations are shown in Fig. 3. Assuming that all the 19 lysine residues present in cytochrome c_n are reactive (Wada & Okunuki, 1968), an absorption coefficient of 12000 litre·mol⁻¹·cm⁻¹ can be calculated for the trinitrophenyl-NH derivative. This compares favourably with the value of 11000 reported by Gibbons (1970). On this base the difference in free amino

Fig. 2. Digestion of the polysaccharide part of cytochrome c_1 and resolubilization of the cytochrome molecules to form cytochrome c_r .

Both the soluble polysaccharides and solubilized cytochrome c (insert) were determined on various samples taken at different times from the enzymic-digestion mixture. Each sample was centrifuged at 15000 rev./min and 4°C. The supernatant was dialysed overnight against water and then assayed for the total sugar content (see under 'Methods') or for the haem c absorption after addition of dithionite. Arrows indicate the addition of fresh dextranase to the reaction mixture.

groups between the two cytochromes is less than one group per molecule. This suggests that, within experimental error, only one amino group is blocked on average, i.e. the number of carbohydrate tails on cytochrome c_r may be one. Thus the average mol.wt. of cytochrome c_r should be approx. 24000 (1 molecule of cytochrome c + 75 molecules of glucose). This is consistent with the sedimentation coefficient $s_{20,w}$ of cytochrome c_r , which is 2.02S in 0.05M-sodium phosphate buffer, pH 7.4, i.e. higher than $s_{20,w} = 1.68S$ found for cytochrome c_n .

Functional properties. (a) Redox reactions with various reagents. Reactions with O_2 , ascorbate and ferrocyanide-ferricyanide of both cytochrome c_n and cytochrome c_r have been investigated in 0.1M-sodium phosphate buffer, pH 7.4.

(1) Reduction by ascorbate (see under 'Methods') was followed spectrophotometrically at 550 nm and

Fig. 3. Titrations of free amino groups on cytochrome c_n and cytochrome c_r .

The A_{335} was measured after reaction of 2,4,6-trinitrobenzenesulphonic acid with cytochrome c_n (○) and cytochrome c_r (Δ) at different protein concentrations (see under 'Methods'). Continuous and broken lines are the result of a least-squares calculation, with slopes of 0.229 and 0.222 respectively.

25°C. The observed process was clearly monophasic for both cytochrome c_n and cytochrome c_r , but in the latter case the half-time ($t_{1/2}$) for reduction (170S) was exactly double that of the former (85S).

(2) The redox potential of the two cytochromes was studied in parallel titrations of oxidized proteins (final concentrations 35 μM) with ferrocyanide (final concentrations 2.5–21 mM). From the equilibrium constants calculated at four different ferrocyanide-ferricyanide concentrations, it was found that the redox potential of the two cytochromes was essentially the same, the value of E'_0 for cytochrome c_r being between 12 mV and 3 mV lower than that of cytochrome c_n . The kinetics of electron transfer with the ferrocyanide-ferricyanide couple was investigated by temperature-jump experiments at different total ferrocyanide concentrations (18–32.8 mM) on two different preparations of cytochrome c_r (13 and 16 μM). The results on cytochrome c_r showed a behaviour either identical with, or similar to, that of cytochrome c_n .

(3) The time course of autoxidation in air for cytochrome c_n and cytochrome c_r after complete reduction by sodium dithionite (see under 'Methods') is reported in Fig. 4. The two proteins behave identically, whereas for the insoluble complex between cytochrome c and Sepharose 4B a much slower velocity of autoxidation compared with the native molecule was observed (Colosimo *et al.*, 1976).

(b) Reaction with O_2 catalysed by cytochrome c oxidase. (1) Steady-state data, in the form of a Lineweaver-Burk plot of initial velocities, are reported in Fig. 5. The number of molecules of

Fig. 4. Time course of autoxidation for cytochrome c_n and cytochrome c_r .

The percentage oxidation calculated from spectral changes at 550nm for cytochrome c_n (○) and cytochrome c_r (△) are plotted as functions of time. The buffer was 0.1M-sodium phosphate, pH7.4; protein concentrations were 68 μM for cytochrome c_n and 20 μM for cytochrome c_r . The temperature was 35°C.

Fig. 5. Lineweaver-Burk plot for initial velocity of oxidation of cytochrome c_n and cytochrome c_r , catalysed by cytochrome c oxidase

Initial reaction velocities were followed spectrophotometrically at 550nm in 0.1M-phosphate buffer (containing 1% Emasol), pH7.4, at 22°C, at different cytochrome c concentrations [S]. ○, Cytochrome c_n ; results are shown for cytochrome c_r before (▲) and after (△) polysaccharide purification. The concentration of cytochrome c oxidase was 0.15 μM .

cytochrome c_r oxidized/s per molecule of cytochrome c oxidase was 1.7, i.e. about one-half that found under the same conditions for cytochrome c_n , whereas the K_m (about 33 μM) is the same for the two proteins.

(2) Pre-steady-state experiments were performed in

parallel by mixing, in the stopped-flow apparatus, cytochrome c oxidase with (ascorbate) reduced cytochrome c_n and cytochrome c_r in the presence of air. The time course of the cytochrome c oxidation (followed at 550nm) and of reduction of oxidase (followed at 605nm) are reported in Fig. 6. The following facts are evident from examination of the data.

The 'burst' of cytochrome c oxidation (at 550nm) and of cytochrome a reduction (at 605nm) is considerably slower with cytochrome c_r than

Fig. 6. Time course of reaction between reduced cytochrome c_n and cytochrome c_r , and cytochrome c oxidase

Cytochrome c oxidase (5 μM) and 10 μM -cytochrome c_n (○) or cytochrome c_r (△), both reduced by 1 mM-ascorbic acid, were mixed in the stopped-flow apparatus and the change recorded in A_{550} (a) or A_{605} (b). The buffer was 0.1M-sodium phosphate (containing 1% Emasol), pH7.4, at 19°C.

with cytochrome c_n . The half-times are approx. 120 ms with cytochrome c_r and 40 ms with cytochrome c_n . This indicates directly that the apparent second-order rate constant for the electron transfer from cytochrome c to cytochrome a is slower when cytochrome c_r is used as an electron donor.

(ii) The number of electron equivalents introduced in the oxidase in the two cases over the same time interval (say 100 ms after mixing) is smaller for cytochrome c_r than for cytochrome c_n .

(iii) The kinetics of cytochrome c oxidation (at 550 nm) after the burst phase is slower for cytochrome c_r than for cytochrome c_n . This is consistent with the finding that, in steady-state experiments, $V_{\max}$ for cytochrome c_r is approximately one-half of that for cytochrome c_n (see Fig. 5).

(iv) Finally the observations at 605 nm over the longer time scale (Fig. 6b) indicate that after 1 s the oxidase is still fully reduced when mixed with cytochrome c_r , whereas it is already being re-oxidized when mixed with cytochrome c_n . This seems consistent with the lower rate of reduction of oxidase in the former case.

Discussion

Colosimo *et al.* (1976) reported that the insoluble complex between cytochrome c and Sepharose 4B is very slowly oxidized by O_2 in the presence of catalytic amounts of cytochrome c oxidase, unless small amounts of soluble cytochrome c are present. Furthermore the apparent rate constant for electron exchange between soluble and insoluble cytochrome c ($125 M^{-1} \cdot s^{-1}$) was two orders of magnitude lower than that reported in the literature for the homonuclear transfer of electrons in the native molecule (Kowalsky, 1965).

The structural origin of these differences in behaviour between cytochrome c_i and cytochrome c_n is, partially clarified in the present paper by the chemical and functional data on the relative influence of chemical modification of specific residues and diffusional effects.

Chemical determination of free amino groups in cytochrome c_n and cytochrome c_r indicates that on average only one amino group is involved in the binding of the polysaccharide tail. In this respect the situation should be identical in cytochrome c_i , since the enzymic digestion by dextranase does not affect protein-matrix bonds. This suggests that, of the 19 free amino groups on the surface of native cytochrome c and easily reactive towards acetic anhydride (Dickerson *et al.*, 1971; Wada & Okunuki, 1968), only one is preferentially coupled to the matrix. Thus it seems that, during coupling, cytochrome c is anchored to the insoluble gel, under our experimental conditions, through only one residue, and that modification is more specific than expected.

The sedimentation coefficient is also not inconsistent with the estimated molecular weight based on chemical measurement of total sugar content. Thus the model that may be proposed from the presently available data is that of a folded cytochrome c molecule to which possibly only one, very long, polysaccharide tail is attached.

The above results have shown that many of the functional properties of native cytochrome c (cytochrome c_n) are restored after resolubilization of the complex by enzymic digestion of the solid matrix. Thus the autooxidation of reduced cytochrome c , the reduction of oxidized cytochrome c by ascorbate and the electron-transfer kinetics with the ferrocyanide-ferricyanide couple, as well as the redox potential, are similar, if not identical, for cytochrome c_r and cytochrome c_n .

Among the above results, those that provide an interesting comparison between the three forms of cytochrome c (cytochrome c_n , cytochrome c_r and cytochrome c_i) are those involving cytochrome c oxidase. The steady-state kinetics, summarized in Fig. 5, clearly indicate that the chemical modification(s) of cytochrome c_r have an effect on the turnover number. This finding is in accord with the results of rapid-mixing experiments, which show that the second-order rate constant for electron transfer from cytochrome c to cytochrome a is smaller for cytochrome c_r than for cytochrome c_n , and that the electron equivalents entering into the oxidase in the burst phase from cytochrome c_r are less than from cytochrome c_n .

However, these differences, albeit significant, cannot possibly account for the inability of cytochrome c oxidase to oxidize the insolubilized cytochrome c (cytochrome c_i), which remains the striking observation reported by Colosimo *et al.* (1976). Thus it may be concluded that the functional behaviour of cytochrome c_i can best be explained by an overwhelming role of diffusional effects that inhibit a proper approach between the bound cytochrome c and its oxidase.

To find a complete and unifying rationale for the structural properties and the functional behaviour of the cytochrome c_r complex, it is of basic importance to get more information about the specificity of covalent linkage between the protein and the polysaccharide moieties.

We gratefully acknowledge the help of Dr. E. Chiancone and Dr. P. Vecchini with the ultracentrifugation experiments, and Dr. F. Martini for valuable discussions.

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