Chromatography of Oxidized and Reduced Cytochrome *c* on Carboxymethylcellulose

By H. B. F. DIXON AND C. M. THOMPSON

Department of Biochemistry, University of Cambridge, and Whatman Research Laboratories, Springfield Mill, Maidstone, Kent

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1. Cytochrome *c* was isolated from horse heart by a chromatographic method.
2. Oxidized and reduced cytochrome *c* were chromatographed on CM-cellulose that was in equilibrium with several buffer systems of constant composition at pH values of 8.4, 6.75 and 4.9.
3. Separation was better at the higher pH values; the oxidized form was retarded more than twice as much as the reduced form, though they differed by only a single charge.
4. Self-competition between cytochrome molecules is suggested to account for the peak distortion observed at high loads (above 20 μ M protein concentration).

Cytochrome *c* can be purified by chromatography in several systems (cf. Paléus & Neilands, 1950; Margoliash, 1954; Margoliash & Schejter, 1966). We describe its chromatography in constant-composition buffers on CM-cellulose for two reasons. One is that the difference of a single charge between the oxidized and reduced forms causes a more-than-twofold difference in partition coefficient. The second is that the system appears to provide a convenient method of studying the mechanism of ion-exchange chromatography of polyelectrolytes and comparing the performance of different types of CM-cellulose. A method of isolating cytochrome *c* from heart muscle is described, since it is based largely on the chromatographic system developed.

METHODS

Preparation of cytochrome c. Step 1: initial extraction (after Paléus, 1960). Horse heart muscle (3.3 kg. from one heart) was minced, stirred with water (2.5 ml./g.), and adjusted to pH 4.1 with 0.5 M-H₂SO₄ (about 250 ml.). After standing for 1 hr., the mince was filtered through muslin and briefly re-extracted with water (1 ml./g. of muscle). The combined filtrate was adjusted to pH 7 with 4 M-NH₃. After standing for 5 min., it was filtered through a fluted Whatman no. 54 paper (a hardened paper of high permeability). The paper had to be changed several times, but by taking liquid for filtration from the top of the vessel, after most of the precipitate had settled, the rate of blocking of the papers was greatly diminished.

Finely ground K₃Fe(CN)₆ was dissolved in the filtrate until the absorption bands of oxyhaemoglobin and reduced cytochrome *c* had disappeared. The oxidation could be carried out on portions of about 1 l., which could then be concentrated.

Step 2: concentration on a fibrous CM-cellulose column.

Whatman CM23 CM-cellulose was prepared for use by treatment with acid and alkali as follows. After suspension in about 20 ml. of 0.5 M-NaOH/g. and standing for 1 hr., the supernatant liquid was poured off and the exchanger resuspended in the same volume of 0.5 M-HCl. Since part of the acid was neutralized by the remaining NaOH, the supernatant liquid was poured off as soon as most of the particles had settled, and the volume restored with 0.5 M-HCl. The order of treatment was sometimes varied, so that the exchanger emerged from the treatment predominantly in the H⁺ form or predominantly in the Na⁺ form. It was then suspended in buffer approx. 50 mM in acetic acid and approx. 50 mM in sodium acetate, carefully mixed and allowed to settle. The settling was timed to allow all particles that moved at over 2 cm./min. to pack down and the supernatant liquid was poured or aspirated off to remove fines. This treatment was repeated until most of the particles that sedimented more slowly were rejected. It was important to carry out this particle-size fractionation in a volume of at least five times the packed volume, else the top of the suspension settled slowly with a sharp boundary. This was presumably because large particles could not sediment rapidly because of collisions with small particles.

The exchanger was suspended in enough buffer to give 1.5 times the final packed bed volume, poured into a column of 4 cm. diam. and packed under an air pressure of up to 4 lb./in.² to a height of 25 cm. It was then washed with about 1 column volume of water. Here and subsequently the column volume is the area of the tube $\times$ the height of packed bed. The heart-muscle extract was then run through the column under the same pressure, at a speed of about 600 ml./cm.²/hr., and the cytochrome could be seen to concentrate at the top. At first the methaemoglobin was also retained, but was eluted when the exchanger equilibrated with the (NH₄)₂SO₄ of the extract. Enough CM-cellulose should be used to leave the bottom 5 cm. clear of cytochrome.

The column was then washed with a solution 100 mM in ammonium acetate and 20 mM in acetic acid until the effluent

Fig. 1. Preparative chromatogram on microgranular CM-cellulose. The column was 48.8 cm. long $\times$ 3.35 cm.² cross-section. The column was equilibrated and developed with 48 mM- Na_2HPO_4 –48 mM- NaH_2PO_4 buffer containing 0.03% toluene. The load was 1.6 g. of crude material.

was Na^+ -free (by flame test). This required about 200 ml. of solution, and the cytochrome did not move appreciably during this wash. The column was then washed with 2 column volumes of water, and the cytochrome was finally displaced with M-NH_3 at a flow rate lowered to 120 ml./cm.²/hr. About 500 ml. was required, and the cytochrome was eluted in a volume of about 200 ml.

Step 3: drying. A few drops of octan-2-ol were added to the column eluate, which was at once concentrated by rotary evaporation at a bath temperature of 30° (the eluate remained below 20°). Evaporation was continued until the volume had been approximately halved, by which time most of the NH_3 had been removed. The remaining solution was freeze-dried to yield 3.3 g. of crude product.

Step 4: preparative chromatography on a microgranular CM-cellulose column (after Thompson, 1967; Thompson, Weaver & Orry, 1967). Whatman CM32 CM-cellulose was treated with acid and alkali and roughly equilibrated with acetate buffer as described above for fibrous CM-cellulose. Lumps were broken in dilute suspension by use of a Potter homogenizer and the fine particles were removed. The CM-cellulose was then suspended in the buffer to be used, 48 mM in Na_2HPO_4 and 48 mM in Na_2PO_4 , pH 6.75, containing 0.03% toluene, and allowed to settle, and the supernatant liquid was discarded. After several repetitions a slurry of about 1.5 volumes/wet settled volume was poured into a column of 2 cm. diam. and packed under an air pressure up to 4 lb./in.² until a bed of about 50 cm. height was obtained. The column was washed with the same buffer until the pH of the effluent was equal to that of the input buffer. A 1.6 g. sample of the dried crude cytochrome, dissolved in 15 ml. of buffer, was applied to the column and washed in with two 2 ml. portions of buffer. The column was eluted with buffer at a flow rate of 35 ml./cm.²/hr. The effluent fractions were analysed for protein and cytochrome by measuring their E_{280} and E_{410} values (after dilution where necessary). The course of the elution is shown in Fig. 1.

The method was also applied to commercial preparations of horse heart cytochrome *c* that were applied directly to a similar column. Equally good results were obtained. The equally satisfactory buffer 24 mM in $\text{Na}_4\text{P}_2\text{O}_7$ and 12 mM in HCl was used (see below) for some early preparative experiments.

Step 5: final concentration and drying. The fractions of column effluent containing cytochrome were pooled, acidified by the addition of 5% of their volume of acetic acid and concentrated on fibrous CM-cellulose as in step 2. When solutions to be concentrated contained over 150 mM- Na^+ ion, dilution to this concentration was necessary in addition to acidification. Particular care was taken to minimize the exposure to alkaline conditions by rapid removal of the NH_3 used for elution and allowing no delay before freezing. The yield was 0.37 g. (0.22 g./kg. of muscle).

Analytical chromatography. Columns of about 30 cm. $\times$ 1 cm. of microgranular CM-cellulose (Whatman CM32) were prepared and operated like the larger columns described for the preparation of the cytochrome. The CM-cellulose was roughly equilibrated before being poured with whatever buffer was to be used, and equilibration was completed by passage of buffer through the packed column. The sample was added in a volume of less than one-thirtieth of the volume of the packed bed. Elution was carried out at about 40 ml./cm.²/hr. Thus 4–5 hr. was normally sufficient for the running of columns when the peaks emerged at less than 5 column volumes. All the buffer solutions contained 0.03% toluene.

Preparation of sample mixtures of oxidized and reduced cytochrome *c*. The cytochrome *c* used was the oxidized freeze-dried material prepared as described by concentration of the main peak from either commercial cytochrome *c* or the material prepared from horse heart. A 50 mg. sample was dissolved in 0.5 ml. of a buffer 1 M in tris and 0.5 M in HCl, and 10 mg. of ascorbic acid was added. The reduced cytochrome *c* was separated from the tris buffer, unchanged ascorbic acid and low-molecular-weight reaction products by gel filtration on a 15 cm. $\times$ 1 cm. column of cross-linked dextran (Sephadex G-25, fine grade) that had been equilibrated with buffer 24 mM in $\text{Na}_4\text{P}_2\text{O}_7$ and 12 mM in HCl. The coloured fraction was collected in a weighed tube and made up to 2.5 ml. with the buffer. A further 50 mg. of the oxidized cytochrome *c* was dissolved in this sample, making it 20 mg./ml. in each form. Then 0.1 ml. of this concentrated solution was mixed with 0.9 ml. of the appropriate buffer before chromatographic analysis.

RESULTS

Chromatography of oxidized and reduced cytochrome *c*: comparison of buffer systems. Fig. 2 compares the elution patterns from columns run in (a) 300 mM-sodium acetate–200 mM-acetic acid buffer, pH 4.9, (b) 50 mM-disodium hydrogen phosphate–50 mM-sodium dihydrogen phosphate buffer, pH 6.75, and (c) 24 mM-tetrasodium pyrophosphate–12 mM-hydrochloric acid buffer, pH 8.4. Much sharper peaks were obtained in the second and third cases than in the first. As would be expected, the lower the pH, and hence the greater the positive charge on the cytochrome, the greater was the concentration of Na^+ ions needed to elute the protein in a given elution volume.

Table 1 gives the elution positions of oxidized and reduced cytochrome *c* in a number of buffer solutions at three pH values: a given fractional change in the concentration of Na^+ ions has more effect at lower

Fig. 2. Chromatograms of samples of 2mg. of oxidized and 2mg. of reduced horse cytochrome *c* on microgranular CM-cellulose. Columns were 31.5–33.5cm. long $\times$ 0.815cm.² cross-section. The columns were equilibrated and developed with the following buffers, each containing 0.03% toluene: (a) 300mM-sodium acetate–200mM-acetic acid; (b) 50mM- Na_2HPO_4 –50mM- NaH_2PO_4 ; (c) 24mM- $\text{Na}_4\text{P}_2\text{O}_7$ –12mM-HCl. Peak 1, reduced form; peak 2, oxidized form.

pH. This is expected, since Boardman & Partridge (1955) showed that the greater the valency of the solute the more effect a small change in the concentration of a univalent competing ion has on its partition coefficient.

Purity of cytochrome *c*. The cytochrome *c* prepared from horse heart as described possessed an E_{410}/E_{280} ratio 4.67. This ratio is 97% of that found by Margoliash & Frohwirt (1959). Rechromatography of the cytochrome *c* (Fig. 3) did not reveal the presence of significant impurities other than reduced cytochrome *c*. Rechromatography of the first and last thirds of the wide peak given in acetate buffer gave peaks in the same position as the centre of the original peak. Microheterogeneity is therefore not the cause of the peaks' widening in this system, unless different forms interconvert during concentration.

DISCUSSION

*Separation of oxidized and reduced cytochrome *c*.*

The horse cytochrome *c* molecule contains 19 lysine and 2 arginine residues, so that at pH 6.75 it will carry 21 positive charges in addition to the charge on the haem group and the 3 partly charged histidine residues. It is therefore noteworthy that change of a single charge affects the elution volume by so much.

If the volume of liquid in the bed of exchanger is subtracted from the elution volume of a peak, a volume proportional to the partition coefficient is obtained. If this volume to be subtracted is taken as 0.5 column volume (the observed position of the first protein peak, presumably unretarded, when crude extracts are chromatographed) the values of Table 1 give about a 3:1 ratio of the partition coefficients of the oxidized and reduced forms for pH 8.4 and 6.75. This corresponds with a difference in ΔG^0 for their adsorption of about 0.6 kcal./mole. This calculation shows that the separation, though apparently large, actually reflects only a small difference in ΔG^0 .

It would be unwise to ascribe the change in partition coefficient solely to the charge difference, in view of the evidence that the oxidized and reduced forms differ in conformation (cf. Margoliash & Schejter, 1966). Particularly noteworthy is the evidence of Rodkey & Ball (1950) that the oxidized form has an additional group of pK 7.8, so that appreciably above this pH the oxidized and reduced forms should not differ in charge. Theorell & Åkesson (1941) found, however, that the two forms differed on titration by 1equiv. up to pH 8 and that only at pH 9.6 did they carry the same charge, but this does confirm the effect of a conformation change on the difference in charge at high pH. In spite of these indications that the difference in chromatographic position may not in this case result solely from a unit difference in charge, it is probable that the difference of a single charge is sufficient to effect separation of the two forms of a cytochrome. Dixon & Moret (1965) found that the change in partition coefficient when the positive charge of a bacterial cytochrome was diminished by transamination of the *N*-terminal residue was similar to that found on reduction of the iron. Also, a single reduction in charge due to the replacement of a lysine residue by glycine in ox cytochrome *c* permits its separation from the horse protein on a carboxylic resin (Margoliash & Schejter, 1966). The consequent changes in conformation do not seem likely to be similar in all these cases.

Sharpness of peaks. The position of the peak of oxidized cytochrome *c* was adjusted to 4–5 column volumes in buffer solutions of constant composition at pH values from 4.9 to 8.4. This was done by adjusting the concentration of the Na^+ ions that

Table 1. *Positions of cytochrome c peaks eluted from CM-cellulose by different eluents*

Concentrations (mm)		No. of column volumes to elute cytochromes		[Na ⁺] (mm)	
Pyrophosphate system (pH 8.4)		Fe(II)	Fe(III)		
[Na ₄ P ₂ O ₇]	[HCl]				
24	12	1.8	4.3	96	
22	11	2.5	6.0	88	
20	10	3.7	10.2	80	
Orthophosphate system (pH 6.75)					
[Na ₂ HPO ₄]	[NaH ₂ PO ₄]				
50	50	2.1	4.3	150	
48	48	2.7	6.8	144	
45	45	3.5	9.5	135	
40	40	5.9	18	120	
Acetate system (pH 4.9)					
[CH ₃ ·CO ₂ Na]	[CH ₃ ·CO ₂ H]	[NaCl]			
30	20	200	*	9.8	230
30	20	240	*	4.1	270
30	20	250	*	3.4	280
30	20	270	*	2.2	300

* In some of these columns the reduced cytochrome *c* became oxidized during chromatography; in others it was not well resolved from the oxidized form.

Fig. 3. Comparison of chromatography at high and low loads. The column was 28.5 cm. long $\times$ 0.815 cm.² cross-section. The microgranular CM-cellulose column was equilibrated and developed with 50 mm-Na₂HPO₄-50 mm-NaH₂PO₄ buffer containing 0.03% toluene. (a) 50 mg. load; (b) 5 mg. load.

compete with the protein for the sites on the CM-cellulose. It was then apparent (Fig. 2) that the peaks of oxidized and reduced cytochrome *c* were wider at pH 4.9 than at the higher pH values. The resolution appears to be as good at the pK₂ of orthophosphate (Fig. 2b) as at the pK₄ of pyro-

phosphate (Fig. 2c), orthophosphate having the advantage of cheapness and availability at high purity. Calculation of the number of theoretical plates by the method of Stack-Dunne (cited by Dixon, 1962) showed over 2 plates/cm. in orthophosphate and less than half of this in the acid system.

The spreading of the peaks in the acid system (300 mm-sodium acetate-200 mm-acetic acid) was not due solely to the high concentrations of acetate ions and acetic acid, since the peaks remained wide in buffers of 30 mm-sodium acetate-20 mm-acetic acid when various concentrations of sodium chloride (Table 1) were used.

Lower resolution in the acid system is not shown for all proteins at acid pH, since 4 plates/cm. were obtained with the smaller, less basic, cytochrome *c*-551 from *Pseudomonas fluorescens* in 25 mm-sodium acetate-30 mm-acetic acid when the work of Dixon & Moret (1965) was repeated with this exchanger.

Since lower pH corresponds to increased positive charge on the protein, we suggest that the peaks are wider with horse cytochrome *c* at acid pH because the multivalent interactions between the protein and the exchanger at this pH equilibrate more slowly than do the interactions of lower valency of either the same cytochrome at higher pH or the less basic bacterial cytochrome at the same pH. The evidence for this suggestion is admittedly slight.

Chromatography at high loads. During the preparative work it was found that the peaks were

skew whenever E_{410} of the effluent exceeded about 2, i.e. when the concentration exceeded about 0.02mM or 0.2mg./ml. This effect is seen particularly clearly in Fig. 3, which shows the skewing of the high load (Fig. 3a) compared with the symmetrical peak obtained with the low load (Fig. 3b). The skewing is presumably due to the fact that a molecule of the cytochrome suffers competition for the sites of the exchanger that is significantly increased above that due to the Na^+ ions by the presence of other molecules of cytochrome. The peak therefore not only possesses a skew shape at high load, but also runs further forward (Fig. 3). All the peak positions recorded in Table 1 refer to the symmetrical peaks obtained at low loads.

Since there do not seem to be other substances in the crude extract that run just in front of the cytochrome, the skewing in the preparative column does not result in diminished purity of the product. A check on the E_{410}/E_{280} ratio ensures that major impurities are not included when the peak is pooled. But, because of the wider peaks at high loads, slightly less eluting conditions were used for the preparative columns (Fig. 1) than those that gave

the most convenient positions of peaks analytically (Fig. 2), i.e. 48mM rather than 50mM of each phosphate.

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