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On the Heterogeneity of Beef Heart Cytochrome c

IV. Isoelectric Fractionation by Electrolysis in a Natural pH-Gradient

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(1) The occurrence of multiple molecular forms (Cy I – Cy IV) of monomeric beef heart cytochrome c previously shown [1,2] was confirmed by the method of stationary electrolysis for isoelectric focusing of proteins. Their isoelectric points (pI) were determined at 4°C. In addition, subfraction Cy II (as defined by disc and moving-boundary electrophoresis) was resolved into two components (Cy II_1 and Cy II_2) which differ only by 0.03–0.04 pH units in their pI.

(2) The resolution of Cy II_1 and Cy II_2 agrees well with the resolution of Cy II previously obtained by chromatography on cation-exchange resin (Duolite CS-101). [1] Thus, additional evidence is given in support of the view [8] that Cy II statistically should be composed of isomers that possess the same number of amide groups, but with one of them positioned on different carboxyl groups.

(3) The limitations of the method of stationary electrolysis for isoelectric focusing and pI measurements of very basic proteins are discussed.

In previous papers of this series [1,2] it was shown that beef heart cytochrome c* is resolved into four molecular forms by means of disc electrophoresis on polyacrylamide gel [1] and moving-boundary electrophoresis;[2] the subfractions were named, in order of their decreasing mobility towards the cathode, Cy I, Cy II, Cy III, and Cy IV. On the other hand, five components were consistently observed by chromatography on a weakly acidic cation exchange resin of the carboxylic acid type, *i.e.* Duolite CS-101,[1] but two of these fractions (denoted R-II and R-III) revealed the same mobility on disc electrophoresis, *i.e.* that of Cy II.[1] In order to get a clearer idea of this discrepancy in resolved subfractions, the method of isoelectric separation and focusing by electrolysis in natural, stable pH gradients [3,4] was tried in the present study. The working principle of this method is to focus the proteins at their respective isoelectric points (pI) in an ampholyte medium at low ionic strength. In addition to the

* The following abbreviation will be used: Cyt. c = cytochrome c.

separation thus obtained, the method permits determination of pI of proteins simply by measuring the pH where the proteins are focused. So far, the method has only been applied to proteins of pI < 7.8, and it was, therefore, also of particular interest to study the behaviour of a more basic protein such as cyt. c.

MATERIALS AND METHODS

Cytochrome c, extracted from beef heart muscle by dilute sulphuric acid at pH 4, was obtained in the monomeric form by gel filtration on Sephadex G-75.[5]

Chemicals. The carrier ampholytes consisted of a mixture of low-molecular aliphatic poly-amino-poly-carboxylic acids with different isoelectric points, which were synthesized as described by Vesterberg. [6,*] Sucrose was obtained from Mallinckrodt Chemical Works, St. Louis, U.S.A. Other chemicals used were of analytical grade.

Preparation and operation of electrolysis column. The electrolysis column ** (110 ml capacity) used was essentially as described by Vesterberg and Svensson.[4] The column was siliconized with a 0.1 % (v/v) aqueous solution of Dow Corning Z-4141 (from Dow Corning, Midland, Michigan, U.S.A.) to give a non-wettable surface in order to minimize tailing of the cyt. c zones while draining the column. An almost linear density gradient of sucrose in an aqueous mixture of carrier ampholytes was prepared as described by Vesterberg and Svensson.[4] The following sucrose gradients were used: 0–60 % (w/v), 0–50 % (w/v), 0–40 % (w/v), and 20–60 % (w/v). The following carrier ampholytes were selected for use in the present study: components with pI's 4.5, 6.5, 7.5, and 8.5 (0.04 g of each), components isoelectric between pH 9.5 and 10.6 (0.8 g) and arginine (0.15 g), which gave a final ampholyte concentration of approx. 1 % (w/v). In order to prevent anode oxidation of the carrier ampholytes 0.05 ml of conc. phosphoric acid was applied at the anode. At the cathode, the following bases were applied: ethylendiamine (0.05 g), tris (0.1 g), and morpholin (0.1 g).

Before its application to the column,[4] the cyt. c preparation was fully reduced by a minimum amount of sodium dithionite,[2] unless otherwise stated. The cyt. c solution was then dialyzed (4°C) free of salts against 1 mM ammonia and finally against a 0.5 % (w/v) solution of carrier ampholytes of pH 8.5.

The hemoprotein was focused at a maximum load of about 1 W with a final potential gradient of about 600 V. The final steady state [4] was reached after 48–72 h. There was no impairment of the focusing of the zones when running the electrolysis for a longer time.

When the electrolysis was completed, the column was immediately drained.[4] Fractions of approx. 0.6 ml were collected under a slow stream of argon in order to prevent absorption of carbon dioxide from the atmosphere.

Disc electrophoresis on polyacrylamide gel was carried out as previously described.[1]

pH measurements. A glass electrode pH-meter – Radiometer, Copenhagen, Denmark, model 25 SE – was used. The glass electrode – Radiometer type GK 2021 C – was standardized against a Beckman standard buffer (pH = 9.40 at 4°C). The pH values of the collected fractions were determined at exactly the same temperature as that applied to the column during focusing, *i.e.* 4.0°C.

Spectrophotometry. The concentration of cyt. c was assayed as previously described; [5] the specific extinction coefficient $E^{1\,\%}_{1\,cm}$ at 550 mμ (red) = 23.94 was used. The percentage of reduced (oxidized) cyt. c was calculated from A_{550} and $A_{550\,red}$ using the ratio $E_{550\,red}/E_{550\,ox} = 3.63$.[5]

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* Available from LKB-Produkter AB, Stockholm-Bromma, Sweden.

** Purchased from Ingenjörsfirman Consulta, Sköndalsvägen 106, Stockholm-Farsta, Sweden. Now available from LKB-Produkter AB, Stockholm-Bromma, Sweden.

RESULTS

The cyt. c preparation used was obtained in the monomeric form and revealed four components (Cy I—Cy IV) on disc electrophoresis on polyacrylamide gel.[1]

The factors influencing the isoelectric separation and focusing of proteins by electrolysis in natural, stable pH gradients have been discussed by Svensson,[3,7] and Vesterberg and Svensson;[4] therefore only the main problems concerned with the present study will be dealt with.

(a) Properties of the pH gradient. The ampholytes were selected to give a rather even distribution of the field strength between anode and cathode during electrolysis. The pH gradient thus obtained is shown in Fig. 1.

(b) The standard procedure. Fig. 1 shows the result of a representative run where the hemoprotein was separated into three distinct peaks which correspond to definite zones on the column. When the pooled peak fractions were

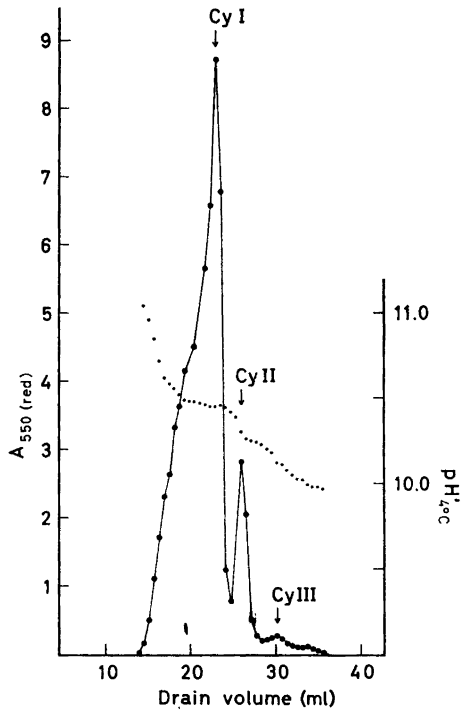

Fig. 1. Diagram showing the concentration of cytochrome c (— ● —) and pH at 4.0°C (●) in the individual fractions (approx. 0.6 ml) run out from the column. The subfractions Cy I — Cy III are indicated by arrows. Approx. 30 mg cyt. c was applied to the column, and the sucrose gradient used was 0—50% (w/v).

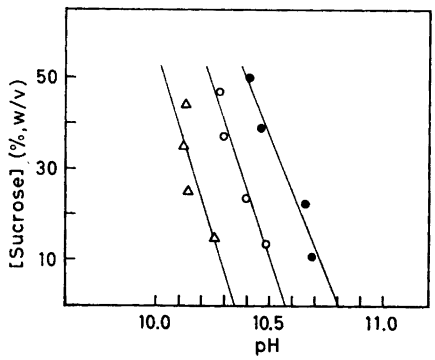

Fig. 2. Effect of sucrose concentration on pI′ of Cy 1 (●), Cy II (○), and Cy III (△). The straight lines were calculated by the least square method.

analyzed by disc electrophoresis, the peaks were found to correspond to Cy I, Cy II, and Cy III, respectively. However, complete separation of these subfractions was not obtained; *i.e.* Cy I (peak I) was slightly contaminated by Cy II (peak II) etc. The smallest subfraction Cy IV was not clearly visible on the column, but was revealed by spectrophotometric measurements on the fractions run out from the column (Fig. 1).

(c) Effect of the sucrose gradient on pI values. A prerequisite for the present technique of isoelectric fractionation is the use of a density gradient of a non-ionic solute in order to prevent thermal convection and to stabilize the protein zones. In the present study sucrose was used. Because sucrose reduces the dielectric constant of aqueous media, a dependence of pI on the sucrose concentration was expected for the very basic protein in this study. Experimentally this effect was investigated by first using the electrode at the top of the column as the anode and then repeating the experiment with the anode at the bottom. The sucrose gradient was also varied as described in a previous section. By making the assumption that a linear density gradient would be obtained in either case, the sucrose concentration at the different protein zones were calculated from their positions in the column. From Fig. 2 it is seen that the higher the sucrose concentration at the focused protein zones the lower their pI′ values. The pI′ values obtained by linear extrapolation to zero sucrose concentration are shown in Table 1.

Table 1. The apparent isoelectric points of subfractions Cy I — Cy III in the ferrous form.

Procedure	T (°C)	Isoelectric points (pI′)		
		Cy I	Cy II[c]	Cy III
Isoelectric fractionation[a]	4	10.80	10.57	10.35
Moving-boundary electrophoresis[b]	0	10.80	10.60	10.22

[a] Extrapolated values (see Fig. 2).
[b] Borate buffer, $\mu \approx 0.1$. Ref. 2.
[c] *i.e.* the main component Cy II_1 (see text).

(d) Resolution of subfraction Cy II into two components. Subfraction Cy II always revealed a double band on the column at the time of steady state. In order to prove the identity of both zones with Cy II as obtained in disc electrophoresis 4 mg of pure Cy II were prepared by this method (Fig. 3, a). When subjected to isoelectric fractionation (Fig. 4) Cy II is resolved into a major and a minor component which will be designated herein as Cy II_1 and Cy II_2, respectively. The exact pI of Cy II_2 was, however, impossible to obtain since this component could not be identified as a definite peak by spectrophotometric measurements on the fractions run out from the column. However, the

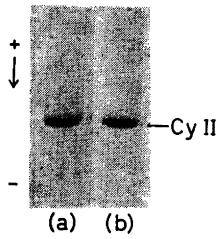

Fig. 3. Photograph of disc electrophoresis pattern of Cy II before (*a*) and after (*b*) electrolysis in a natural pH-gradient (shown in Fig. 4).

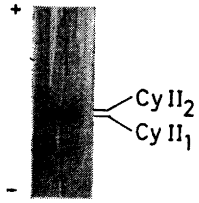

Fig. 4. Photograph of electrolysis column showing the resolution of Cy II (as defined by disc electrophoresis) into two components, Cy II_1 and Cy II_2.

difference in pI′ between Cy II_1 and Cy II_2 was calculated from the pH-gradient in this region to be only 0.03—0.04 pH units. When all the fractions run out from the column were pooled, the hemoprotein moved as a single fraction with the original mobility in disc electrophoresis (Fig. 3, b).

(e) Effect of the state of oxidation-reduction of cytochrome c. When ferri-cyt. c (ferricyanide oxidized) [2] was applied on the column, a gradual reduction was observed. Thus, at the time of steady state (72 h), Cy II and Cy III were found in the fully reduced form, whereas Cy I was reduced to about 70 %. In the standard procedure cyt. c was reduced by means of dithionite, and in this case the hemoprotein also was fully reduced at the end of the electrolysis.

(f) Stability of cytochrome c. The step-wise deamidation of Cy I, and its conversion to subfraction Cy II — Cy IV, has been studied in a previous paper.[8] From the pH-dependence of these reactions at 4°C, a conversion of Cy I was also to be expected in stationary electrolysis due to the high pI of cyt. c. This was actually the case. Thus, when Cy I, as obtained by preparative disc electrophoresis, was subjected to electrolysis for 72 h, a gradual conversion to Cy II and Cy III was observed (Fig. 5); preparative disc electrophoresis revealed

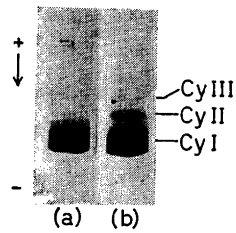

Fig. 5. Photograph of disc electrophoresis patterns showing the conversion of Cy I (*a*) into the subfractions Cy II — Cy III (*b*) during electrolysis in a natural pH-gradient for 72 h; 4.0°C.

their relative percentages as: Cy I 95.3 %, Cy II 4.3 %, and Cy III 0.4 %. The pseudo first-order rate constant for the conversion of Cy I → Cy II was calculated to be $k_1 = 2.1 \times 10^{-7}$ (sec^{-1}).

DISCUSSION

During the development of the technique of isoelectric fractionation it has been shown [4] that the high resolving power of the method makes it possible to obtain a complete separation of proteins which differ only by 0.06 pH units in their pI. In their study, Vesterberg and Svensson [4] calculated a resolving power of 0.02 pH units. Thus, as expected from the moving-boundary electrophoresis experiments,[2] the subfractions of beef heart cyt. c were resolved into four distinct zones by isoelectric focusing. Due to the rapid diffusion of cyt. c molecules (mol. wt. 12 130)[2] when the current was switched off, and partly due to tailing of the zones during the draining procedure, no complete separation of the components was obtained by running out the column. The asymmetry of the main peak (Cy I) in Fig. 1 is in accord with the appearance of the corresponding zone during focusing on the column. Thus, the zone was sharply focused at the anodic side, but showed a picture of droplet sedimentation [3] at the cathodic side due to the high protein concentration of the zone. This effect, however, did not make any trouble either for the separation or for the pI determination of this zone.

Detailed comparison and discussion of the nature of the differences between the cyt. c subfractions have been made elsewhere[1,2,8] and only one point needs to be further emphasized here. Thus, the results obtained confirm those previously found by chromatography on Duolite CS-101,[1] *i.e.* that fraction Cy II (as defined by disc and moving-boundary electrophoresis) is resolved into two components, denoted Cy II_1 and Cy II_2, by the present method; the percentage of Cy II_1 being $\gg$ that of Cy II_2. From previous physico-chemical studies of the main subfractions,[2] as well as from studies of the kinetics of the step-wise conversion of Cy I to Cy II — Cy IV,[8] this result is not unexpected. Thus, to explain the heterogeneity of monomeric beef heart cyt. c on the basis of a difference in amide content, three labile or accessible amides need to be present in Cy I.[8] Hence, statistically one should expect that Cy II is a mixture of isomers containing one of the total 7 amide groups on different carboxyls.[8] The resolution of CyII_1 and Cy II_2 obtained by stationary electrolysis indicates that there is a difference in pI of 0.03—0.04 pH units between these variants.

Two main difficulties are encountered when the present technique of isoelectric fractionation is applied to the very basic protein cyt. c. The first is the fact that the presence of a high concentration of a non-ionic solute (sucrose) in the pH gradient will decrease the dielectric constant of water.[9] This causes the pK's of the protein amino groups to shift,[10] and, consequently, the pI of the cyt. c subfractions to change also. Though no linear relationship exists between the dielectric constant and the sucrose concentration, a linear plot has been used between the latter and pI in Fig. 2 in order to be able to extrapolate from the observed pI values to zero sucrose concentration. The values thus obtained are in good agreement with those previously found by moving-boundary electrophoresis in borate buffer, $\mu \approx 0.1$ at 0°C (Table 1). Thus, the discrepancy in pI values obtained for hemoglobin [3] and myoglobin [4] by comparing these two analytical methods, is not observed in the case of cyt. c. This fact can be explained by a negligible binding of anions to cyt. c at its high

isoelectric point as compared to the situation for hemoglobin and myoglobin in moving-boundary electrophoresis.

Another problem is the fact that a certain amount of each subfraction initially present in the cyt. c preparation undergoes deamidation at the pH values of their high isoelectric points.[8] This effect results in a conversion of Cy I to Cy II and of Cy II to Cy III, and apparently the rate of conversion is slightly greater ($k_1 = 2.1 \times 10^{-7}$ sec^{-1}) than is to be expected from previous results obtained in borate buffer of almost the same pH, ionic strength and temperature ($k_1 = 1.1 \times 10^{-7}$ sec^{-1}).[8]

Finally, the reduction of ferri-cyt. c observed in the method of stationary electrolysis is worth mentioning. This result was to be expected inasmuch as there is a slow autoreduction of this hemoprotein in alkaline solution (below pH 12),[11] which proceeds at a faster rate during different electrophoretic procedures.[1,2,12]

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