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EVIDENCE FOR ENDOGENOUS PROTEOLYTIC SOLUBILIZATION OF HUMAN RED-CELL MEMBRANE NADH-CYTOCHROME b_5 REDUCTASE

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1. Introduction

We have presented evidence that in human red blood cells (RBC) NADH-cytochrome b_5 reductase (EC 1.6.2.2.) is not only soluble, but also tightly bound to the inner face of the membrane [1]. The membrane-bound enzyme is: (i) unmasked by Triton X-100 treatment; and (ii) solubilized by cathepsin D treatment. A structural relationship between the membrane-bound enzyme and the native soluble enzyme has been established on immunologic and genetic evidence [1]. On those grounds we have postulated that the soluble cytochrome b_5 reductase of the human RBC, already known to play a major role in methemoglobin reduction, derives from the membrane-bound entity through proteolytic cleavage.

Here we show that, indeed, the human red-cell membrane contains a proteolytic activity which transforms the membrane-bound form into the soluble one. By high-speed gel permeation chromatography (GPC) on TSK 250 gel, we compared the molecular weight (or M_r) of:

(i) The native membrane-bound enzyme treated by Triton X-100;
(ii) The spontaneously soluble form;
(iii) The soluble form released by cathepsin D treatment of the membrane;
(iv) The soluble form released by endogenous membrane protease(s).

We found that the membrane bound enzyme was M_r 45 000 whereas the spontaneously soluble enzyme was M_r 29 000. This value was similar to that obtained after solubilization of the membrane bound enzyme by cathepsin D treatment and by endogenous autolysis.

2. Material and methods

2.1. *Cytochrome* b$_5$ *reductase preparation from human RBC*

(i) *Spontaneously soluble enzyme* The red cell soluble cytochrome b_5 reductase was partially purified as in [2].

(ii) *Membrane-bound enzyme* The RBC membranes were prepared according to [3]. The ghosts were suspended in a 10 mM Tris–HCl buffer (pH 7.4) containing 2% Triton X-100. After 30 min incubation at 4°C the suspension was frozen and thawed 3 times and centrifuged for 10 min (105 000 × g) at 4°C (Airfuge Beckman). The supernatant was collected for further analysis.

(iii) *Cathepsin D-released enzyme* The membranes were incubated for 2 h at 37°C in 0.1 M Tris–maleate buffer (pH 5.6) in the presence of cathepsin D, Sigma (5 μg/100 μg membrane proteins). The solubilized enzyme was studied in the 105 000 × g supernatant obtained as above.

(iv) *Enzyme solubilized by endogenous membrane protease activity* The membrane suspension was incubated for 2 h at 37°C in Tris–maleate 0.1 M (pH 5.6) buffer in the presence of 1% Triton X-100. After incubation the 105 000 × g supernatant was analysed.

2.2. *Enzyme assays*

NADH-cytochrome b_5 reductase activity was assayed according to [4], in which the rate of reduction of a ferrocyanide–methemoglobin complex is followed.

2.3. M$_r$ *determination*

Gel permeation chromatography (GPC) was per-

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formed on a Chromatem 38 (Touzart et Matignon, Ivry) liquid chromatograph equipped with a 300 X 7.5 mm Bio Silk TSK 250 column (Biorad). The eluent was Tris–HCl 0.05 M, EDTA 0.001 M (pH 7.4) with or without 1% Triton X-100. The flow rate was 0.7 ml/min. The column was calibrated by: cytochrome *c* (M_r 12 500); α-chymotrypsinogen A (M_r 25 000); ovalbumin (M_r 45 000); and bovine serum albumin (M_r 68 000).

Each marker protein (5 μg in 5–20 μl) was applied onto the column. Detection was performed at 280 nm (Altex). For the analysis of the different samples containing cytochrome b_5 reductase, 10 μg protein was applied onto the column. Fractions of 350 μl were collected each 30 s and the enzymatic activity was determined on aliquots of 100 μl [4].

3. Results

GPC analyses were done to compare the M_r of the spontaneously soluble, solubilized and membrane-bound red cell cytochrome b_5 reductase.

The calibration of the column eluted with buffer with or without 1% Triton X-100 gave identical results, using the above markers. Over of 45–12.5 X 10^3 M_r the elution volume was linearly correlated to log M_r (fig.1).

The results obtained with the different enzymatic species were the following (fig.2A):

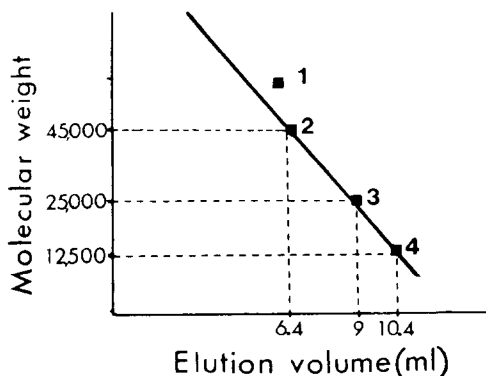

Fig.1. M_r calibration of Bio Silk TSK 250 column: Elution buffer was 50 mM Tris–HCl, 1 mM EDTA (pH 7.4) without or with Triton X-100 1% final. The flow rate was 0.7 ml/min and the pressure 15 bars. Each protein (5 μg) was applied onto the column. The protein concentration in the effluent was monitored at 280 nm. (1) Bovine serum albumin (68 000); (2) ovalbumin (45 000); (3) α-chymotrypsinogen (25 000); (4) cytochrome *c* (12 500).

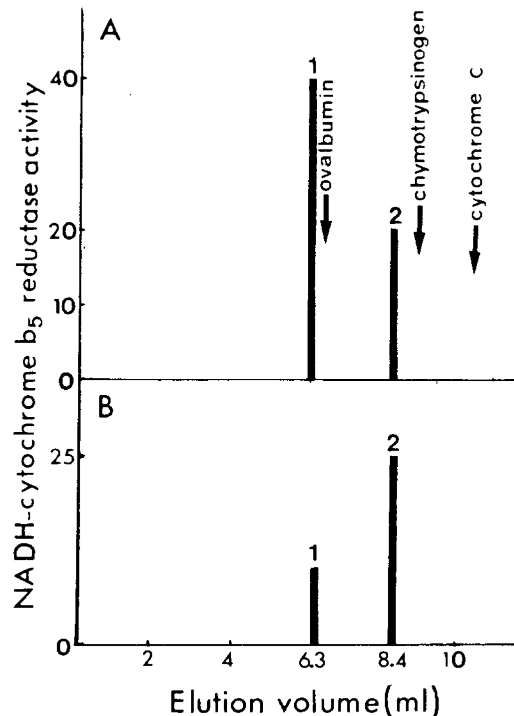

Fig.2. Gel permeation chromatography of the different samples on a Bio Silk TSK 250 column. Experimental conditions are in fig.1. (A) (1) Membranes treated by Triton X-100 2% final; (2) cathepsin D released enzyme, and semi-purified spontaneously soluble cytochrome b_5 reductase. Of each sample 20 μl (~10 μg protein) were applied to the column. The detection of the enzyme was performed by enzymatic activity assay in each fraction [4]. Results were expressed as $A \cdot \text{min}^{-1} \cdot 100\ \mu\text{l}^{-1} \times 10^2$. (B) Membranes treated by Triton X-100 1% final and incubated 2 h at 37°C at pH 5.6: (1) residual unmodified enzyme; (2) solubilized enzyme. Of suspension 20 μl (~10 μg protein) were applied on the column. Enzymatic activities were expressed as $A \cdot \text{min}^{-1} \cdot 100\ \mu\text{l}^{-1} \times 10^2$.

(i) The Triton X-100-treated membrane enzyme was the larger species eluted at a volume corresponding to M_r 45 000.

This result is in good agreement with an experiment in which we had determined a M_r of 44 000 (unpublished) by ultracentrifugation in a 5–20% sucrose gradient according to Martin and Ames.

(ii) The soluble semi-purified enzyme was demonstrated to be smaller than the membrane-associated form.

By GPC we found M_r 29 000. After treatment of the ghosts by cathepsin D, the activity was eluted at the same volume (8.4 ml). Experiments in which the soluble and the cathepsin D-solubilized enzyme were

mixed and applied together on the column only demonstrate a single peak containing the enzymatic activity.

The analysis of the phenomenon of spontaneous release of a soluble species from the red cell membrane upon incubation at 37°C is shown in fig.2B. Two distincts peaks containing enzyme activity were observed. The first one corresponded to the residual unmodified membrane species (M_r 45 000) while the second showed a M_r identical to that of the soluble forms above (29 000).

4. Discussion

Since cathepsin D treatment of the red cell membrane results in the release of a soluble and active species, we had postulated, that in vivo, the spontaneously soluble enzyme might be produced by endogenous proteolysis of the amphipathic molecule [1]. These results reported support that hypothesis.

4.1. *Methodologic interest of gel permeation chromatography*

This study shows the great interest of the gel permeation chromatography method. This method is very rapid and requires very small quantities of material of the same order as those used in SDS–PAGE. Gel permeation chromatography offers the additional advantage to allow the recovery of the enzyme in a native form, therefore detectable by its activity. The use of Triton X-100 does not modify the elution pattern of the markers and the samples.

4.2. M_r *value*

The M_r of 44–47 × 10^3 is in agreement with that in [5] for calf liver microsomes, and [6] for rabbit liver microsomes. However, it is significantly higher than the value of 33 000 observed in [7] on a highly purified fraction devoid of detergent.

Solubilization of the membrane-bound enzyme by either cathepsin D or autoincubation at 37°C in the presence of Triton X-100 yielded species of the same M_r 29–30 × 10^3. This value is in good agreement with data from the literature concerning the proteolytic cleavage of the amphipathic liver cytochrome b_5 reductase by lysosome or cathepsin D in various animals [5–9].

Interestingly, the phenomenon of spontaneous proteolysis of the red cell membrane-bound enzyme does not occur in the absence of Triton X-100. The role of the detergent is not known. It might activate a membrane protease or/and unfold the cytochrome b_5 reductase and render it more accessible to endogenous proteolysis. In [10] Triton X-100 solubilized and activated red-cell membrane protease(s). The supernatant of red cell membranes treated by Triton X-100 2% final, contains 70% of the total proteolytic activity [10].

There is no indication that the point of cleavage by endogenous or exogenous (cathepsin D) proteolytic attack is rigorously identical. Further studies are required to clarify the break point produced between the two domains by the endogenous RBC membrane protease(s) [5–7].

The question concerning the proteolytic processing of the membrane cytochrome b_5 reductase during the life-time of the red cell is very important. At which precise step of differentiation this occurs remains to be determined. The soluble cytochrome b_5 reductase is reduced by 50% in cord blood and new-born red cells [11] whereas their membrane-bound activity is normal (unpublished). It would be interesting to test whether this is due to a decreased membrane proteolytic activity of the cord blood red-cells. This is now under investigation.

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