Biochimica et Biophysica Acta, 471 (1977) 118—124

BBA 77844

TRANSFERRIN AND IRON UPTAKE BY RABBIT RETICULOCYTES

T.T. LOH, Y.G. YEUNG and D. YEUNG

Department of Physiology and Department of Biochemistry, Faculty of Medicine, University of Hong Kong (Hong Kong)

(Received May 3rd, 1977)

Summary

Whether, during iron uptake by rabbit reticulocytes, transferrin-iron molecules penetrate the membrane to the cell interior before release of iron has been investigated. Uptake of ^{125}I-labelled transferrin and ^{59}Fe by the stroma and cytoplasm fractions from reticulocyte haemolysates does not suggest that the protein accompanies iron into the cell. The greater part (75%) of the ^{125}I-labelled transferrin remained on the membrane while iron concentration in the cytoplasm fraction increased linearly with time during incubation. The 25% of ^{125}I-labelled transferrin in the supernatant may have been derived from contaminated lighter membrane fragments, since approximately the same proportion of membrane fragments as shown by acetylcholinesterase activity was found in the supernatant.

When ^{59}Fe-labelled transferrin-Sepharose was incubated with reticulocytes and cells and then lyzed, ^{59}Fe activity in the supernatant increased with the time and increasing reticulocyte count; yet no trace of ^{125}I-labelled transferrin appeared in this fraction when reticulocytes were incubated with ^{125}I-labelled transferrin-Sepharose. Due to the size of Sepharose (particle size, 40—190 μm) and its covalent binding to transferrin, it is unlikely that transferrin-Sepharose could have penetrated deeply into the cell either by endocytosis or microtubular mechanisms. Our results suggest that transferrin molecules do not need to penetrate the membrane and reach the cell interior before releasing iron.

Introduction

The mammalian reticulocyte is well known to remove iron from transferrin and to incorporate it into newly synthesized haemoglobin. During this process

Address correspondence to: Dr. T.T. Loh, Department of Physiology, Faculty of Medicine, University of Hong Kong, Hong Kong.

transferrin molecules are taken up by the cell, presumably becoming bound to specific receptors on the cell membrane. The precise mechanism whereby transferrin-bound iron is taken up by proerythroid cells remains unresolved. Morgan and his co-workers suggest that transferrin is first adsorbed to specific membrane receptors [1] followed by a slower, temperature-sensitive stage of transferrin uptake [2]. It is in this second stage that the transferrin-iron molecules diffuse well into or through the reticulocyte plasma membrane to mitochondria probably via surface endocytosis [3] or microtubular mechanism [4]. This hypothesis is supported further by chromatographic evidence that the reticulocyte cytosol haemolysate exhibits a transferrin-like peak [5]. On the other hand, others believe that iron is removed from transferrin while the latter is attached to the specific receptor site. The protein itself does not penetrate into the cell interior [6,7]. Iron may then combine with a membrane iron-carrier [8] and subsequently become mobilized from the stroma by an additional intracellular iron-binding protein [9]. The present work was undertaken to attempt to clarify the course of events, i.e. whether combination with transferrin is a requisite for iron to enter the cell milieu.

Materials and Methods

Proteins and protein-labelling

Rabbit serum transferrin was prepared using ammonium sulfate fractionation and column chromatography on DEAE-Sephadex (Pharmacia, Uppsala, Sweden) and crystallization from aqueous solution as previously described [10,11]. Its purity was checked with electrophoretic studies. Apotransferrin, the iron-free derivative of transferrin, was prepared by dialyzing the iron-saturated protein successively against two changes of 0.02 M disodium ethylenediaminetetraacetate (EDTA) in 0.05 M sodium acetate buffer (pH 5.0) and distilled water, whereafter transferrin was again dialyzed and equilibrated against two changes of phosphate-buffered saline, pH 7.4. When the protein was labelled with ^{59}Fe, care was taken to ensure the radioactive iron was bound only by the specific binding sites of the transferrin. Radioactive ^{59}Fe was first chelated to nitrilotriacetic acid before the iron was donated to the transferrin. This was done by adding a sufficient quantity of the 1 : 2 complex of $^{59}Fe(III) \cdot$ nitrilotriacetate, at pH 5.0, to apoprotein at pH 4.7 to achieve 50% saturation of the iron-binding sites, as described by Aisen and Leibman [12]. In order to assure that the protein complex was entirely in the bicarbonate form, 50 μl of 1 M $NaHCO_3$ was added to 2 ml of solution containing 20 mg of protein. The mixture was then incubated at 37°C for 1 h. After incubation, it was again dialyzed against two changes of phosphate buffered saline. It could be demonstrated that more than 90% of the ^{59}Fe was transferrin-bound, as shown by electrophoresis on cellulose acetate. The concentration of iron in the transferrin and its total iron binding capacity were determined [13]. The specific activity of the iron in the final solution was about 0.2 Ci/g iron. The transferrin was subsequently labelled with ^{125}I ($Na^{125}I$, carrier-free, Amersham, England) by the Chloramine-T method [14]. Rabbit γ-globulin was obtained from Sigma Chemical Co., St. Louis, U.S.A. and labelled with ^{131}I as described.

Reticulocytes

Reticulocytosis was induced in adult rabbits either by repeated bleeding from the marginal ear vein or by phenylhydrazine injection (25 mg/kg). The reticulocyte count obtained from the first method varied from 15 to 35%. Phenylhydrazine-treated rabbits yielded reticulocyte counts up to 90%. The cells were washed three times at 4°C with phosphate-buffered saline and then suspended in Hanks and Wallace solution to yield an haematocrit of approximately 50%. No attempt was made to separate reticulocytes from mature erythrocytes as it has been demonstrated adequately that uptake of transferrin-iron by erythrocytes is practically nil [6].

Incubation

Washed reticulocytes were incubated with the labelled proteins for selected period of time. The methods are described elsewhere [11,15]. The cells were then washed three times with ice-cold phosphate buffered saline. They were lyzed for 10 min with 2 ml of 5 mM Tris · HCl buffer, pH 7.5 containing 1 mM trisodium citrate. The membrane was then separated from the cytoplasm by centrifugation (20 000 × *g* for 20 min), as described by Borova et al. [16]. The radioactivity in the aliquots of the two fractions, i.e. stroma and supernatant, were assayed for radioactivity in a well-type gamma detector.

Coupling of transferrin to sepharose

20 mg of rabbit transferrin which had been labelled with ^{59}Fe, as described before, were coupled to 2 g freeze-dried CN Br-activated Sepharose 4B [17]. Small Sepharose particules of less than 10 μm diameter were removed by filtering the suspension of Sepharose through a Pharmacia nylon net (pore size 10 μm). To aliquots of 400 μl of washed cells, were added 200 μl of the ice-cold ^{59}Fe-labelled transferrin-sepharose (about 2 mg protein/ml). They were allocated for incubation at 37°C for 0, 30 and 60 min in each series of measurements. For the 0 min preparation, cells and transferrin-Sepharose were mixed and filtered quickly through a Pharmacia nylon net (pore size 10 μm) to remove transferrin-Sepharose particles. The other aliquots were incubated with shaking at 37°C for either 30 min or 60 min. At the end of each incubation period, reticulocytes were freed from larger transferrin-Sepharose molecules by seiving, as described, and washed three times with cold phosphate-buffered saline. They were then haemolyzed with 2 ml of 5 mM Tris · HCl buffer, pH 7.5 containing 1 mM trisodium citrate. The membrane (stroma) fraction was separated from the cytoplasm fraction (supernatant) by centrifuging the haemolysate at 20000 × *g* for 20 min. Radioactivities in the individual supernatants were assayed.

In another series of experiments, ^{125}I-labelled rabbit transferrin was coupled to Sepharose particles, as described. The ^{125}I-labelled transferrin-Sepharose suspension was then incubated with rabbit reticulocytes. Incubation and haemolysis procedures were repeated as well as radioactivity measurements of ^{125}I in the supernatants of the haemolysates.

Membrane enzyme identification

The extent of contamination of membrane fragments in the supernatant was

related percentagewise to the acetylcholinesterase activity in the supernatant based on its relationship with total acetylcholinesterase activity. Acetylcholinesterase, a well known membrane marker [18], was assayed according to the method described by Ellman et al. [19].

Results

As previously shown in incubation studies of rabbit reticulocytes [11,13], iron uptake increases at an approximately constant rate for at least the first half hour of incubation (Fig. 1). During this interval two phases of transferrin uptake are seen, viz., an initial absorption followed by a slower and progressive uptake which reaches a plateau (Fig. 2). Fig. 1 also shows that ^{59}Fe concentration in the cytoplasm parallels uptake by whole cells. However, ^{59}Fe activity in stroma remained relatively constant throughout incubation and contributed little iron to total uptake, especially in the prolonged periods of incubation. Conversely, about 75% of ^{125}I-labelled transferrin appeared in the stroma and about 25% in the cytoplasm (Fig. 2). When an assay of acetylcholinesterase activity was made for the two compartments, fairly constant concentration (17—24%) of the enzyme was detected in the cytoplasm fraction. This experimental information does not preclude the assumption that the [^{125}I]transferrin remaining in the cytoplasm may have remained attached to the membrane debris which was initially an integral part of the membrane.

When ^{131}I-labelled γ-globulin was incubated in a medium with reticulocytes, the greater part of the radio-iodine sequestered in the stroma. As with transferrin, only about 20% activity appeared in the supernatant. The absolute

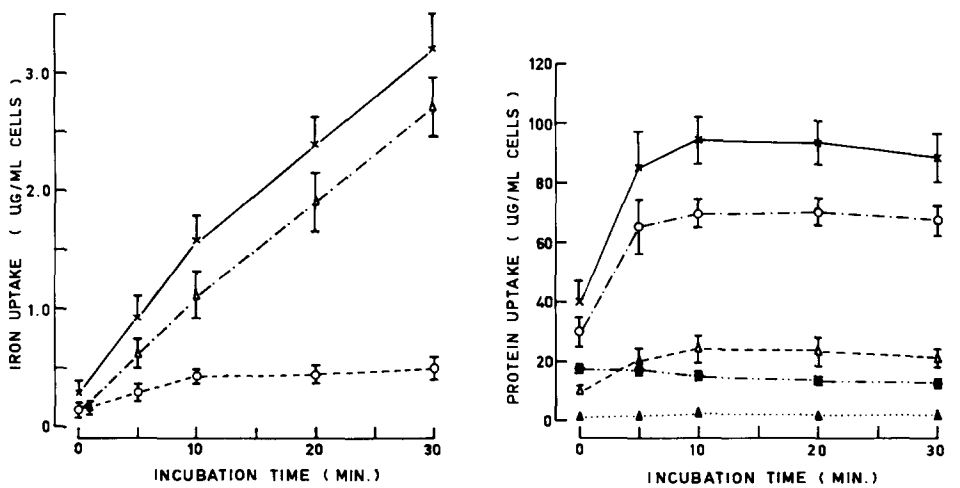

Fig. 1. Iron uptake by rabbit reticulocytes (X———X) and its haemolysate fractions, stroma (○- - - - -○) and supernatant (△— · — ·△). Each point represents mean ± S.E. with n = 12.

Fig. 2. The uptake of [^{125}I]transferrin by rabbit reticulocytes (X———X) and its haemolysate fractions, stroma (○— · — ·○) and supernatant (△———△). Each point represents mean ± S.E. with n = 12. The uptake of ^{131}I-labelled γ-globulin by the reticulocyte stroma (■— · — ·■) and supernatant (▲· · · · ·▲) was included.

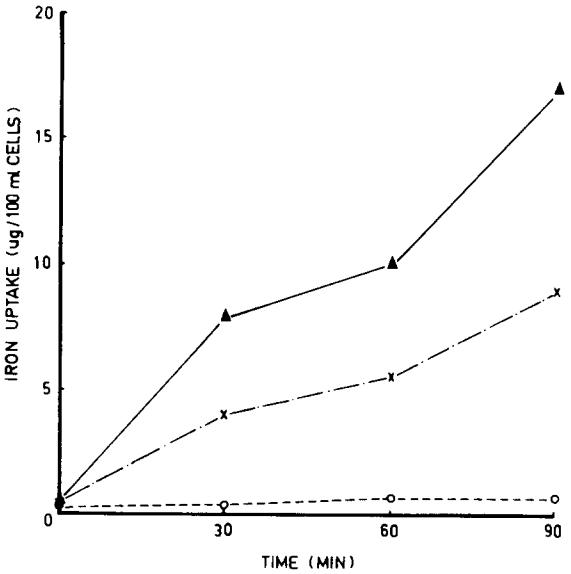

Fig. 3. Radioactivity of ^{59}Fe found in the supernatant of the reticulocyte haemolysate after the whole cells had incubated with ^{59}Fe-labelled transferrin-Sepharose for various intervals. Reticulocyte count: ▲———▲, 94%; X— · — ·X, 48%; o- - - - -o, 1.2%.

γ-globulin uptake was 20% of the transferrin uptake by reticulocytes.

Fig. 3 shows results of experiments in which reticulocytes were incubated in a suspension with ^{59}Fe-labelled transferrin-Sepharose for selected time intervals and then lyzed. ^{59}Fe appeared in the supernatant of the haemolysate, (i.e. the cytoplasm fraction) and continued to rise with time. As expected, ^{59}Fe uptake in cells increased with increasing reticulocyte concentration. Conversely, only a minute amount (0.1%) of ^{125}I-labelled transferrin appeared in the supernatant fluid obtained from the haemolysate after reticulocytes were incubated at selected time intervals in ^{125}I-labelled transferrin-Sepharose solution (Table I).

Discussion

During uptake of iron by the reticulocyte, the binding affinity between iron and transferrin is yet to be clarified. According to Morgan and co-workers, the transferrin penetrates deeply into the reticulocyte cell interior possibly by

TABLE I

^{125}I ACTIVITY IN SUPERNATANT OF THE HAEMOLYSATE AFTER RETICULOCYTES WERE INCUBATED WITH ^{125}I-LABELLED TRANSFERRIN-SEPHAROSE

Incubation (min)	^{125}I activity in incubating medium (cpm)	^{125}I activity in supernatant (cpm)	% of ^{125}I in supernatant
0	13 060	4	0.03
30	13 060	12	0.09
60	13 060	14	0.10

endocytosis [3] or one of several microtubular mechanisms [4]. Others believe that the iron is removed from transferrin while the protein is attached to a membrane or surface receptor [6,7]. Results obtained in the present study support the latter hypothesis since the experimental data indicate that movement of transferrin and iron into the cell are not coordinated. Cytoplasmic ^{59}Fe radioactivity closely followed the iron uptake curve by whole cells and advanced with incubation time. The remaining 25% of the transferrin found in the supernatant, may have been associated with the lighter membrane fragment debris not spun down. The similar relative quantity of acetylcholinesterase remaining in the supernatant supports this contention. Furthermore, similarity of the patterns of distribution of γ-globulin and transferrin in the stroma and cytoplasm fractions suggests contamination of the supernatant by fragments of the cell membrane, since γ-globulin is not normally taken up by reticulocytes [2].

As shown in Fig. 3, rabbit reticulocytes were able to take up a significant amount of iron from the iron-transferrin which had previously been coupled to Sepharose. Sepharose size ranged from 40 to 190 μm in diameter. The magnitude of iron transport from Sepharose-coupled transferrin in the first half hour amounted to approximately 8 μg per 100 ml cells which is only 3% of the normal iron uptake value. Nonetheless, the results were not all together unexpected in view of the configuration of the Sepharose-coupled transferrin molecules which must have obstructed the receptor affinity for transferrin by the cells.

Due to micellar size, it seems unlikely that transferrin-Sepharose molecules could have penetrated deeply into the cell by endocytosis or microtubular mechanisms. The possibility that the transferrin could have become uncoupled from Sepharose during incubation is remote since the sepharose is known to be covalently bound to protein [20]. The interpretation of data shown in Table I do not support the thesis that ^{125}I-labelled transferrin entered the cell. Moreover, the amount of ^{59}Fe uptake by the cells in the incubating medium of ^{59}Fe-labelled transferrin-Sepharose increased with the incubation time and the concentration of reticulocytes suggests that one of the iron uptake mechanism can take place without the entry of transferrin into the cell interior. However, it is not certain how significant this mechanism is in the normal iron uptake process by reticulocytes. In conclusion, our results suggest that transferrin molecules do not necessarily penetrate deeply into the cell before iron is released. Iron seems to be bound to transferrin only during part of its transition into the cell.

Acknowledgements

We would like to thank Professor A.C.L. Hsieh and Professor J.W. Archdeacon for constructive criticism while the manuscript was in preparation. This work was supported by Wing Lung Bank Medical Research Fund and the University of Hong Kong Research Grants.

References

1 Baker, E. and Morgan, E.H. (1969) Biochem. 8, 1133—1141
2 Baker, E. and Morgan, E.H. (1971) J. Cell. Physiol. 77, 277—384

3 Morgan, E.H. and Appleton, T.C. (1969) Nature 223, 1371—1372
4 Hemmaplardh, D., Kailis, S.G. and Morgan, E.M. (1974) Br. J. Haematol, 28, 53—65
5 Sly, D.A., Grohlich, D. and Bezkorovainy, A. (1975) Biochim. Biophys. Acta 385, 36—40
6 Jandl, J.H. and Katz, J. (1963) J. Clin. Invest. 42, 314—326
7 Speyer, B.E. and Fielding, J. (1974) Biochim. Biophys. Acta 332, 192—200
8 Garrett, N.E., Burris-Garrett, R.J. and Archdeacon, J.W. (1973) Biochem. Biophys. Res. Commun. 52, 466—474
9 Workman, E.F. Jr., and Bates, G.W. (1974) Biochem. Biophys. Res. Commun. 58, 787—794
10 Baker, E., Shaw, D.C. and Morgan, E.H. (1968) Biochem. 7, 1371—1378
11 Loh, T.T. and Morgan, E.H. (1975) Expt. Cell Res. 91, 87—94
12 Aisen, P. and Leibman, A. (1973) Biochim. Biophys. Acta 304, 797—804
13 Morgan, E.H. and Carter, G. (1960) Aust. Ann. Med. 9, 209—213
14 Hunter, W.M. and Greenwood F.C. (1962) Nature 194, 495
15 Morgan, E.H. and Laurell, C.-B. (1963) Br. J. Haematol. 9, 471—483
16 Borova, J., Ponka, P. and Neuwirt, J. (1973) Biochim. Biophys. Acta 320, 143—156
17 Porath, J. (1974) Methods in Enzymology (Jakoby, W.B. and Wilchek, M., eds.), Vol. 34, pp. 13—30, Academic Press, New York
18 Augustinsson, K.B., Axenfors, B. and Anderson, I. (1973) Biochim. Biophys. Acta 293, 424—433
19 Ellman, G.L., Courtney, D.K., Andres, Jr., V. and Featherstone, R.M. (1961) Biochem. Pharmacol. 7, 88—95
20 Cuatrecasas, P. (1970) J. Biol. Chem. 245, 3059—3065