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Iron-binding lipids of rabbit duodenal brush-border membrane

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Rabbit duodenal brush-border membrane contains chloroform/methanol (2:1, v/v) extractable Fe-binding lipids (27.2 ± 6.7 nmol/mg protein, mean ± S.E. ($n = 5$)). Thin-layer chromatography in two solvent systems reveals that the major Fe-binding component(s) co-migrate with free fatty acids. Fe-binding by pure lipids reveals that phosphatidic acid, phosphatidylserine, oleic and stearic acids all show apparent Fe-binding in filtration assays, although oleic acid shows the highest apparent binding (5–10-fold) on a molar basis. The free fatty acid content of brush-border membrane vesicles is sufficient to account for the chloroform/methanol extractable Fe-binding observed in vesicle preparations. The pH dependence of Fe-binding by oleic acid is similar to that reported for the detergent extractable Fe-binding lipid which has been implicated in transport of Fe from Fe/ascorbate solutions by rabbit duodenal brush-border membrane vesicles (Simpson, R.J. and Peters, T.J. (1986) Biochim. Biophys. Acta 859, 227–236).

Introduction

The molecular mechanism and regulation of the transport of inorganic iron across the brush-border membrane is currently obscure. Cox and O'Donnell have shown that uptake of Fe from Fe/ascorbate solutions by rabbit brush-border membrane vesicles is a regulable process [1]. We have shown that the Fe uptake process represents transport across the brush-border membrane followed by intravesicular binding [2,3].

We recently demonstrated that transport of Fe from Fe/ascorbate solutions, by rabbit brush-border membrane vesicles in vitro, has a pH optimum of approximately 7.0 and is inhibited by NaCl [2]. Cholate extracts of rabbit brush-border membrane vesicles contain lipid(s) with Fe binding properties (pH optimum, reversible binding) consistent with a membrane carrier [2].

In this paper we report that lipid extracts (chloroform/methanol (2:1, v/v)) of rabbit brush-border membrane vesicles contain Fe-binding components. The identity of these components is reported.

Materials and Methods

Materials

Sepharose CL6B and Sephadex G-50 (medium) were obtained from Pharmacia (Uppsala, Sweden), sodium cholate, crude soybean lipid extract (type II-S, 'phosphatidylcholine'), dipalmitoylphosphatidylethanolamine, phosphatidylcholine (egg yolk, type III E), dog brain lipid extract (type VI, 'phosphatidylethanolamine'), *Escherichia coli* phosphatidylethanolamine, were obtained from Sigma and used without further purification.

Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

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Chloroform and methanol (AnalaR grade, BDH) were redistilled before use. Thin layer chromatography plates (Silica gel G, 250 μm) were from Analtech (Neward, U.S.A.). Other phospholipids and lipids were the purest material obtainable from Sigma. Radiochemicals were from Amersham International (U.K.). Other materials were as in Ref. 2.

General methods

Protein concentrations were determined by the modified Lowry method [4]. Phospholipid suspensions were prepared by evaporating phospholipids (dissolved in chloroform/methanol (2 : 1, v/v) or hexane) under N_2 or under partial vacuum. The phospholipids were suspended in the appropriate buffer by agitation with glass beads, or vortexing, followed by sonication with an MSE probe-type sonicator (MSE Mk 2, Crawley, Sussex, U.K.) until the suspension clarified. Dipalmitoylphosphatidylethanolamine suspensions were obtained by sonicating solid material in chloroform, adding pure phosphatidylcholine in hexane, then evaporating the solvent and sonicating as above. Neutral lipids were sonicated in 150 mM NaCl/10 mM Na Hepes buffer (pH 7.0)/11 mM sodium cholate (3 × 10 s bursts).

Human erythrocyte membranes were prepared essentially as described in Ref. 5 except that 5 mM Tris-HCl (pH 7.4) was substituted for 5 mM phosphate buffer (pH 8.0). Brush-border membrane vesicles were prepared from frozen rabbit duodenum by the Kessler method [6] as described in Ref. 2.

Apparent ^{59}Fe binding was measured by Millipore filtration as in Ref. 2. This technique depends on retention of ^{59}Fe-binding compounds by 0.22 μm Millipore filters. Previous studies showed this method to be suitable for studies of cholate soluble and insoluble components of brush-border membrane, provided cholate concentrations in the assay were kept below approx. 5 mM [2]. Retention of ^{14}C-labelled oleic acid and cholesterol was investigated in dummy Fe-binding assays lacking ^{59}Fe (^{56}Fe was substituted) at pH 7.2. Values of 35.7 ± 2.7% and 80.7 ± 5.0%, respectively, (mean ± S.E. for three determinations) were obtained. These values mean that the assay method is only semiquantitative; however, the technique is suitable for multiple determinations of, for example, column [2] or thin-layer chromatography fractions. Care was exerted to employ similar media (especially low cholate concentrations) as in Ref. 2. Generally representative results that were reproducible on at least three separate occasions are presented.

Gel filtration analysis of ^{59}Fe/ascorbate/lipid mixtures was performed by adding solid sodium cholate to 10% (w/v) and mixing until a clear suspension resulted. 0.5 ml of the mixture was applied to Sephadex G-50 (30 × 1 cm) or Sepharose CL6B (85 × 1.6 cm) columns equilibrated and eluted (17 ml/h and 13 ml/h, respectively) with 150 mM NaCl/11 mM sodium cholate/10 mM Na-Hepes (pH 7.0). Elution and fraction counting were as described in Ref. 2.

Thin-layer chromatography of lipid extracts

All glassware employed for phospholipid analysis was washed in acetone/ethanol (1 : 1, v/v). Thin-layer chromatography plates (Silica gel G, 250 μm) were activated for 1 h at 100 °C. Chloroform/methanol extracts were prepared by vortexing with 20 vol. of chloroform/methanol (2 : 1, v/v) for 10 s followed by addition of 5 vol. of 0.15 M NaCl and revortexing. The aqueous layer and insoluble material was discarded and the organic phase was evaporated under a stream of oxygen-free N_2. The resulting extracts were dissolved in 200 μl chloroform/methanol (see above) and stored at −20 °C. Extract (equivalent to 25 μl of original sample) or approx. 25 μg of various lipid standards (dissolved in same solvent as extract) was spotted on the plates which were developed in chloroform/methanol/water (65 : 25 : 4, by vol.) or in hexane/diethyl ether/water (80 : 20 : 1, by vol.). Plates were visualised by spraying with H_2SO_4 and charring at 200 °C. Glycolipids were visualised with α-naphthol/H_2SO_4 [7]. For studies of Fe binding, extract from 0.5 ml of vesicle suspension was applied as a 10 cm streak and developed as above. The plate was scraped to give nine fractions which were extracted twice with 1 ml of chloroform/methanol (2 : 1, v/v). The extracts for each fraction were combined, evaporated to dryness, then resuspended by sonication (10 s) in 200 μl of 0.15 M NaCl, 11 mM sodium cholate in 10 mM Na-Hepes buffer (pH 7.0).

Results and Discussion

Apparent ^{59}Fe-binding by lipids

Crude lipid extracts of whole brush-border membrane vesicles (chloroform/methanol (2:1, v/v)) when resuspended by sonication in 0.15 M NaCl/10 mM Hepes (pH 7.0)/11 mM sodium cholate, were found to contain large quantities of Fe-binding material, as do cholate extracts (27.2 ± 6.7 nmol/mg vesicle protein (mean ± S.E., n = 4) compared with approx. 50 nmol/mg protein in cholate extracts [2]). Fe-binding by a variety of lipid extracts and pure lipids was therefore investigated (Table I). It can be seen that crude lipid extracts from various sources bind Fe to differing degrees while specific lipids, especially phosphatidic acid and fatty acids, appear to bind large amounts of Fe. Binding demonstrated with synthetic phosphatidylethanolamine may be due to impurities as chromatographically purified phosphatidylethanolamine from *Escherichia coli* and brush-border membrane vesicle phosphatidylethanolamine (see below) does not bind comparable amounts of Fe. The time, pH and Fe concentration dependence of Fe binding by soybean lipids was very similar to the dependences shown in Ref. 2 for the cholate-extractable brush-border membrane vesicle Fe binder.

TABLE I

APPARENT Fe BINDING FROM Fe/ASCORBATE SOLUTIONS BY NATIVE AND PURIFIED LIPIDS

Lipids were suspended either in 0.1 M mannitol/0.1 M NaCl/20 mM Na-Hepes (pH 7.4) or 0.15 M NaCl/10 mM Na-Hepes/11 mM sodium cholate (pH 7.0) as described in Materials and Methods. No difference in binding was noted when the same lipids were assayed in both buffers. ^{59}Fe binding was determined by Millipore filtration with final assay lipid concentrations of (a) 9 μg/ml and (b) 0.9 μg/ml. Fe and ascorbate concentrations were 91 μM and 1.8 mM, respectively. The figures are presented as means ± S.E. for (n) experiments.

Lipid	Apparent Fe binding (nmol Fe/mg lipid)
(a)	
Cholesterol	1.44 ± 0.44 (3)
Phosphatidylcholine	1.50 ± 0.20 (3)
Diolein (dioleoyl diglyceride)	4.39 ± 1.84 (3)
E. coli phosphatidylethanolamine	7.30 ± 0.43 (3)
Synthetic phosphatidylethanolamine	31.9 ± 2.7 (6)
Human erythrocyte lipids [a]	3.47 ± 0.71 (3)
Dog brain lipids	26.5 ± 3.8 (3)
Cerebrosides	7.6 ± 1.4 (4)
Rabbit brush-border membrane lipids [b]	54 ± 14 (3)
(b)	
Soybean lipids	119 ± 11 (3)
Phosphatidic acid	403 ± 34 (4)
Oleic acid	1210 ± 336 (6)
Stearic acid	117 ± 56 (4)
Phosphatidylserine	270 ± 88 (3)

[a] Calculated assuming 0.5 mg lipid per mg vesicle protein [8].
[b] Calculated assuming 6 mg lipid per ml of erythrocytes [9].

The large amount of Fe binding by brush-border membrane vesicle cholate and chloroform/methanol extracts can only be explained by Fe binding with a stoichiometry of at least one ligand molecule per Fe if one of the predominant lipids reported to be present in brush-border membrane (glycolipids, cholesterol, fatty acids, phosphatidylethanolamine and phosphatidylcholine (plus sphingomyelin)) [8] is responsible. It appears from the data in Table I that free fatty acids may be responsible for the Fe binding in brush-border membrane extracts. Data in Ref. 8 suggest that rabbit brush-border membrane vesicles contain approx. 200 nmol of free fatty acid per mg of vesicle protein. Phosphatidic acid, though a potent Fe binder, is present in only minor quantities [8] and displays a different Fe concentration and pH dependence for Fe binding (not shown) from that observed with brush-border membrane vesicle extracts. All of the major lipid classes of rabbit brush-border membrane, except free fatty acids, are present in human erythrocyte membranes [10]. Human erythrocyte lipids show much lower Fe binding than do brush-border membrane lipids (Table I).

Fe binding by oleic acid appears to represent Fe^{2+} binding (as opposed to Fe^{3+} which may also be present in air-equilibrated Fe/ascorbate solutions) as experiments conducted using $FeSO_4$ in place of $FeCl_3$, N_2-bubbled solutions and incubations under N_2, revealed similar, high levels of Fe-binding as those reported in Table I.

Thin-layer chromatographic analysis of ^{59}Fe-binding lipids of brush-border membrane vesicles

In order to confirm the identity of the Fe

binding material, thin-layer chromatography coupled with Fe binding analysis was performed (Fig. 1). The solvent systems were selected in order to avoid relatively non-volatile constituents (e.g., acetic acid, NH_3) likely to interfere with subsequent Fe binding assays. Fig. 1A shows that fatty acids and cholesterol correspond with the major Fe binding peak. The total apparent Fe^{2+} binding capacity recovered from this thin-layer chromatography analysis was high (65.5 ± 6.9% (mean ± S.E., $n = 3$)) with most of the binding (73.3 ± 4.1% (mean ± S.E., $n = 3$)) in the main peak. Similar findings were obtained with a second solvent system (Fig. 1B). Phospholipids and glycolipids remained at the origin in this solvent system. The main ^{59}Fe-binding peak is broader than most standards in both solvent systems, perhaps suggesting some heterogeneity. Various fatty acids were found to run in slightly different positions in solvent system B. From this analysis, free fatty acids appear to be primarily responsible for the Fe binding by chloroform/methanol extracts of brush-border membrane vesicles.

Characteristics of ^{59}Fe-binding by oleic acid

Fig. 2 shows the time dependence of apparent Fe binding by oleic acid. The apparent Fe binding occurs rapidly and shows a pH optimum of about 7.1 (Fig. 3). The retention of ^{14}C-oleic acid by filters in dummy assays substituting ^{56}Fe for ^{59}Fe was found to increase from 35.7 ± 2.7% to 87.5 ± 6% (S.E., $n = 3$) going from pH 7.2 to pH 6.55, reflecting the more detergent-like properties of oleate compared with oleic acid. This change in retention means that the true optimum for binding of Fe by oleate will be greater than 7. The dependence on Fe concentration (Fig. 4) suggests an apparent K_d of less than 5 μM. Qualitatively similar properties of apparent binding were observed with stearic acid, although the level of binding observed was quantitatively lower (Table I). The characteristics of apparent ^{59}Fe binding by fatty acids noted above are very similar to those found with cholate extractable Fe-binding material from rabbit duodenal brush border membrane vesicles (see Ref. 2). This binding presumably represents the formation of insoluble fatty acid soaps which are retained by the Millipore filters. The overall apparent binding may thus be a pro-

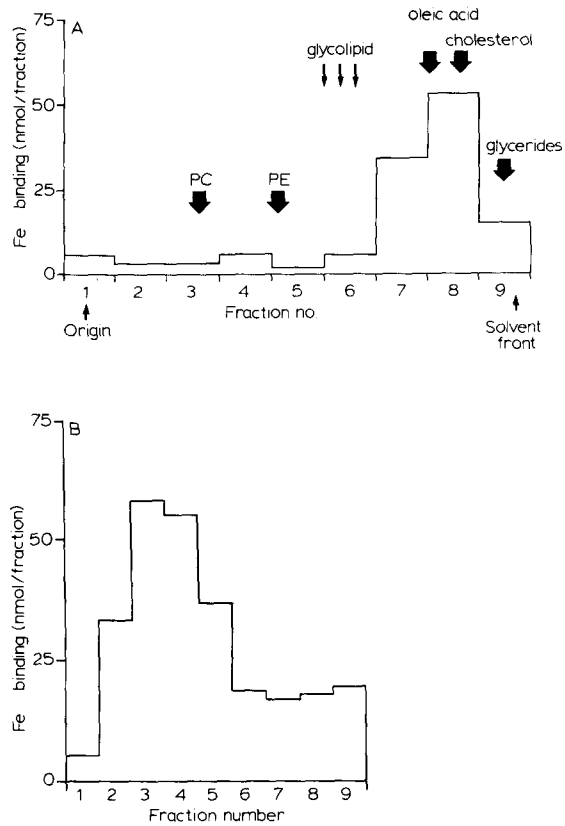

Fig. 1. Thin-layer chromatography and Fe-binding analysis of chloroform/methanol extracts from brush-border vesicles. Silica gel plates were streaked (10 cm) with extract from 0.5 ml of vesicle suspension (5 mg of vesicle protein) and developed in (A) chloroform/methanol/water (65:25:4, by vol.) or (B) hexane/diethyl ether/water (80:20:1, by vol.) and dried in air. The entire extract lanes were scraped in (A) equal 2 cm steps or (B) steps of 0.5 cm (fractions 2–5), 1 cm (fractions 1 (origin material) and 6) or 5 cm (fractions 7–9). Fractions were extracted and extracts were resuspended in 200 μl 0.15 M NaCl/10 mM Na-Hepes (pH 7.0)/11 mM sodium cholate and assayed for Fe binding from Fe/ascorbate as described in Table I. Standards, extract aliquots and mixtures of standards plus extract were spotted on the same or parallel plates. Spots were visualized (after scraping) with α-naphthol/H_2SO_4 [7] or by charring. Standards are: PC, phosphatidylcholine; PE, phosphatidylethanolamine; glycolipids, cerebrosides (Sigma types I and II) from bovine brain. Phosphatidic acid and phosphatidylserine ran as streaks from R_F 0.15–0.7 and 0.0–0.4, respectively, in system (A). All phospholipids and cerebrosides remained at the origin (fraction 1) in system (B). Various fatty acids ran with R_F values of 0.076 (arachidonic acid) – 0.35 (linoleic acid) in system (B). In system (A) palmitic, stearic, linoleic, linolenic and arachidonic acids all overlapped with oleic acid, although individual R_F values varied slightly. In system (B) cholesterol and diolein were not resolved from the main Fe-binding peak.

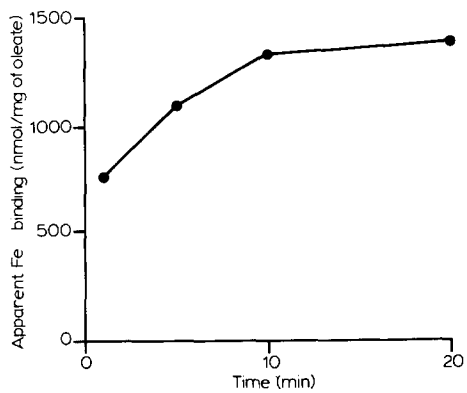

Fig. 2. Time dependence of apparent Fe binding by sonicated oleic acid suspensions. Oleic acid (1 mg/ml) was resuspended and sonicated in 0.15 M NaCl/10 mM Na-Hepes (pH 7.0)/11 mM sodium cholate as described in Materials and Methods. Uptake was determined by Millipore filtration with medium containing 91 μM ^{59}Fe/1.8 mM sodium ascorbate/0.1 M NaCl/0.1 M mannitol/20 mM Na-Hepes (final pH 7.25) with final assay oleic acid and cholate concentrations of 9.1 μg/ml and 0.1 mM, respectively. Incubation was at 37°C.

cess of complex formation with subsequent aggregation.

When oleic acid or soybean lipids were solubilised with 10% cholate and labelled with ^{59}Fe equivalent to 50–90% of the binding capacity (as measured by filtration assays), and subjected to analysis by Sephadex G-50 chromatography as in Ref. 2, none of the radioactivity was recovered in the position of the Fe-binding peak (Fig. 5), suggesting that the complex dissociates readily. The ^{59}Fe-binding measured in column eluates by millipore filtration was in a single peak in a position corresponding to an apparent molecular weight of approx. 10000 and was distinct from the position of the ^{59}Fe-label. When an oleic acid/^{59}Fe mixture was solubilised as above and chromatographed on Sepharose CL6B as in Ref. 2, recovery of ^{59}Fe was low (12%) and most of the recovered ^{59}Fe eluted at a position similar to that of Fe/ascorbate, which is also poorly recovered from these columns [2]. This behaviour is similar to that

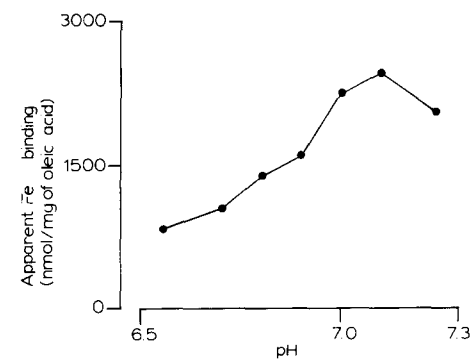

Fig. 3. pH dependence of apparent ^{59}Fe binding by oleic acid. Uptake determinations were performed with oleic acid suspensions as in Fig. 2 incubated for 10 min at 37°C in media as in Fig. 2 except the pH was varied as shown (see Ref. 3). Binding was determined by Millipore filtration.

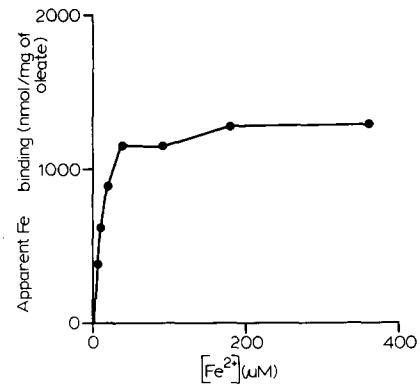

Fig. 4. Dependence of apparent ^{59}Fe binding by oleic acid on medium Fe^{2+} concentration. Oleic acid suspension, prepared and diluted as in Fig. 2, was incubated for 10 min at 37°C with various medium ^{59}Fe concentrations and constant ^{59}Fe ascorbate ratios of 1:20. Binding was determined as in Fig. 2.

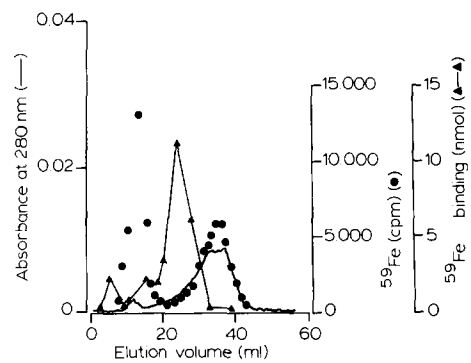

Fig. 5. Gel filtration analysis of oleic acid-^{59}Fe/ascorbate complexes. Oleic acid (0.2 mg) was suspended in 0.25 ml of 0.2 M NaCl/0.1 M mannitol/20 mM Na-Hepes (pH 7.25) by sonication then incubated for 10 min at 37°C with 91 μM Fe/1.8 mM Na ascorbate (final concentrations). Solid sodium cholate (10% final concentration (w/v)) was added and the mixture applied to a 1×30 cm column of Sephadex G-50. Fractions were collected and analysed for ^{59}Fe or Fe binding from Fe/ascorbate as described in Materials and methods.

exhibited by the cholate extractable ^{59}Fe binding component of brush-border membrane vesicles [2]. In both gel filtration analyses, some high-molecular weight ^{59}Fe was observed (M_r approx. $5 \cdot 10^5$ on Sepharose CL6B). This may represent iron hydroxide formed by oxidation and hydrolysis or it may be undissociated ^{59}Fe-soap aggregate.

The significance of fatty acid binding of ^{59}Fe in ^{59}Fe uptake by intact brush-border membrane vesicles

We previously showed that the Fe-binding lipids of brush-border membrane, here identified as fatty acids, may be responsible for transport of Fe from Fe/ascorbate solutions by brush-border vesicles [2]. The affinity of fatty acid for Fe and its ability to give up Fe once bound are consistent with a carrier function. Erythrocytes, which contain very low free fatty acid levels compared to the brush-border membrane [10], and lower chloroform/methanol extractable Fe binding capacity (Table I) exhibit a slow uptake of Fe from Fe/ascorbate [11]. Further studies to determine whether fatty acids can act as membrane carriers of Fe are clearly necessary.

Conclusions

Non-esterified fatty acid is a major Fe binding component of rabbit duodenal brush-border membrane vesicles. Fatty acids may mediate membrane transport of Fe from Fe/ascorbate by brush-border membrane vesicles.

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