BioMetals 1999, **12**: 53–62

The ferric-reducing activity of duodenal brush-border membrane vesicles is associated with a *b*-type haem

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Rabbit brush-border membrane vesicles possess ferricyanide reducing activity. This activity is preferentially dependent on NADH as reductant, and can be stimulated by the addition of FMN. The latency of activity observed following vesicle solubilisation suggests that the responsible component is transmembranous, and partially sequestered on the inner-face of the vesicles prior to full solubilisation. Subsequent increases in detergent concentration (>0.3% w/v lauryl maltoside) were found to be inhibitory. Ferricyanide reducing activity was effectively inhibited by the sulphydryl modifying reagents N-ethyl malemide and *p*-chloromercuribenzoate, but not by the flavin analogue diphenylene iodonium. The ferric-reducing activity co-purified with a *b*-type haem when applied to Sephacryl S-200 columns. The putative cytochrome was found to be immunologically distinct from neutrophil cytochrome b_{558}.

Keywords: brush-border membrane vesicles, ferricyanide, duodenum, reductase.

Abbreviations: BBMV- Brush-border membrane vesicles; CHAPS- 3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonate; ferricyanide- potassium hexacyanoferrate (III); ferrocyanide- potassium hexacyanoferrate (II); FMN- flavin mononucleotide; lauryl maltoside- *n*-Dodecyl α-D-maltoside; lauroyl sarcosine- N-dodecanoyl-N-methylglycine; NEM- N-ethyl malemide; PCMBS- *p*-chloromercuriphenylsulphonate

Introduction

Multiple mechanisms for iron uptake are known to exist for various microorganisms. Due to the essential biochemical functions of iron, its complex chemistry, the many factors affecting bioavailability, and the requirement that iron homeostasis is regulated at the intestinal level (McCance & Widdowson, 1937), it would seem likely that more than one mechanism of acquisition exists in higher organisms. Indeed in mammals, recent literature describes a free-fatty acid mediated pathway, and a putative carrier-mediated mechanism dependent on both cellular metabolism and the brush-border membrane potential gradient (Raja *et al.* 1989), The latter mechanism requires the cleavage of iron from the ferric complex at the duodenal mucosal surface prior to uptake. This carrier-mediated pathway has been shown to be markedly inhibited by ferrous-trapping agents (such as ferrozine), and the non-permeant oxidising agent ferricyanide, thereby indicating that reduction of ferric iron is a prerequisite for uptake (Raja *et al.* 1992).

Recently, the gene coding for a probable human duodenal divalent metal transporter (including iron) and termed DCT1 has been identified and cloned (Gunshin *et al.* 1997). DCT1 is a member of the 'natural-resistance-associated macrophage protein (Nramp) family (Gruenheid *et al.* 1995). A mutation in the gene of this candidate iron transporter has subsequently been reported as the genetic basis for microcytic anaemia in homozygous mk/mk mice (Fleming *et al.* 1997). Since DCT1 has been characterised as a transporter of ferrous rather than ferric iron (Gunshin *et al.* 1997), the existence of an epithelial cell-surface reducing activity could be important in the duodenal absorption of dietary non-haem

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iron. Crane first hypothesised that transplasma membrane redox activity could be involved in mucosal Fe^{3+} uptake (Crane *et al.* 1985), but until recently, the role of enzymatic reduction in intestinal iron absorption had not been widely considered.

We have previously demonstrated a duodenal mucosal-surface FE(III) reducing activity in mouse (Raja *et al.* 1992, Pountney *et al.* 1996), and man (Raja *et al.* 1996). Furthermore, we have shown that there is a persistent abnormality in the reduction of iron by the intestine in genetic haemochromatosis (Raja *et al.* 1996). In the mouse, the rates of mucosal reduction are paralleled by changes in the rate of iron uptake induced by changes in body iron stores (iron deficiency) or hypoxia (Raja *et al.* 1992, Pountney *et al.* 1996). Recently, reduction of $Fe^{3+}(NTA)_2$ in the presence of human HuTu80 cells or human duodenal microvillus membranes has been reported (Riedel *et al.* 1995). A ferric reductase activity in brush-border membrane vesicles isolated from Caco-2 cells has also been demonstrated (Ekmekcioglu *et al.* 1996).

The general existence of plasma membrane ferric reductase activity has been described in both prokaryotic and eukaryotic cells (Crane *et al.* 1995). In mammals, an NADH-dependent di-ferric transferrin reductase in liver plasma and endosomal membranes has been reported (Thorstensen *et al.* 1988), but this remains controversial. In *Saccharomyces cerevisiae*, the *fre-1* gene encoding the reductase has been cloned and sequenced (Dancis *et al.* 1992, Roman *et al.* 1993), and there is significant similarity in the amino acid sequence to the large subunit of cytochrome b_{558} of phagocytic cells.

The aim of the present study was to characterise ferric iron reduction in the presence of brush-border membrane vesicles, and in doing so a *b*-type haem that co-purified with the reducing activity was observed and further investigated.

Materials and methods.

Reagents.

Chemicals and biochemicals were obtained at the highest possible grade from either BDH Laboratory Supplies, Merck (Poole, UK) or Sigma (Poole, UK). Diphenylene iodonium was from Pfaltz and Bauer, Connecticut, USA.

Preparation of Duodenal Brush-border Membrane Vesicles.

Brush-border membrane vesicles (BBMV) were prepared from rabbit duodenal and upper jejunal scrapings, essentially by the method of Kessler *et al.* (1978), as adapted and described by Simpson & Peters, (1984). Briefly, intestinal tissue was perfused with saline, the mucosa scraped and suspended in 50mM mannitol, 2mM HEPES-NaOH pH 7.1 (30 ml g tissue^{-1}). The mucosal suspension was homogenised for 2 minutes in a chilled Waring Blender and solid $MgCl_2$ added to a final concentration of 10mM, and the homogenate stirred on ice for 20 minutes before centrifugation at 3000 g for 10 minutes. The resulting supernatant was recentrifuged at 40,000g for 40 minutes and the resulting pellet resuspended in resuspension buffer (100 mM mannitol, 100mM NaCl, 100mM $MgSO_4$, 20mM HEPES-NaOH, pH 7.4; 20ml g^{-1} mucosa weight). The suspension was centrifuged at 6000 g for 20 minutes and the resulting supernatant recentrifuged at 40,000g for 40 minutes. The final vesicle pellet was resuspended in resuspension buffer at an approximate concentration of 10 mg ml^{-1} and stored at –70ºC. Protein concentration was determined using the Pierce BCA kit (Pierce Chemicals, IL, USA). Bovine serum albumin was used as standard.

Ferricyanide Reduction Assay.

Ferricyanide reducing activity was determined at room temperature by measuring the disappearance of the chromogenic substrate ferricyanide (Grebing *et al.* 1984), coupled with NAD(P)H, at 410 nm. A 3ml capacity cuvette contained 1.66mM ferricyanide and 100mM NaOH-HEPES, pH 7.1, to an amount that the final volume attained would be 3ml after all subsequent additions. 50 μl of a freshly prepared NAD(P)H solution was added, and the basal ferricyanide reduction rate recorded. 50 μl aliquots of BBMV (~1mg protein) were then added, and using an extinction coefficient of 1020 mol^{-1} cm^{-1} for ferricyanide, the specific activity was calculated in μmoles e^- transferred min^{-1} mg protein^{-1}.

Brush-border membrane and microsomal marker enzyme assay- Zn^{2+}- and Tris-resistant α-glucosidase, respectively.

The assay, in the presence of tris or zinc, was identical to that described previously, using 4-methylumbeliferyl α-D-glucopyranoside as substrate (Peters, 1976).

Spectroscopy.

Visible spectra were recorded at room temperature using a Cary Varian spectrophotometer connected to an Apple Computer. Typically, the spectral bandwidth, scanning rate and scan resolution and chart speed were 1nm; 2nm sec^{-1}; 1 reading per 0.5nm; and 10cm min^{-1}, respectively. Electron paramagnetic resonance spectra were recorded using a Bruker ESP300 spectrometer fitted with an Oxford Instruments liquid helium flow cryostat ESR900. Spectra were baseline corrected by subtraction of a spectrum of buffer alone.

Potentiometric titrations of haem-b in BBMV.

Redox titrations were carried out using a platinum electrode under anaerobic conditions using a glucose/glucose oxidase system in the presence of catalase (Wrigglesworth *et al.* 1988). The medium (3.5ml), contained 94mM Na-HEPES, 200μl BBMV preparation, 5mM glucose, plus trace amounts of glucose oxidase and catalase. Methylene blue (10μM) was used as a redox mediator and the potential was varied with the addition of sodium dithionite or potassium ferricyanide. After stabilisation of the potential, the spectrum was scanned between 400nm and 600nm. Only spectral changes in the Soret region were sufficiently large to derive the titration curves.

SDS-Polyacrylamide gel electrophoresis and Western Blotting.

SDS-PAGE was performed according to Laemmli, (1970) using a BioRad Mini-Protean II gel system (BioRad Laboratories, CA. USA). Proteins were transferred onto nitrocellulose (Hybond) and probed with polyclonal antibodies raised against various subunits of the neutrophil respiratory burst NADPH oxidase (p22-*phox*, p47-*phox*, p67-*phox*, gp91-*phox*). Bands were visualised using alkaline phosphatase conjugated anti-rabbit IgG (BioRad) according to the manufacturers instructions.

Gel filtration.

All procedures were carried out at 4°C. A Sephacryl S-200 column with a bed-height of 30cm was used. The column was washed with 3 bed-volumes of 100mM Na-HEPES (pH 7.4) containing 1% w/v lauroyl sarcosine. 1ml BBMV (~20mg protein) was solubilised with lauryl maltoside (1% w/v). Following application of the sample, the column was eluted under gravity with the above buffer. 1.2ml fractions were collected and subsequently monitored at 280nm for protein content, and at 500nm for light-scattering due to reconstituted lipid micelles. Protein-containing fractions were scanned as described above, and assayed for ferricyanide reducing activity.

Statistical analysis.

Data are presented as means ± SEM, unless indicated otherwise. Where appropriate, data was analysed by a series of two-sample student t-tests. The assumption of normality for the t-tests was valid since Minitab's normal score test gave no evidence that the data differed significantly from normal distributions. K_{max} and V_{max} determinations were performed by fitting the original data to a hyperbola described by using the Biosoft Fig-P program (Biosoft, Cambridge, U.K.).

Results and discussion.

Duodenal brush-border membrane vesicles were prepared by the method of Kessler, (1978), who reported vesicles to be enriched in plasma membranes and essentially free from intact basolateral, mitochondrial and microsomal membranes. Ekmekcioglu *et al.* (1996) measured succinate dehydrogenase (EC 1.3.99.1) and NADH-cytochrome *c*-reductase (EC 1.6.99.2) to show that mitochondrial or microsomal contamination respectively, could not fully account for the ferri-reducing activity of vesicles prepared by the same procedure. Table 1 shows that several inhibitors of mitochondrial electron transport did not significantly inhibit BBMV reducing activity. In addition, Na-dithionite reduced-minus-oxidised spectra of the preparation (see Figure 3 for an example) did not show any absorbance bands characteristic of mitochondrial cytochromes. To further assess the purity of the vesicle preparation, tris- and zinc-resistant α-glucosidase activity was measured (marker enzyme of microsomal and brush-border membrane respectively). This assay (Table 2) shows that the final pellet was enriched in plasma membranes (×15.7 ± 3.2), and that there was a decrease in endoplasmic reticulum activity by a factor of 0.85 ± 0.08.

The ferric-reducing activity of BBMV was found to be dependent on NADH as an intracellular electron donor (Table 3). In the presence of NADH the activity was further stimulated by the addition of 20μM flavin mononucleotide (FMN). No stimulation by FMN was observed when NADPH was used as a reductant. Flavin was not detected in the purified vesicles by spectroscopy (see below), indicating that if a flavin is involved *in vivo* it may become separated from the brush-border membrane during the centrifugal preparation. Figure 1 demonstrates the results of two experiments in which the concentration of NADH or NADPH was varied. Increases in NADPH concentration only slightly increased the

Table 1. The effect of several inhibitors of mitochondrial electron transfer on ferricyanide reducing activity.

Ferricyanide reduction by rabbit duodenal BBMV was determined using optimal conditions (1.6mM ferricyanide, 0.85mM NADH, 0.03% w/v (~1mM) lauryl maltoside, 20μM FMN) in the presence of the inhibitors at the specified concentrations. Data are means (SEM for (n) determinations.

Inhibitor:	Specific activity: μmoles e^- transferred min^{-1} mg $protein^{-1}$
Control activity (no additions)	0.67 ± 0.13 (5)
Rotenone (10μM)	0.61 ± 0.19 (4)
Antimycin-A (10μM)	0.58 ± 0.15 (3)
KCN (5μM)	0.64 ± 0.16 (4)

Table 2. Brush-border membrane and microsomal marker enzyme assay- Zn^{2+} and Tris-resistant α-glucosidase.

α-glucosidase activity was assayed by measuring the production of the fluorescent 4-methylumbeliferone α-glucopyranoside (Peters, 1976). In both assays, 0.1% Triton X-100 was included to solubilise the membrane proteins. The differential sensitivity is used to distinguish between the brush-border and endoplasmic reticulum activity. Fluorescence was measured at an excitation wavelength of 365nm and an emission wavelength of 490nm. The enrichment factor (x) is the ratio of the specific activity in the BBMV: initial homogenate. The yield or 'recovery', is the % of the total activity of the homogenate activity that is recovered in the final pellet. The data are means ± SEM for 3 experiments, each of which was performed in duplicate.

	Specific activity (mU mg protein^{-1})	enrichment (x-fold)	Total enzyme (mU)	Yield (%)
Zn^{2+} -resistant: (Zn^{2+} inhibits activity associated with endoplasmic reticulum).				
Homogenate	0.35 ± 0.05	1.0	402.7 ± 57.6	100
BBMV	5.30 ± 0.54	15.72 ± 3.2	167.7 ± 20.4	41.4 ± 1.27
Tris-resistant: (Tris inhibits activity associated with brush-border).				
Homogenate	2.24 ± 0.21	1.0	2894.0 ± 215	100
BBMV	1.90 ± 0.21	0.85 ± 0.08	79.8 ± 8.74	2.7 ± 0.23

Table 3. Ferricyanide reduction by BBMV.

Reducing activity was determined spectrophotometrically (at room temperature, 25°C by monitoring the disappearance of ferricyanide at 410 nm. A slow basal rate of reduction attributable to direct reduction of ferricyanide by NAD(P)H (0.85 mM) was subtracted from the assay rate prior to calculation of specific activity. Data are means ± SEM for (n) determinations. *$p < 0.02$, NADH compared with NADH + FMN.

Substrate	Specific activity (μmoles e^- transferred min^{-1} mg protein $^{-1}$)
NADH (0.85mM)	0.51 ± 0.08 (7)
NADH (0.85mM) + FMN (20μM)	0.72 ± 0.07 (7)*
NADPH (0.85mM)	0.20 ± 0.04
NADPH (0.85mM) + FMN (20μM)	0.21 ± 0.05 (5)

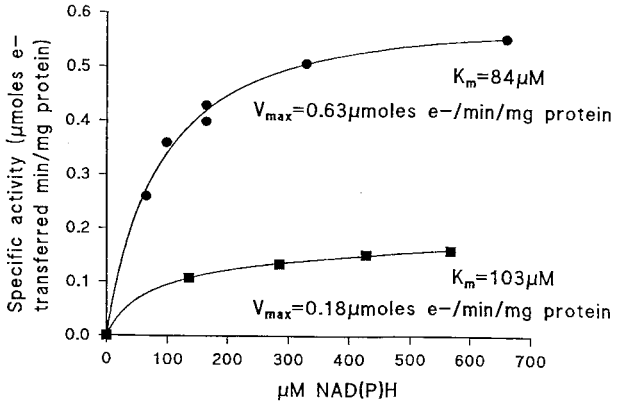

Figure 1. The effect of [NAD(P)H] on ferricyanide reducing activity of rabbit BBMV.
Reducing activity was determined by monitoring the disappearance of ferricyanide at 410nm in the presence of increasing concentrations of either (●—●) NADH, or (■—■) NADPH. Values for the apparent K_m (μM) and V_{max} (μmoles e^- min^{-1} mg protein^{-1}) were determined by fitting the data to a hyperbola. Further experimental details are given in the methods section.

specific activity, but NADH was more effective in stimulating activity at increased concentrations. In all subsequent assays reported, NADH was used at a concentration of 0.85mM.

In the mouse, BBMV ferri-reducing activity in the absence of FMN has been found to be preferentially dependent on NADH. However, in the presence of FMN no preference for either NADH or NADPH as electron donor was observed (R. J. Simpson, unpublished data). Riedel *et al.* (1995) reported that the reduction of ferric iron by human duodenal microvillus membranes is NADH-dependent. Although the mid-point potentials for the NAD^+/NADH and the $NADP^+$/NADPH couples are the same (E°' –0.32V), it is not established if these different observations are due to species variation in electron donors, or reflect fundamental mechanistic differences.

The ferric-reducing activity was increased when solubilised with lauryl maltoside (Figure 2). The latent activity observed upon vesicle solubilisation suggests that the responsible component is transmembrane and partially sequestered on the inner face of the vesicles prior to full solubilisation. The inhibitory effect observed at higher detergent concentrations may suggest a specific orientation in

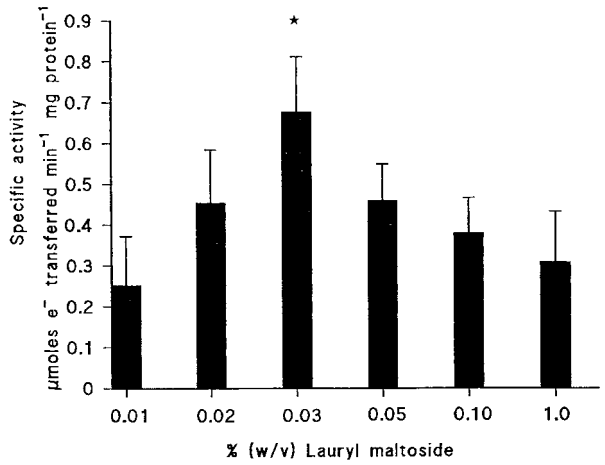

Figure 2. The effect of lauryl maltoside on BBMV ferricyanide reducing activity.
Ferricyanide disappearance was monitored at 410 nm. The cuvette contained 1.6mM ferricyanide, 50 µl 100 mM NADH, 50 µl BBMV (0.92 mg protein), and lauryl maltoside at the concentrations shown. The final cuvette volume was adjusted to 3 ml with 100 mM NaOH-HEPES, pH 7.1. Data are means ± SEM for 5 determinations. *p<0.05 compared with 0.01% w/v lauryl maltoside.

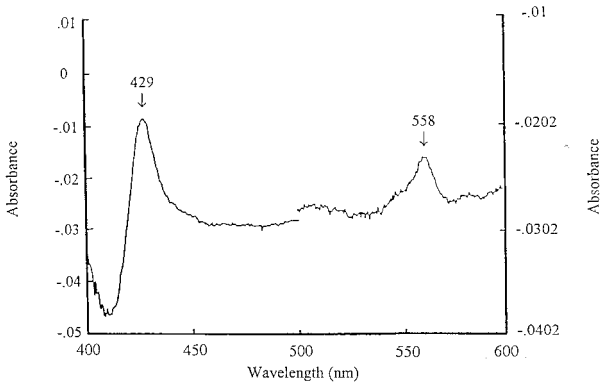

Figure 3. Optical spectrum of duodenal BBMV.
Dithionite reduced-minus-oxidised spectrum. BBMV were solubilised by the addition of lauryl maltoside (0.03% w/v). The spectral bandwidth, scanning rate and scan resolution were 1 nm, 2 nm sec^{-1}, and 0.5 points nm^{-1}. Full experimental details are given in the methods section.

the lipid environment may be necessary for reductase activity. These results are in agreement with those of Riedel *et al.* (1995) who reported that concentrations of CHAPS exceeding 1% w/v significantly inhibited reducing activity. Similar effects have been described upon solubilisation of the NADH-diferric transferrin reductase in liver plasma membranes (Sun *et al.* 1987). In several experiments the vesicles were pre-heated to 70°C for 5 minutes with the effect that reducing activity was diminished by approximately 65% (0.19 ± 0.03 (3) e^- transferred min^{-1} mg $protein^{-1}$, as compared with control activity: 0.49 ± 0.05 (3), p<0.05) further implicating the involvement of protein.

The reduced-minus-oxidised spectrum of rabbit duodenal BBMV (Figure 3) is typical of a *b*-type cytochrome, and appears similar to that of the cytochrome b_{558} of the neutrophil oxidoreductase (Lutter *et al.* 1985), and the putative oxygen sensitive haem of erythropoietin-producing HepG2 cells (Görlach *et al.* 1993). Similar spectra (not shown) were obtained using mouse and human BBMV preparations. The maxima are relatively broad, with a Soret peak measured at around 422nm and the α-peak at 557nm. Using a *b*-cytochrome extinction coefficient of 21.6mM^{-1} cm^{-1} (Grebing *et al.* 1984) for the α-band, the concentration of *b*-haem obtained for rabbit-BBMV was estimated to be 0.39 ± 0.03 (SEM, n=5) nmoles mg $protein^{-1}$. This is within the range reported for other plasma-membranes (Goldenberg, 1982).

Figure 4 shows potentiometric measurements on the *b*-type cytochrome of the vesicles using the Soret band at 420nm to monitor the redox changes. The results could be approximately fitted to a Nernst equation curve for a single component with a mid-point potential of –50mV. Difficulties of equilibration of the membrane-associated cytochrome make it possible only to place the mid-point potential between 0 and –100mV with any accuracy. Similar problems of non-equilibration are reported for other transmembrane reductases , for example the FRE1 protein (cytochrome *b*) of the yeast *Saccharomyces cerevisiae* (Shatwell *et al.* 1996). More reliable values have been produced for the two b-cytochromes of the neutrophil superoxide generating system (–225mV and –265mV), (Cross *et al.* 1995). However, the unusually low redox potential of the neutrophil cytochromes may reflect the requirement for this system to generate superoxide (E_m= –200mV). Reduction of non-haem iron by the intestinal reductase may not require such negative potentials.

Due to the similarity reported to exist between the yeast *fre*-1 ferri-reductase and the large subunit of cytochrome b_{558}, (Shatwell *et al.* 1996), (an essential component of the oxidoreductase found in the plasma membrane of human phagocytic cells), polyclonal antibodies raised against various subunits of the neutrophil oxidoreductase (p22-*phox*, p47-*phox*, p67-*phox* and gp91-*phox*) were used to probe Western Blots. There was essentially no cross reac-

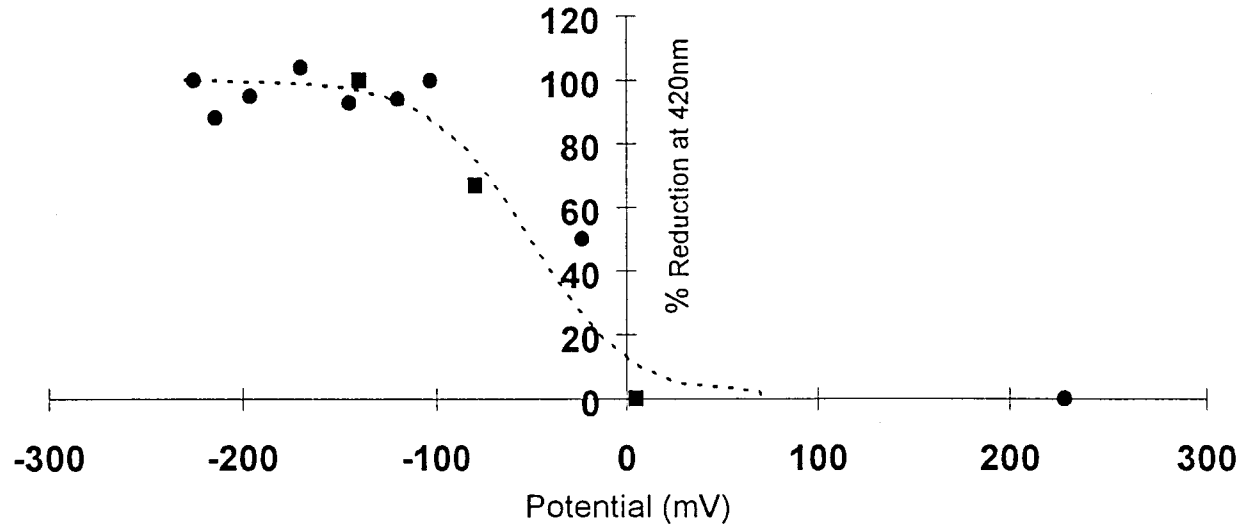

Figure 4. Potentiometric titrations of cytochrome-*b* in brush-border membrane vesicles.
Redox measurements were performed as described in the methods section. Dark circles represent reductive titrations (sodium dithionite) from two different vesicle samples. Dark squares represent oxidative titrations (potassium ferricyanide) from one sample. The dotted line is the theoretical curve for a single species (n = 1) with a mid-point potential of -50 mV.

tivity with either mouse, rabbit, rat or human BBMV preparations. The existence of a low potential *b*-type cytochrome with spectral similarity to the neutrophil cytochrome has been observed in fibroblasts. However, despite a spectral similarity, the fibroblast and neutrophil cytochromes were found to be immunologically distinct (Meier *et al.* 1993). The present results suggest that a similar distinction applies to the intestinal ferric reductase system. The negative result also indicates that there is no significant contamination of vesicle preparations with neutrophils or macrophages which contain the respiratory burst cytochrome b_{558}. It is well established that these cells are present in the intestinal subepithelial region (lamina propria) where they serve to sequester iron (Refsum & Schreiner, 1984).

The *b*-haem shows some reactivity with CO (Figure 5), the Soret peak of the species shifting to the blue. Assuming the CO-reactive haem to be due to contaminating cytochrome *P*-450 with an extinction coefficient of 91.0 mM^{-1} cm^{-1} (Omura & Sato, 1964), the concentration of cytochrome *P*-450 was determined to be 0.16 ± 0.01 nmoles mg protein $^{-1}$. This represents 40% of the *b*-type haem in the sample, but is likely to be an overestimation of cytochrome *P*-450 concentration, since any damaged haem-*b* would also be expected to react with CO. Cytochrome *P*-450 may be reduced by either NADH or NADPH, with NADPH being the more effective electron donor (Omura & Sato, 1964). However in the present study, the BBMV activity is mainly

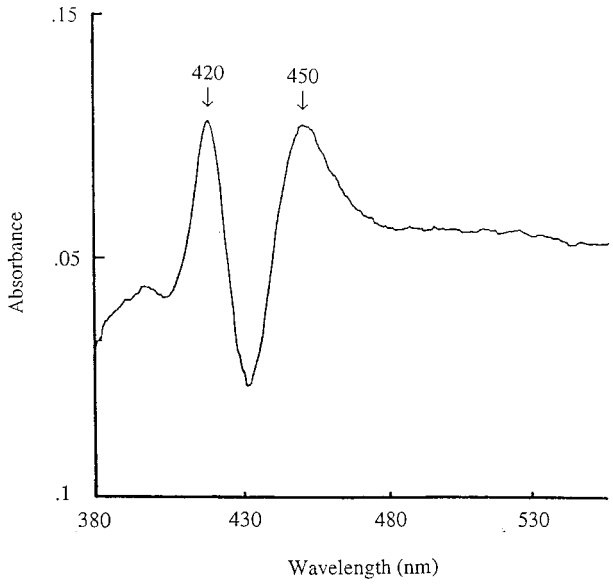

Figure 5. CO reduced-minus-reduced difference spectrum of rabbit BBMV.
BBMV were solubilised by the addition of lauryl maltoside (0.03% w/v). A dithionite reduced spectrum was then recorded, and subsequently subtracted from the spectrum obtained following CO saturation in the presence of dithionite. The spectral bandwidth, scanning rate, and scan resolution were 1nm, 2 nm sec^{-1}, and 0.5 points nm^{-1} respectively.

NADH-dependent. Several earlier studies have concluded that a cytochrome *P*-450 is an intrinsic component of the plasma membrane, although the isolation techniques used in these earlier studies did not necessarily avoid microsomal contamination (Jarasch *et al.* 1979, Bruder *et al.* 1980).

In order to quantitate the amount of cytochrome *P*-450 present in the preparation, the electron paramagnetic resonance (EPR) spectrum of the air-oxidised preparation was examined. Figure 6 shows signals at g=2.40, 2.25, and 1.92 which are characteristic of low-spin oxidised *P*-450 (Rich *et al.* 1975). After signal integration using a copper-EDTA standard (1mM), the spin concentration was calculated to be 0.1 nmoles mg protein^{-1}. This represents approximately 25% of the total *b*-haem concentration determined by optical spectroscopy. We conclude that up to one third of the *b*-type haem in the sample arises from *P*-450, and two thirds represents other cytochromes present in the plasma membranes. Despite the inclusion of EDTA to chelate free manganese ions, there are several weak signals in the g=2 region, indicating the presence of an Fe-S centre, possibly that of mitochondrial succinate dehydrogenase. At the present stage of investigation it is not possible to unequivocally assign signals in EPR spectra to specific haem components, since the plasma membrane may contain several related or similar species, or even a haem that undergoes modification at alkaline pH as is the case with the neutrophil cytochrome b_{558} (Fujii *et al.* 1995).

Table 4 shows the effect of sulphydryl modifying agents and diphenylene iodonium on ferricyanide reducing activity. None of the inhibitors were found to inhibit the basal non-specific reduction of ferricyanide in the absence of BBMV. Both N-ethyl maleimide and *p*-chloromercuribenzoate inhibited the formation of ferrocyanide in the presence of BBMV, suggesting the involvement of essential thiol groups. These observations are similar to the inhibitory effects observed on the reducing capacity of membrane vesicles isolated from Caco-2 cells (Ekmekcioglu *et al.* 1996). The dithionite reduced-minus-oxidised spectrum in the presence of NEM (not shown) was identical to the control spectra, indicating that while the *b*-type haem is able to be reduced in the presence of NEM, a thiol group at a site involved in final electron transfer to substrate may be modified. Diphenylene iodonium (DPI) is a potent inhibitor of both mitochondrial NADH dehydrogenase and the neutrophil respiratory burst oxidase (Gatley & Sherratt, 1976; Cross & Jones, 1986), with the flavoprotein component of each enzyme being modified. The lack of effect of DPI

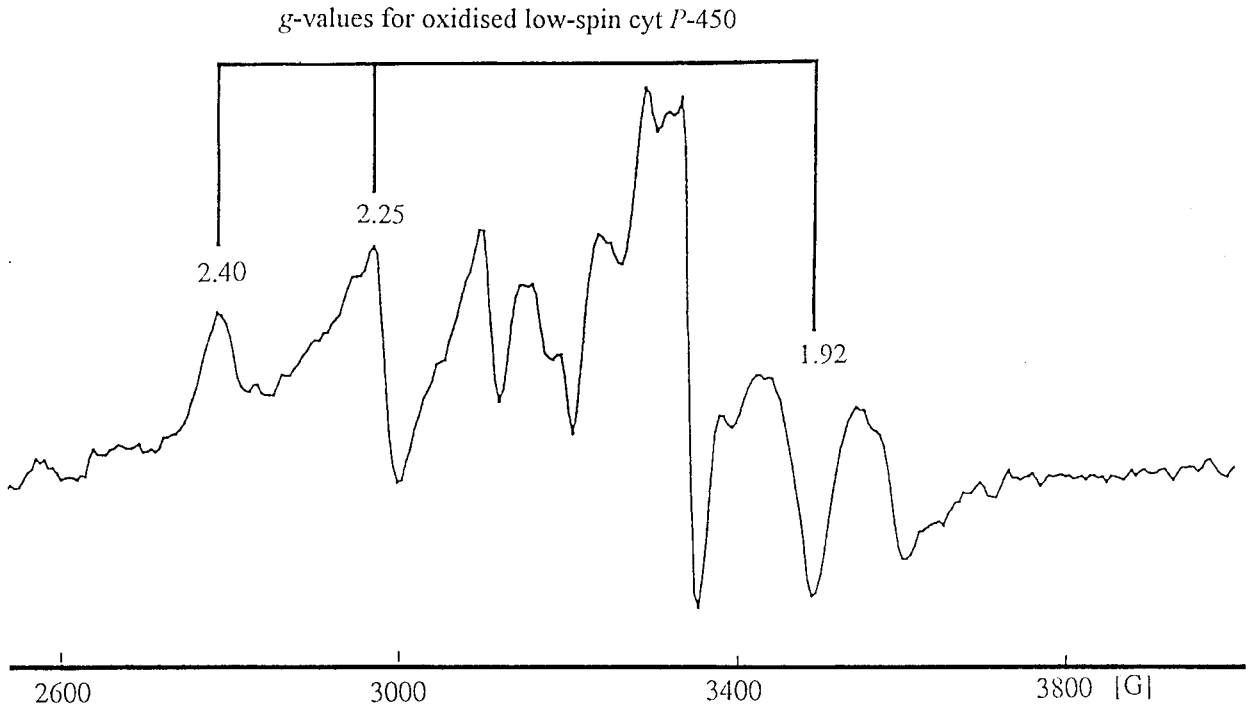

Figure 6. EPR spectrum of native, concentrated rabbit BBMV.
BBMV were concentrated in an EPR tube by centrifugation to 81.5mgml^{-1}. EPR spectra were recorded using a Bruker ESP300 spectrometer at 10db/30K. The field sweep width was 1500G, and the receiver gain 2 x 10^5.

Table 4. The effect of sulphydryl modifying agents, and diphenylene iodonium on BBMV ferricyanide reducing activity.

Ferricyanide reduction by rabbit BBMV was determined using optimal conditions (0.85 mM NADH, 0.03% w/v (~1 mM) lauryl maltoside, 20 μM FMN) in the presence of the inhibitors at the specified concentrations. Data are ± SEM for 3 determinations. *$p < 0.01$ compared with the control group.

Inhibitor:	Specific activity: (μmoles e^- transferred min^{-1} mg $protein^{-1}$)
Control (no addition)	0.48 ± 0.038
NEM (100 μM)	0.23 ± 0.032*
PCMBS (100 μM)	0.21 ± 0.022*
DPI (1 mM)	0.47 ± 0.057

on BBMV reducing activity is consistent with the absence of flavoprotein in the visible and EPR spectra, which may have been lost during the initial vesicle preparation. We have previously shown diphenylene iodonium to be an effective inhibitor of ferricyanide reducing activity in incubations of intact mouse duodenal tissue (Pountney, 1996). The lack of effect of DPI on BBMV reducing activity also excludes NADH coenzyme-Q reductase as a contaminant of BBMV preparations since this latter enzyme of the mitochondrial respiratory chain is sensitive to the inhibitor.

A Sephacryl S-200 gel filtration column was used in an attempt to extract and enrich the ferri-reducing activity. Figure 7 shows how the *b*-type haem eluted in a peak separate to other protein-containing fractions and lipid micelles. Visible spectra of this fraction showed a weak, but characteristic haem spectrum, and only this fraction retained ferricyanide reducing activity (0.16μmoles e^- transferred min^{-1} mg $protein^{-1}$).

The recent cloning of the DCT1 Fe^{2+} transporter (Gunshin *et al.* 1997, Fleming *et al.* 1997) focuses attention on the sources of the Fe^{2+} ions for this carrier. The BBMV ferric-reductase is likely to be an important contributor to the flow of Fe^{2+} ions to the carrier, as we have previously shown that changes in the rate of duodenal iron uptake *in vitro* are paralleled by changes in the rate of mucosal surface reduction (Raja *et al.* 1992, Pountney *et al.* 1996). Certainly ferricyanide and the other unphysiological iron-chelates used *in vitro* are not natural electron acceptors, but the essential requirement of iron together with the poor bioavailability of ferric iron, would suggest that the duodenal reductase warrants further study.

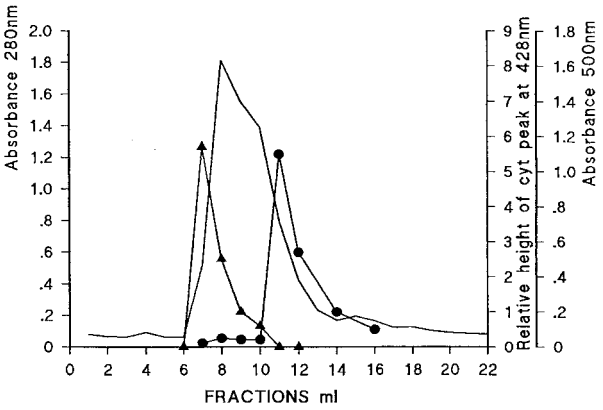

Figure 7. Sephacryl S-200 elution profiles of duodenal BBMV.
A Sephacryl S-200 filtration column was loaded with rabbit BBMV solubilised with lauryl maltoside (1% w/v). The absorbance profile at 280nm (—) represents protein separated from lipid micelles: the later being indicated by the profile at 500nm (●—●). Reduced-minus-oxidised spectra (380–600nm) were recorded for each fraction, and the peak in absorbance at 428nm was taken to represent the amount of cytochrome present (▲—▲). Fractions 6 and 7, which possessed reducing activity, measured by determining ferricyanide disappearance were eluted before the large protein peak. Full experimental details are given in the methods section.

In the present study, in addition to characterising the ferri-reducing activity we have attempted to purify the responsible component. While the initial purification indicates an association of ferric-reducing activity with a *b*-type haem, the complete purification of the duodenal reducing activity remains elusive. Riedel *et al.* (1995) reported an initial ammonium sulphate/isoelectric focusing purification from human duodenal enterocyte membranes, but again much of the activity was lost. Experimentation with whole cells and tissue is problematic as the ferric-reducing component cannot be easily identified, but equally, work with BBMV is complicated by the fact that iron uptake into vesicles does not fully represent the adaptive mechanism observed *in vivo*, and *in vitro* using whole duodenal fragments (Simpson & Peters; 1985, 1986; Raja *et al.* 1987).

In addition to the above observations, the lability of reducing activity in the detergent concentrations necessary for efficient purification, further compounds the problem. Effective solubilisation is necessary since BBMV contain in their core components of the microvillus cytoskeleton such as actin, villin and fibrin (Bretscher, 1983), which tend to aggregate and block chromatography columns even

at relatively high detergent concentrations. The results of the present study indicate a higher stability of reducing activity in lauryl maltoside, which may be useful for future purification studies.

Here we report for the first time the existence of a *b*-type haem that co-purified with the reducing activity in an initial purification. Future functional studies using antibodies raised against this putative component will be necessary to investigate the function of this cytochrome in the reductive assimilation of iron.

Acknowledgements

This work was funded by a project grant from the Wellcome Trust and is a contribution from the King's College Centre for the Study of Metals in Biology and Medicine. We gratefully acknowledge the help of Dr J. Shergill and Professor R. Cammack in performing the EPR experiments. Polyclonal antibodies raised against various subunits of the human neutrophil oxidoreductase were a gift from Dr J. Curnutte of the Scripps Research Institute, La Jolla, USA.

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