Characterization and partial purification of a ferrireductase from human duodenal microvillus membranes

Hans-Dieter RIEDEL, Alvaro J. REMUS, Barbara A. FITSCHER and Wolfgang STREMMEL*

Department of Medicine, University Hospital of Heidelberg, Bergheimer Strasse 58, 69115 Heidelberg, Germany

Reduction of ferric iron in the presence of HuTu 80 cells or duodenal microvillus membranes (MVMs) was investigated. With both systems, NADH-dependent reduction of Fe^{3+} /NTA (nitrilotriacetic acid) was demonstrated, using the ferrous iron chelator ferrozine. Uptake of Fe^{3+} from Fe^{3+} /NTA by HuTu 80 cells was strongly inhibited by addition of ferrozine, indicating that Fe^{2+} is the substrate for the iron uptake system. With isolated plasma membranes it is shown that the reductase activity

is sensitive to trypsin and incubation at 65 °C. The reductase activity could be extracted from the plasma membrane and partially purified by ammonium sulphate precipitation and isoelectric focusing. From the purification and inhibition characteristics we conclude that reduction of ferric iron on the surface of duodenal plasma membranes is catalysed by a membrane protein.

INTRODUCTION

In man and other mammals body iron concentration is maintained through control of duodenal iron absorption. Approximately 50% of the dietary iron is present as haem iron, which is taken up by the mucosal cell via endocytosis [1,2]. The uptake mechanism for non-haem iron is not yet completely understood. Following the identification of several new iron-binding proteins from rat and human duodenal homogenates, a model for ferric iron uptake has recently been proposed [3–8]. According to this model ferric iron, which is soluble in the acidic milieu of the stomach, is first bound to mucins and thus kept soluble in the more alkaline milieu of the upper small intestine. Integrins in the membranes of the absorptive cells facilitate uptake and transfer to mobilferrin, a 56 kDa cytosolic iron-binding protein. A possible association of this pathway with flavin could maintain iron in the appropriate redox state [8,9]. It has been proposed that the membrane potential of the absorptive cell could be the driving force for iron uptake [10], and thus the redox state of iron would be a critical factor. In several cell culture systems it was shown that uptake rates for ferrous iron are 10–200-fold higher than those for ferric iron [11,12] and it was therefore proposed that reduction of ferric iron could be the first step in iron uptake. Reduction of Fe^{3+} in the presence of mouse duodenal fragments and an NADH-dependent diferric transferrin reductase activity in rat liver plasma membranes and endosomal membranes have been reported [13–16]. However, so far the protein responsible for Fe^{3+} reduction has not been identified. There is also a contradictory report claiming that there is no evidence for a membrane-associated diferric transferrin reductase. It has been speculated that the presence of negatively charged membranes and ferrous iron chelators in the incubation assays could shift the ferric/ferrous iron equilibrium and thus mimic an enzymic activity [17].

In this report we examine ferric iron reduction in the presence of intact cells and isolated duodenal plasma membranes. A ferric

iron reductase activity was identified in both experimental systems and partly purified from the membranes using standard protein purification procedures. In addition, inhibition studies supported our finding that iron reduction in the presence of plasma membranes is most probably catalysed by a membrane protein.

MATERIALS AND METHODS

Materials

The cell line HuTu 80 (human duodenal adenocarcinoma) was obtained from A.T.C.C. L-Lysine, L-arginine, ampholine pH 3.5–10.0, nitrilotriacetic acid (NTA), Sephadex G-75 and ferrozine [3-(2-pyridyl)-5,6-bis-(4-phenylsulphonic acid)-1,2,4-triazine] were from Sigma (Munich, Germany). $^{59}\text{FeCl}_3$, specific radioactivity 110–770 MBq/mg of Fe, was from Amersham-Buchler (Braunschweig, Germany). All other chemicals were obtained from Merck (Darmstadt, Germany) at the highest quality available.

Preparation of duodenal microvillus membranes

Microvillus membranes (MVMs) were prepared from freshly obtained duodenum from multi-organ donors as described previously [6].

Reductase assay

Fe^{3+} /NTA solutions (molar ratio 1:4) were freshly prepared before each experiment as described previously [6]. Briefly, a stock solution consisting of 1 mM FeCl_3 and 4 mM NTA in 100 mM HCl was titrated to neutral pH by dropwise addition of 100 mM NaOH and then diluted to the desired Fe^{3+} concentration in the incubation assay. Standard incubation assays

Abbreviations used: MVM, microvillus membrane; NTA, nitrilotriacetic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulphonate hydrate.

* To whom correspondence should be addressed.

contained 50 μM Fe^{3+} /NTA, 200 μM ferrozine, 100 μM NADH and the protein fraction to be tested in a final volume of 1 ml of buffer A (140 mM NaCl, 5 mM KCl, 1 mM CaCl_2 , 1 mM MgCl_2 , 5 mM NaH_2PO_4 and 5 mM Hepes, pH 7.4). For reduction assays with MVM or soluble protein fractions, the reactions were started by addition of the protein samples to the preheated (37 °C) incubation mix. For reduction assays with HuTu 80 cells, culture dishes of 60 mm diam. were used. Cells were grown to approximately 70 % confluence, the culture medium was removed and the cell monolayer washed twice with buffer A. The reaction was started by addition of 1 ml of incubation mix, preheated to 37 °C. Following incubation at 37 °C for the desired time the probes were centrifuged for 1 min at 10000 g and the A_{562} of the supernatants was determined. An A_{562} standard curve (obtained by determining the A_{562} of Fe^{2+} /ferrozine complexes of known concentrations) was used to quantify the results.

Iron uptake studies

For determination of iron uptake HuTu 80 cells grown to about 70 % confluence in 60-mm-diam. culture dishes were used. The cells were washed twice with PBS and then preincubated for 10 min in buffer A containing 6 μM NADH. Radioactive ^{59}Fe /NTA solution was added to give a final Fe^{3+} concentration of 1.5 μM and cells were incubated at 37 °C. The reactions were terminated by washing with ice-cold PBS. Cells were lysed in 0.1 M NaOH and incorporated ^{59}Fe was determined as previously described [6].

Protein purification

Extraction of proteins

Duodenal MVMs were incubated in buffer A with 0.1 % CHAPS for 2 h on ice with gently stirring. The suspension was centrifuged for 5 min at 10000 g and the supernatant containing the solubilized membrane proteins was dialysed against buffer A.

Ammonium sulphate backwash

Solid ammonium sulphate was slowly added to the dialysed supernatant to give 90 % saturation (w/v). After 2 h on ice the sample was centrifuged for 20 min at 10000 g . The supernatant I was discarded, the pellet I resuspended in 60 % saturated ammonium sulphate in buffer A. After 2 h on ice the centrifugation step was repeated. The supernatant II was dialysed against buffer A, the pellet II was resuspended in 40 % saturated ammonium sulphate in buffer A. After another 2 h on ice the sample was centrifuged and the supernatant III dialysed against buffer A. The pellet III was resolved in buffer A and also dialysed against buffer A.

Isoelectric focusing

The ammonium sulphate 40–0 % fraction (pellet III) was dialysed against PBS and applied to an isoelectric focusing gel. The gel was composed of 2.2 g of Sephadex G-75, 70 mg of L-lysine, 70 mg of L-arginine, 5 ml of Ampholines pH 3.5–10.0, 5 ml of protein sample and 38 ml of water. The slurry was distributed on a clean glass plate and electrofocusing was carried out at 4 °C in an LKB 2117 Multiphor chamber for 20 h at 780 V (50 mA). The gel matrix was scraped off the glass plate in 10 fractions (2 cm each), the pH of each fraction was determined and the proteins were eluted with buffer A (2 ml per fraction) for 1 h on ice.

RESULTS

In a first set of experiments Fe^{3+} reduction was determined in the presence of HuTu 80 cells or isolated duodenal plasma membranes. As shown in Table 1, ferrireductase activity could be detected for both experimental systems, using Fe^{3+} /NTA as substrate. With duodenal membranes, reduction of Fe^{3+} was to about 90 % dependent on the addition of NADH, whereas with the cell line HuTu 80, reduction was only stimulated 2.5-fold upon addition of NADH. Obviously, intact cells provide endogenous NADH (or other reducing factors), so that they do not depend on exogenous NADH. The ferrireductase activity of isolated plasma membranes was inhibited by about 80 % following preincubation of the membranes in the presence of trypsin. Similar inhibition ratios were obtained after heat inactivation for 5 min at 65 °C (Table 1).

The results indicated that cell plasma membranes are capable of reducing Fe^{3+} in the presence of NADH. The question whether this ferrireductase activity is important for cellular iron uptake was addressed in the following experiments. HuTu 80 cells were incubated in a buffer containing $^{59}\text{Fe}^{3+}$ /NTA and iron uptake was measured as described earlier [6]. Radioactive iron accumulated in the cells and uptake was linear with time (Figure 1). However, when the ferrous iron chelator ferrozine was added

Table 1 Ferrireductase activity under various conditions

Ferric iron reduction was determined for 30 min at 37 °C as described in the Materials and methods section. The complete incubation mix was composed of 50 μM Fe /NTA (molar ratio 1:4), 100 μM NADH and 200 μM ferrozine in buffer A. The 100% values correspond to 42 nmol/min per mg of MVM and 27 nmol/min per mg of HuTu 80 cells, respectively. Abbreviation: n.d., not determined.

Incubation mix	MVM (% activity)	HuTu 80 (% activity)
Complete	100	100
– NADH	8	37
– Fe /NTA	< 1	< 1
Complete + trypsin	19	n.d.
Complete + 5 min, 65 °C	17	n.d.

Figure 1 Rate of ^{59}Fe uptake from ^{59}Fe /NTA

HuTu 80 cells were incubated for the indicated times in buffer A with 10 μM NADH and 1.5 μM ^{59}Fe /NTA (molar ratio 1:4). The incubation temperature was 37 °C ($\blacktriangle$) or 4 °C ($\blacksquare$). The data are expressed as means of three experiments with triplicate samples.

Table 2 Iron uptake of HuTu 80 cells from $^{59}\text{Fe}/\text{NTA}$

Cells were incubated for 30 min at 37 °C unless otherwise indicated. The complete incubation mix was composed of 1.5 μM $^{59}\text{Fe}/\text{NTA}$ (molar ratio 1:4) and 10 μM NADH in buffer A. Incorporated ^{59}Fe was determined after alkaline lysis of the cells as previously described [6]. All incubations were carried out in triplicate.

Incubation mix	Uptake (pmol of Fe/mg of protein)
Complete	0.185 $\pm$ 0.030
— NADH	0.080 $\pm$ 0.010
+ 10 μM Ferrozine	0.030 $\pm$ 0.010
Complete (4 °C)	0.025 $\pm$ 0.005

Table 3 Purification of the ferrireductase activity

(a) Fractionated ammonium sulphate precipitation. Reductase activity was determined for 60 min at 37 °C in a total volume of 1 ml of buffer A. All assays contained 50 μM Fe/NTA (molar ratio 1:4), 100 μM NADH, 200 μM ferrozine and 10 μg of the protein sample. (b) Isoelectric focusing (IEF). Reductase activity was determined as described above, except that 5 μg of protein of each fraction were used. All incubations were carried out in triplicate. For detailed description of the ammonium sulphate precipitation and the isoelectric focusing steps see the Materials and methods section.

Fraction	Fe^{3+} reduced (nmol/min per mg)
(a) Ammonium sulphate (% satn.)	
(1) 90–60	1.2 $\pm$ 0.4
(2) 60–40	2.8 $\pm$ 0.9
(3) 40–0	35.0 $\pm$ 2.7
(b) IEF (pI range)	
(1) 10.0–8.5	1.5 $\pm$ 0.4
(2) 8.5–7.0	1.0 $\pm$ 0.3
(3) 7.0–6.0	2.5 $\pm$ 0.9
(4) 6.0–4.5	30.5 $\pm$ 3.0
(5) 4.5–4.0	7.5 $\pm$ 1.5

to the incubation buffer, uptake of iron was almost completely inhibited (Table 2). This result indicates that in fact Fe^{2+} could be the substrate for the low-molecular-mass iron uptake system. If so, then reduction of Fe^{3+} at the cell membrane would be the first step in uptake of iron from ferric iron substrates.

The results described so far, especially the inhibition studies, indicated that most probably a protein is involved in the reduction step. To extract the reductase, isolated plasma membranes were incubated in a buffer containing 0.1% CHAPS. In a control assay membranes were incubated in a buffer without CHAPS. Both incubation mixtures were centrifuged and the supernatants and pellets were tested separately for reductase activity. With the CHAPS-treated membranes, approximately 60% of the total activity was found in the supernatant, indicating that in fact a reductase activity can be extracted from the membranes. In the control without CHAPS almost the entire activity remained membrane associated, with less than 5% being in the supernatant. Similar results have been described for an NADH diferric transferrin reductase in liver plasma membranes [13].

To enrich further the ferrireductase of duodenal plasma membranes, the extracted proteins were concentrated by a fractionated ammonium sulphate backwash. The reductase activity was almost entirely found in the 0–40% fraction. This fraction was dialysed and applied to an electrofocusing gel under non-denaturing conditions. The gel was fractionated, the pH of each fraction determined, and the proteins eluted and extensively

dialysed. Reductase activity was found within a pI range of 4.5–6.0 (Table 3). This activity again was NADH-dependent and could be inhibited by preincubation with trypsin or at 65 °C.

DISCUSSION

In this paper we present evidence for a ferrireductase activity in human duodenal plasma membranes. In lower eukaryotes like the yeast *Saccharomyces cerevisiae*, reduction is an essential step for uptake of iron from ferric iron substrates. The enzyme responsible for iron reduction has been characterized by mutant selection and complementation studies, and finally the cDNA of the so-called *frel* gene has been cloned and sequenced [18–21]. Compared with the wild type, mutants in the *frel* locus can only grow in medium with high ferric iron concentrations (400 μM versus 20 μM , respectively). On the other hand, both mutant and wild-type strains grow equally well in media containing ferrous iron. From these data it was concluded that ferrous iron is the substrate for the iron transport system and that the ferrireductase is not identical to the iron transport protein [22]. Whereas the ferrireductase of yeast has been cloned and sequenced, the existence of an analogous enzyme in the plasma membranes of higher eukaryotes is still under debate. It has been shown that reductive mechanisms are important for intestinal uptake of iron from ferric maltol and ferric NTA. Uptake from these substrates could be enhanced in the presence of ascorbic acid, whereas it was inhibited by the ferrous iron chelator bathophenanthroline sulphate [15]. Both observations indicate that, like with yeast, reduction is a prerequisite for iron uptake from ferric iron substrates. Iron reduction in the presence of intact cells has also been observed in a number of *in vitro* studies [11,23–26]. To answer the question whether or not a membrane protein is involved in ferric iron reduction we have first studied reductive activities in the presence of intact cells and isolated cell membranes. Direct evidence for the importance of ferric iron reduction for uptake came from the finding that the ferrous iron chelator ferrozine almost completely blocked iron uptake of HuTu 80 cells when ferric NTA was used as substrate (Table 2). HuTu 80 cells showed ferrireductase activity which was only partly dependent on exogenously added NADH, indicating that in fact cellular transmembrane electron donors contribute to the reducing activity. Not unexpectedly, with isolated and purified duodenal plasma membranes, reduction of iron was completely dependent upon addition of NADH. The reductase activity was strongly inhibited by either preincubation at 65 °C or with trypsin. Both findings, heat inactivation and inactivation by a protease, indicated that most probably a protein was involved in the membrane-associated reducing activity. To substantiate these findings further we attempted to extract and enrich this activity from the duodenal plasma membranes. A reductase activity could partly be extracted by incubation in a buffer containing low concentrations (0.1%) of the detergent CHAPS. Higher concentrations (1%) resulted in an almost complete loss of activity in the extracted membranes. However, under these conditions no reductase activity was found in the supernatants (H. D. Riedel, A. J. Remus, B. A. Fitcher and W. Stremmel, unpublished work). It had to be concluded that high concentrations of CHAPS on the one hand effectively solubilize the reductase, and on the other hand irreversibly inactivate enzyme activity. The 0.1% CHAPS-extracted enzyme activity was further purified and concentrated by ammonium sulphate precipitation and electrofocusing. A protein fraction within the pI range of 4.5–6.0 showing ferrireductase activity was finally recovered. As with intact membranes, the solubilized

enzyme was heat labile and could be inactivated by tryptic digestion.

In conclusion, our data strongly suggest that reduction at the surface of the cell membrane is an enzyme-catalysed process. This enzyme can be extracted from the membrane and still exhibits reductase activity in the solubilized form. Extraction of the membranes is a critical step in the purification procedure. Even under the mild conditions described, a rapid loss of enzyme activity was sometimes observed. We are currently searching for conditions to stabilize the solubilized enzyme, so that further purification and characterization of the enzyme can be achieved.

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