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Biochimica et Biophysica Acta 1195 (1994) 276–280

BBA Biochimica et Biophysica Acta

A plasma membrane NADH oxidase is involved in copper uptake by plasma membrane vesicles isolated from rat liver

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Received 31 January 1994; revised 4 July 1994

Abstract

The accumulation of copper (Cu) by hepatocytes is initiated by the binding of Cu in either a $CuHis_2$ complex or as a CuHisAlb ternary complex, followed by transfer of the metal alone across the cell membrane. In this paper, we provide evidence that the transfer involves reduction of cupric (Cu(II)) copper to cuprous (Cu(I)) copper and further we show that membrane-bound NADH oxidase can provide the electron required for the reduction. ^{64}Cu uptake by rat liver plasma membrane vesicles is stimulated by the addition of NADH, but not NAD^+. The stimulation increases the V_{max} from 4.75 ± 0.02 to 8.38 ± 0.40 nmol Cu/mg protein per min ($P < 0.05$, mean $\pm$ S.E., $n = 3$) without significantly altering the $K_{0.5}$ (1.52 ± 0.17 and 2.10 ± 0.22 μmol/l; with n values of 1.30 ± 0.01 and 1.43 ± 0.10, respectively; analysing by the Hill equation). Correspondingly, addition of $CuHis_2$ stimulated NADH-oxidase activity by a maximum of 7.4 ± 2.1 nmol/mg protein per min ($P < 0.01$, mean $\pm$ S.E., $n = 5$) at 5 μmol/l and a NADH concentration of 150 μmol/l. Ascorbic acid also stimulated copper uptake, and points to a reductive dissociation of copper prior to its movement into the cell. Our data indicate that membrane bound enzymes can provide an electron for the reduction of copper prior to uptake and suggest a physiological role for the plasma membrane NADH oxidase.

Keywords: NADH oxidase; Plasma membrane; Copper reduction; Copper transport; (Rat hepatocyte)

1. Introduction

The nutritional trace element copper (Cu) is essential to cellular functioning of all living systems, as a cofactor for several intracellular enzymes [1]. Following absorption across the intestine, copper is taken up by the liver and is later released attached to the protein, ceruloplasmin.

The copper that is taken up by the hepatocyte from portal blood is believed to derive from either the Cu-histidine ($CuHis_2$) [2,3], or the Cu-histidine-albumin (CuHisAlb) complex [4] in the plasma. It is possible that the complexes look similar, with the Cu atom coordinating with the imidazole-N and amino-N of the histidine residues and the remainder of the binding moiety being irrelevant [4]. Before uptake, the Cu is removed from the complex and transferred alone into the cell [2–4].

How the Cu is removed from its ligand is not known. Preliminary data have shown that ascorbic acid can stimulate uptake in cultured rat hepatocytes ([5]; Bingham, M.J. and McArdle, H.J., unpublished results) and mouse hepatocytes (McArdle, H.J., unpublished results). The process has also been studied in relation to Cu uptake from ceruloplasmin by K562 cells. Percival and Harris [6] have shown that ascorbic acid increases Cu uptake, and that cuprous (Cu(I)) copper chelators block the effect.

We hypothesize, therefore, that reduction of cupric (Cu(II)) copper is a necessary prerequisite for Cu uptake by the hepatocyte. A reductase has been described which could fulfil this function [7]. However, there are no experimental data linking the activity of this enzyme with Cu uptake.

In this paper, we have tested this hypothesis. We have isolated plasma membrane vesicles from rat liver and have incubated them with $[^{64}Cu]CuHis_2$ in the presence of NADH, to determine whether uptake is stimulated. Similarly, we have investigated the effect of $CuHis_2$ on NADH-oxidase activity, using the same system described by Brightman et al. [8]. Our results support our hypothesis that the plasma membrane NADH oxidase can provide electrons for the reduction of Cu^{2+} to Cu^+ prior to uptake by the cell.

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SSDI 0005-2736(94)00203-7

2. Materials and methods

2.1. Rat liver vesicle preparation

The membrane vesicles were prepared from the liver of adult male Wistar rats using the method of Van Amelsvoort and co-workers [9], as modified by Rosenthal et al. [10] and Lindsay, Duthie and McArdle (unpublished data). Briefly, the animals were anaesthetized by Nembutal (Sanofi Sante Animale SA, Paris, France), and the liver was perfused, after severing the inferior vena cava, with approx. 25 ml of isotonic sucrose buffer A (250 mM sucrose, 0.2 mM $CaCl_2$, 10 mM Hepes-KOH (pH 7.5)) heated to 37°C, through the portal vein with a 23G needle to remove blood. All subsequent steps were carried out at 4°C.

The liver was removed, weighed, washed and homogenized in three volumes of Buffer A. It was centrifuged at $1000 \times g$ for 10 min and the pellet was discarded. The supernatant was then centrifuged at $20\,000 \times g$ for 30 min. The pellet was resuspended in sucrose Buffer A and layered on top of a sucrose step gradient prepared from 210 and 410 g/l sucrose in 10 mM Hepes-KOH. The membranes were centrifuged at $50\,000 \times g$ in a SW 41 Ti rotor in a Beckman Spinco L50 centrifuge. Membranes isolated at the 210/460 g/l interface were collected, diluted with isotonic sucrose and sedimented at $80\,000 \times g$ in a 70.1 Ti rotor. This produced a cloudy, buff coloured layer overlaying a reddish brown pellet. Only the cloudy layer was taken and resuspended in isotonic Buffer B (as buffer A but with 5 mM $MgCl_2$). The membranes were vesiculated by passing through a 19G needle, aliquoted and stored at −70°C at a concentration of about 5 mg protein/ml. Control experiments have shown that vesicles stored at −20°C lost transport activity.

2.2. Assay of enzymes and purity

Succinate dehydrogenase (mitochondrial membrane enzyme marker) activity was measured using the method of Van Amelsvoort et al. [9] adapted for use in in a Bio-Rad Model 3550-UV plate reader. 2.5 μl homogenate or vesicles were added to individual wells of Dynatech microplates. The reaction was started by adding 250 μl reaction buffer containing 3 mM sodium succinate, 0.3 mM KCN, 0.1 mM cytochrome *c* in 50 mM phosphate buffer (pH 7.5). Data were analysed using the Bio-Rad Kinetic Collector version 2 on a Macintosh computer. Data are presented as absorbance change (ΔA) per mg protein per min. NADPH-dependent cytochrome-*c* reductase (microsomal marker enzyme) activity was measured as described above except that 0.1 mM NADPH replaced sodium succinate in the reaction mixture. In all experiments, homogenates and their vesicles were measured on the same microplate.

5′-Nucleotidase (EC 3.1.3.5) activity was assayed according to Avruch and Wallach [11]. The 0.4 ml assay medium contained 1.8 mM $MgCl_2$, 500 mM Tris-HCl (pH 8.0), 4 μM [U-^{14}C]AMP (0.5 μCi/ml) and 200 μg protein, i.e., vesicle suspension or homogenate. Incubation at 37°C was continued for 60 min. Blank control samples contained no enzyme. The reaction was stopped by the addition of 0.3 ml of 0.25 M $ZnSO_4$. Protein and unhydrolysed AMP were precipitated by adding 0.3 ml $Ba(OH)_2$. The tubes were centrifuged for 10 min at 8000 rpm in an Eppendorf 5415 centrifuge. A 0.5-ml aliquot of supernatant was added to 5 ml Ecoscint scintillation fluid (National Diagnostics, Manville, NJ) and counted in an LKB liquid scintillation counter.

2.3. Preparation of ^{64}Cu-labelled medium

^{64}Cu was prepared by irradiation of 3 mg copper wire (99.999%, Ventron, Karlsruhe, Germany) for 24 h in a thermal neutron flux of $10^{17}/m^2$ per s in the IRI reactor. The ^{64}Cu solution produced (diluted in 150 mM sodium acetate buffer (pH 5.6) had a specific activity of about 150 MBq/mg.

Unless otherwise stated, the copper was added as 2 μM copper dihistidine ($^{64}CuHis_2$) at 5 MBq/l diluted in Balanced Salt Solution (BSS), (136 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 4 mM $NaHCO_3$, 18 mM Hepes, 5.5 mM glucose (pH 7.4)). ^{64}Cu solution was mixed with histidine (pH 7.0) at a ratio of at least 1:10 (2 μM Cu^{2+}, 20 μM histidine) before being added to the incubation medium.

2.4. Experimental procedures

Stimulating agents were added to the medium with vesicles (final protein concentration 250 μg/ml) at the concentrations given in the figure legends, and the reaction was started by adding the [^{64}Cu]$CuHis_2$. All uptakes were carried out at 25°C for 1 min. Aliquots of the incubation medium (80 μl) were filtered through 0.45 μm Millipore filters (pre-washed with 9 g/l NaCl containing 0.5 mM $CuCl_2$ and washed three times with 2 ml of ice-cold incubation medium with 10 mM EDTA [12]. The filters were removed from the filtration apparatus (Hoefer Scientific Instruments, San Fransisco, CA), and radioactivity measured in a Packard 5000-gamma counter. Control experiments (filtering 80 μl without vesicles) demonstrated that background binding to filters was neglible, but appropriate blanks (incubation medium with appropriate additions and no vesicles) were always included.

Uptake measurements were made by incubating the vesicles with a constant amount of [^{64}Cu]$CuHis_2$ and increasing amounts of unlabeled copper-dihistidine (0 to 15 μM), in the absence or presence of 1 mM NADH (Boehringer, Mannheim, Germany). Estimates of apparent K_m or $K_{0.5}$ and V_{max} were made on the untransformed data of each preparation using an iterative nonlinear least-

squares method (Ultrafit, Biosoft, Cambridge, UK), and considered significantly different when $P < 0.05$. Correlation coefficients always exceeded 0.98 and did not fit equations that included nonspecific binding parameters.

2.5. NADH-oxidase assay

Plasma membrane NADH oxidase was assayed at 25°C, in BSS buffer (pH 7.4), 150 μM NADH (Boehringer, Mannheim, Germany) with 1 mM KCN [8], to inhibit any mitochondrial NADH oxidases contaminating the plasma membranes. The assay was started with the addition of 0.1 mg of plasma membrane protein in a final volume of 2.0 ml. The reaction was monitored by the decrease in the absorbance at 340 nm. A blank rate was determined for 5 min. $CuHis_2$ was then added, and the rate was measured for a further 10 min. The absorption coefficient used for NADH was 6.21 $mM^{-1}\,cm^{-1}$. Protein assays were carried out using the Bio-Rad protein method as described by Bradford [13], using bovine serum albumin as standard.

3. Results

Vesicles membrane purity was assessed by marker enzyme analysis. 5'-Nucleotidase activity increased 28-fold (from 5.4 ± 0.3 cpm/mg protein to 154 ± 46 cpm/mg protein; mean ± S.E., $n = 4$), while succinate dehydrogenase activity (a mitochondrial marker) was not detectable in the vesicles. NADPH–cytochrome-*c* reductase also increased (from 0.3 ± 0.07 ΔA/mg protein per min to 1.05 ± 0.44 ΔA/mg protein per min, mean ± S.E., $n = 4$), indicating a slight enrichment with endosomal membranes, as has been reported by others [9].

Addition of NADH stimulated uptake of ^{64}Cu from [^{64}Cu]$CuHis_2$ (Fig. 1). The concentration dependence of NADH in the ^{64}Cu uptake was most prominent using concentrations up to 50 μmol/l, with slighty greater stimulation with higher NADH concentrations. The stoichiometry of the process (0–50 μmol/l) can be calculated and was found to be 0.91 ± 0.19 ($P < 0.001$, mean ± S.E., $n = 5$), suggesting that 1 mol of NADH was oxidized per Cu^{2+} transported.

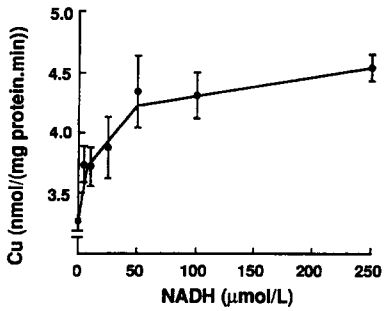

Fig. 1. Effect of NADH on copper uptake by rat liver plasma membrane vesicles at 25°C for 1 min. Vesicles were incubated with various concentrations of NADH and a [^{64}Cu]$CuHis_2$ concentration of 2 μM. The results are the means ± S.D. of five preparations of vesicles.

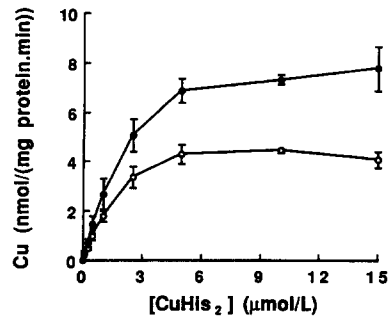

Fig. 2. Uptake of Cu in the absence (○) or presence of 1 mM NADH (●). The uptake rate was measured over 1 min at 25°C in the presence of increasing concentrations of [^{64}Cu]$CuHis_2$. The results are the means ± S.D. of five preparations.

To determine how NADH altered Cu uptake, we incubated the vesicles with increasing $CuHis_2$ concentrations in the presence and absence of 1 mmol/l NADH. The results are shown in Fig. 2. NADH stimulated Cu uptake, increasing V_{max} without altering the $K_{0.5}$ (Table 1). As has previously been shown [18], the n value is greater than 1, indicating co-operativity in the transport process, but n was not altered by the presence of NADH.

The effect is specific for NADH, since adding NAD^+ had no effect on Cu uptake (results not shown).

The corollary of the effect was assessed by incubating vesicles with NADH in the presence of increasing $CuHis_2$ concentrations. First, we measured background oxidation and NADH oxidation in the presence of diferric transferrin [7,8]. There was a slow oxidation of NADH (1% loss of absorbance at 340 nm over a 15-min period) without vesicles and diferric transferrin. The addition of diferric transferrin, but not apotransferrin, to plasma membrane vesicles in the presence of NADH resulted in an increased rate of NADH oxidation (data not shown), as has been reported by other groups [7,8]. Oxidation of NADH was dependent on the presence of membrane, NADH and $CuHis_2$ (Table 2), although a significant basal activity (without added $CuHis_2$) was measured.

In the presence of $CuHis_2$, NADH-oxidase activity was stimulated, reaching an apparent maximum at about 2 μmol/l $CuHis_2$, with an NADH concentration of 0.15 mM (Fig. 3). Due to a broad range in the basal activities

Table 1
Kinetic parameters, apparent V_{max}, $K_{0.5}$ and n values, for vesicles incubated in the absence or presence of 1 mM NADH at 25°C

NADH (mM)	V_{max} (nmol Cu/ mg protein per min)	$K_{0.5}$ (μM)	n
0	4.75 ± 0.02	1.52 ± 0.17	1.30 ± 0.01
1	8.38 ± 0.40 [a]	2.10 ± 0.22	1.43 ± 0.10

Values are means ± S.E., $n = 5$. Data were fitted to the Hill equation using a nonlinear iterative computer program (see Materials and methods).
[a] Significantly (Student's t-test; $P < 0.05$) different.

Table 2
Requirements for NADH oxidation by rat liver plasma membrane vesicles

Medium	NADH oxidation (nmol min^{-1} mg^{-1})
Complete assay medium [a]	34.5
Minus NADH	0.0
Minus membrane protein	0.7
Minus $CuHis_2$	21.2

[a] BSS buffer (pH 7.4), 150 μM NADH, 1 mM KCN, 0.1 mg membrane protein, 2 μM $CuHis_2$.

(in the absence of $CuHis_2$) of the NADH oxidase in the various vesicle preparations, the differences just failed to reach statistical significance. However, as with the analysis of Fig. 1, the stoichiometry was determined to be close to 1 (1.1 ± 0.5, $P < 0.01$, mean ± S.E., $n = 5$).

On the other hand, some inhibitors (adriamycin and atebrin) of the transplasmalemmal electron transport in intact cells inhibited the ^{64}Cu uptake (Table 3). Mitochondrial inhibitors azide, cyanide and heptylhydroxyquinone *N*-oxide (HOQNO) did not inhibit (Table 3).

To determine whether other electron donors could also stimulate uptake, we incubated vesicles in the presence of increasing concentrations of ascorbate and 2 μM [^{64}Cu]$CuHis_2$. As expected, uptake was also increased (Fig. 4) showing that the transport system does not have an absolute requirement for NADH-derived electrons.

4. Discussion

Several cell types, including hepatocytes, have been shown to reduce impermeable oxidants such as ferricyanide outside the cell [14] and it was demonstrated that this occurred without the secretion of large quantities of reducing agents. An NADH-oxidizing system in plasma membranes of rodent liver was first identified cytochemically by Morré et al. [15] and the finding was supported biochemically by Crane and Low [16].

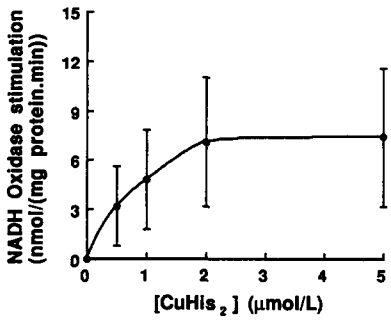

Fig. 3. Oxidation of NADH by rat liver plasma membrane vesicles in the presence of $CuHis_2$. In the values presented in the figure, the NADH oxidase activity, prior to the $CuHis_2$ addition was subtracted from the rate of NADH oxidation after $CuHis_2$ addition. Basal activity (in the absence of $CuHis_2$) of the NADH oxidase in vesicle preparations ranged from 12.9 to 23.0 nmol/min per mg protein when measured in BSS buffer (pH 7.4), at 25°C, with 150 μM NADH and in the presence of 1 mM KCN. After about 5 min $CuHis_2$ was added and the accelerated rate was monitored. Absorbance was measured at 340 nm.

Table 3
Inhibition of the NADH stimulating effect on the ^{64}Cu uptake by rat liver plasma membrane vesicles in the presence of some specific inhibitors (cf. [7])

Agent	Concentration	Control activity (%)
Amiloride	0.2 mM	69 ± 10
Adriamycin	1 μM	79 ± 5
Azide	1 mM	107 ± 8
Cyanide	1 mM	95 ± 6
HOQNO	1 μM	139 ± 15

HOQNO is heptylhydroxyquinoline *N*-oxide (Sigma). The ^{64}Cu uptake was measured over 1 min at 25°C in the presence of 2 μM [^{64}Cu]$CuHis_2$, 0.5 mM NADH and inhibitors with indicated concentrations. The results are expressed as % control activity in the absence of inhibitors, and are the mean of three preparations.

For many years, the function of this enzyme has remained unclear. Originally, it was thought that it may act as to reduce transferrin iron (cf. [17]), but nowadays this is not generally accepted. The activity can be modulated by growth factors and hormones, indicating that it may be involved in cell growth regulation [8,14]. We can now suggest that the enzyme is also involved in Cu uptake by the liver.

Copper is assumed to be delivered to the liver as either $CuHis_2$ or as a CuHisAlb ternary complex [2–4]. Either way, the complex has an extremely high affinity for Cu^{2+}. The way the copper can be removed is to reduce it. How this may be accomplished has previously been unclear.

In K562 cells, Percival and Harris [6] have shown that ceruloplasmin copper can be accumulated through a reduction process, but have suggested that ascorbic acid may play the central role or that electrons can be transported across the cell membrane. Our data show that NADH can provide the electrons for the reduction of Cu^{2+}, as the first step in the Cu uptake process. The stoichiometry of 1:1 fits well with each NADH oxidized providing one electron for each Cu^{2+}. Whether the enzyme involved is NADH oxidase or NADH–ferricyanide reductase, which have been shown to be separate enzymes [8], is not so clear. The

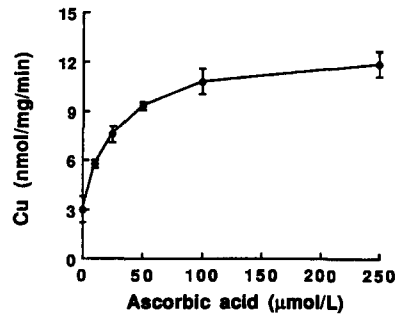

Fig. 4. The effect of ascorbate on copper uptake by rat liver plasma membrane vesicles at 25°C for 1 min. Vesicles were incubated with various concentrations of ascorbic acid and a [^{64}Cu]$CuHis_2$ concentration of 2 μM. The results are the means ± S.D. of four preparations.

inhibition of the NADH stimulating effect on the ^{64}Cu uptake by adrimycin and atebrin is consistent with the observation by Sun et al. [7], when studying the properties of the NADH diferric transferrin reductase. Although the inhibitions effects were somewhat smaller than observed by Sun et al. [7], inhibition provides evidence that a reduction step by a transferred electron precedes the uptake of copper.

From the data it is clear that a substantial binding and uptake takes place in the absence of electron donors. There are two possibilities to explain such an effect. Firstly, it should be born in mind that a large proportion of the copper associated with the membrane under these conditions is bound rather than accumulated [18]. Secondly, it is feasible that other reducing equivalents are present in the hepatocyte membrane. Ascorbate has been shown to catalyze the dissociation of copper and bathocuproine, a chelator of cuprous copper, inhibits uptake [6]. Both steps point to a reductive dissociation of copper prior to its movement into the cell.

How the transport system is associated with the enzyme(s) remains to be investigated. Since ascorbic acid also stimulates uptake, it is unlikely that the enzyme itself is the transporter. Rather, we would suggest that they are in close proximity on the plasma membrane. A copper transport protein was recently characterized in *Saccharomyces cerevisiae* [19], and the data also show a reduction of copper as part of the uptake process.

In Fig. 5 we propose our model which although speculative, does have considerable heuristic value. It incorporates not only data presented in the present paper but uses also information collected by others [20,6]. The transporter is a dimer, linked by at least one disulfide bridge. There is

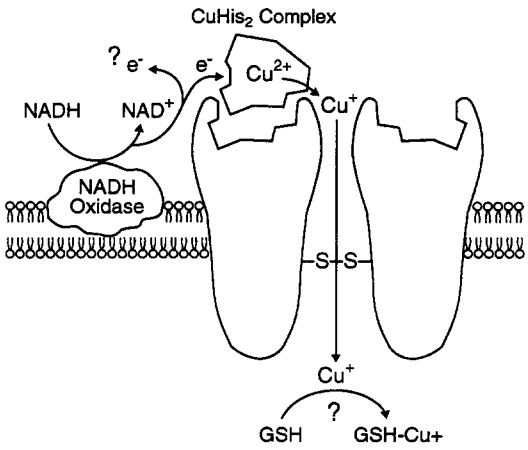

Fig. 5. Suggested model for copper uptake by mammalian cells. Shown are the binding of $CuHis_2$ complex to a specific membrane component. The binding site on the transporter recognizes the Cu-complex [20]. Following binding, the cupric ion is reduced by an electron donated by the oxidation of NADH, released from its associated ligand and it is moved through the membrane. As an alternative copper atoms can be discharged from the complex in response to electrons being donated by ascorbate [6].

co-operativity between the binding sites, although the mechanism at the molecular level is not known. The oxidation of NADH provides the electron required for releasing Cu^{2+} from its associated ligand and it is moved through the membrane into the cell. The intracellular events are not yet definitive, but we speculated that within the cell copper is first bound to glutathione as has been demonstrated by Freedman and collaborators [21].

Clearly, the model is preliminary but does explain most of our current knowledge of Cu uptake across the liver, in fact it may well be that this mechanism underlies uptake of copper from all the ligands available to the metal in plasma [6,20].

Acknowledgements

This work was supported by the Wellcome Trust and the British Council.

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