Cupric Ion-mediated Active Transport of Amino Acids in Membrane Vesicles of \textit{Mycobacterium phlei}*

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In the absence of substrate oxidation, membrane vesicles of \textit{Mycobacterium phlei} were shown to accumulate proline, glutamine, and glutamic acid mediated by Cu$^{2+}$. The Cu$^{2+}$-mediated uptake of amino acids was found to be an active process and required the presence of amino acid binding protein(s). Thus, membrane vesicles which lack active transport of a particular amino acid because of the loss of specific binding protein(s) do not accumulate these amino acids even in the presence of Cu$^{2+}$. The Cu$^{2+}$-mediated uptake of proline exhibited a specific requirement for Na$^+$. The Cu$^{2+}$-mediated uptake process was not inhibited by anaerobiosis and respiratory inhibitors, such as sodium azide and $m$-chlorocarbonylcyanide phenylhydrazone. However, atebrenin and o-phenanthroline were found to inhibit the uptake process. The results with various sulfhydryl blocking agents suggest that oxidation of available sulfhydryl residues of the membrane protein(s) is required for the Cu$^{2+}$-mediated uptake process. The uptake of amino acid was inhibited by $p$-chloromercuzbenzene sulfonic acid or N-ethylmaleimide when added prior to the addition of Cu$^{2+}$. In addition, efflux of amino acids, previously accumulated to the steady state level occurred when sulfhydryl reducing agents such as glutathione, cysteine, or diithiothreitol were added. Evidence is presented that disulfide configuration of the carrier or membrane protein(s) favors the transport of amino acids against the concentration gradient, whereas the sulfhydryl state of these proteins induces the efflux process. Studies show that there was 1:1 stoichiometry for the oxidation of available sulfhydryl residues in the membrane to the reduction of Cu$^{2+}$, and the proton gradient generated as a result of the oxidation of sulfhydryl groups is presumably the driving force for the uptake of these amino acids.

Numerous studies have been carried out to delineate the relationship between active transport of metabolites and energy yielding processes in bacterial systems (1-10). Studies carried out with membrane vesicles of \textit{Mycobacterium phlei} have shown that the uptake of proline, glutamine, and glutamic acid is coupled to electron transfer (11-14). However, there was no apparent correlation between the rate of substrate oxidation and the level of accumulation of these amino acids (11-14). Moreover, it has been observed that the energy requirements for the uptake of proline differed from that of glutamine and glutamic acid in membrane vesicles of \textit{M. phlei} since proline uptake exhibits a strict requirement for Na$^+$ ions (14). The uptake of proline, in contrast to glutamine and glutamic acid in membrane vesicles of \textit{M. phlei}, has been shown to require specific phospholipids (13). Recently, it has been shown that the mechanism of active transport of proline differs from that of other amino acids in whole cells of \textit{M. phlei} (15). Studies by Berger and Heppel (16) in \textit{Escherichia coli} cells have also indicated that the nature of energy coupling to the active transport of various amino acids differs depending upon whether the permease is shock-sensitive or shock-resistant. Shock-resistant transport systems were coupled directly to an energized state of the membrane, whereas shock-sensitive systems required the synthesis of ATP.

Recently, Yankofsky and Brodie (17) observed that the uptake of proline in membrane vesicles of \textit{M. phlei} can occur in the absence of exogenous substrates and can be stimulated by chemical agents such as Cu$^{2+}$. The results presented in this communication demonstrate that, in addition to proline, Cu$^{2+}$ mediated the uptake of glutamine and glutamic acid against a concentration gradient. In addition, evidence is presented that the Cu$^{2+}$-mediated uptake of these amino acids in membrane vesicles of \textit{M. phlei} is a carrier-dependent process since leucine and other amino acids, whose binding or carrier proteins have been lost during the preparation of these vesicles (sonic oscillation), were not accumulated by Cu$^{2+}$. The Cu$^{2+}$-mediated uptake of proline exhibited a specific requirement for sodium ion, as has been observed for substrate-mediated transport. Studies indicate that the oxidation of the sulfhydryl group of either carrier or membrane protein(s) is essential for the uptake of amino acids, and the pH gradient generated may provide the driving force for the uptake of these amino acids.

**EXPERIMENTAL PROCEDURES**

Preparation of Membrane Vesicles—\textit{Mycobacterium phlei} (ATCC 354) whole cells were grown and the membrane vesicles (electron transport particles) were prepared by sonic disruption of washed cells (18).
Measurement of Oxidation—The effect of cupric ions on endogenous oxidation as well as on substrate-induced oxidation was measured polarographically at 30°C with an oxygen monitor (19) and by manometric techniques with a Gilson respirometer.

Assay of [14C]Proline, [14C]Glutamine, and [14C]Glutamic Acid Transport—The method for measuring transport of proline, glutamine, and glutamic acid in the membrane vesicles was essentially the same as described in earlier publications (13, 14). The 1.5-ml assay system contained 25 mM potassium/4-(2-hydroxyethyl)l-piperazineethanesulfonic acid buffer (pH 7.0), 5 mM MgCl2, 10 mM NaCl, and 10 μM [14C]-labeled amino acids.

Assay of Transport under Anaerobic Conditions—To study the cupric ion-mediated transport of proline, glutamine, and glutamic acid under anaerobic conditions, Thunberg tubes were used. Oxygen was removed from the reaction system with a vacuum pump and the sealed environment was saturated with argon gas. This was repeated three times to ensure anaerobic conditions. The reaction was initiated by the addition of the indicated amount of [14C]-labeled amino acids from the side arm. The tubes were incubated in a shaking water bath at 30°C for various time intervals, and at the end of the indicated time interval, the incubation mixture was diluted 10 times with 0.05 M potassium phosphate buffer, which was deoxygenated (pH 7.0), and filtered through a millipore filter, as described previously.

Kinetics of Uptake—The Km and Vmax of different amino acids in the presence of varying concentrations of cupric chloride were determined by standard Lineweaver plots.

Sulfhydryl Group Determination—The sulfhydryl groups were determined spectrophotometrically by the method of Ellman (20) using 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB).

Estimation of Cuprous Ions—Assay of Cu++ concentration under different assay conditions was carried out using specific cuprous ion chelating agent, bathocuproine disulfonate sodium salt (Sigma), similar to the method of Yonetani (21).

Measurement of Internal pH and pH Gradient—The internal pH of membrane vesicles was calculated from the Henderson-Hasselbalch equation by measuring the distribution of ionized and unionized [2-14C]DMO in the membrane vesicles, essentially according to the published procedure (22). The external pH was measured at the end of incubation with DMO using a Corning digital pH meter with an expanded scale. The pH gradient was then calculated from the difference between internal and external pH.

The intravesicular volume was calculated from the difference between the total \( [\text{H}_2\text{O}] \)-permeable space and \( \langle \text{carboxyl-14C} \rangle \)extramembrane impermeable space (23, 24).

Identification of Radioactive Materials Accumulated by Membrane Vesicles—Following a 10-min incubation, the membrane preparations were removed from the reaction mixture by Millipore filtration. The Millipore filter was washed with 50 ml potassium phosphate (pH 7.0) buffer and extracted three times with boiling water. The extracts were combined, lyophilized, and chromatographed on Whatman No. 1 filter paper using cold amino acids as markers and an expanded scale. The pH gradient was then calculated from the difference between internal and external pH.

The intravesicular volume was calculated from the difference between the total \( [\text{H}_2\text{O}] \)-permeable space and \( \langle \text{carboxyl-14C} \rangle \)extramembrane impermeable space (23, 24).

Materials—The radioactive chemicals were purchased from New England Nuclear. All other reagents were of reagent grade purity and were purchased from Sigma.

RESULTS

Uptake of Proline, Glutamine, and Glutamic Acid Mediated by Cu++ Ions in Membrane Vesicles—The uptake of proline, glutamine, and glutamic acid in the membrane vesicles of \( \text{M. phlei} \) has been shown to require substrate oxidation and to proceed against a concentration gradient (14). Of the various substrates used, ascorbate-TPD was found most effective in supporting the uptake of these amino acids, following in decreasing order of effectiveness were exogenous NADH, generated NADH, and succinate. However, no correlation between the rate of substrate oxidation and the level of amino acid accumulation has been observed (13, 14). As shown in Fig. 1, the addition of Cu++ in the absence of exogenous substrate stimulated the uptake of proline, glutamine, and glutamic acid in the membrane vesicles of \( \text{M. phlei} \). The Cu++-stimulated uptake of these amino acids was found to be an active process since there was a 6- to 10-fold concentration gradient established. The intravesicular space of membrane vesicles used for the calculation of concentration gradient was 1.7 μl/mg of membrane protein as described earlier (11). The amino acids accumulated in membrane vesicles were found not to be chemically modified or incorporated into proteins. Cupric sulfite and cupric chloride were found to stimulate the uptake of amino acids to the same extent with maximum effect at 150 μM. Cupric gluconate showed maximum effect at 105 μM. However, cupric salts were ineffective.

Cupric ion-mediated uptake of amino acids other than glutamine, glutamic acid, and proline was also examined in these membrane vesicles. It was observed that the other amino acids, e.g. leucine, were not actively accumulated into the membrane vesicles by cupric ions (Fig. 1). It is pertinent to mention that these membrane vesicles are incapable of accumulating other amino acids, e.g. leucine, tryptophan,

![Fig. 1](http://www.jbc.org/)

**Fig. 1.** Cupric-ion-mediated uptake of proline, glutamine, glutamic acid, and leucine under aerobic and anaerobic conditions. The reaction mixture in a 1.5-ml system contained 25 mM potassium/4-(2-hydroxyethyl)l-piperazineethanesulfonic acid (pH 7.0), 5 mM MgCl2, 10 mM NaCl, 10 μM U-14C-labeled different amino acids, and membrane vesicles (2 mg of protein/ml). 150 μM cupric chloride was also added to the system prior to the addition of labeled amino acids. The dotted line represents cupric mediated uptake under anaerobic conditions. ○, amino acid uptake in absence of cupric ions; —, amino acid uptake in presence of cupric ions under aerobic conditions.
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histidine, lysine, etc. in the presence of exogenously added substrate (14) since these membrane vesicles have presumably lost carrier protein(s) during preparation from whole cells (14).

Effect of Various Inhibitors on Cu"+ Ion-mediated Uptake -
The uptake of proline, glutamine, and glutamic acid mediated by Cu"+ was found to be insensitive to various inhibitors such as azide and arsenate (Table I). The uptake was sensitive to cyanide (10 mM), but this inhibition was probably due to the known chemical interaction of cupric ions with cyanide (21). Irradiation of membrane vesicles at 360 nm has earlier been shown to inactivate the natural quinone MK-9 (II-H) and a light-sensitive component of the respiratory chain (25). The irradiated membrane vesicles are unable to oxidize succinate and NAD+ linked substrates (25). As shown in Table I, the cupric ion-stimulated uptake of proline, glutamine, and glutamic acid remained unaffected in irradiated membrane vesicles. However, the uptake of these amino acids was inhibited by atebrin or o-phenanthroline (Table I), which have been shown to block electron flow at the non-heme iron level in the respiratory chain of M. phlei (26). At the steady state levels of accumulated amino acids, the addition of atebrin or o-phenanthroline elicited a rapid efflux of these amino acids (Fig. 3). This may suggest that the effect of Cu"+ occurs before the naphthoquinone (MK-9(II-H)) level, but after or at the flavoprotein-non-heme iron level of the respiratory chain. It is conceivable that o-phenanthroline may form a complex with Cu"+; however, studies have shown that the Cu"+-o-phenanthroline complex formation does not occur to any significant extent at pH 7.4 which was used in these studies. The Cu"+-o-phenanthroline complex requires a high pH and concentration of ClO"+ (3 mM). This concentration of Cu"+ is higher than that used in these studies.

Effect of Anaerobiosis on Transport - Studies with various respiratory inhibitors showed that the complete respiratory chain was not required for the cupric ion-stimulated uptake of amino acids. It was, therefore, of interest to determine whether oxygen itself was an obligatory requirement for the Cu"+ stimulated uptake. As shown in Fig. 1, the cupric mediated uptake of proline, glutamine, and glutamic acid was insensitive to anaerobiosis. It should be pointed out, however, that the presence of Cu"+ did inhibit succinate and NAD+-linked oxidation in these membrane vesicles.

Effect of Sulfhydryl Inhibitors - Cu"+ is a known oxidizing agent of the sulfhydryl groups of various proteins. The possibility that the oxidation of sulfhydryl groups of membrane proteins was associated with the stimulated uptake of proline, glutamine, and glutamic acid by Cu"+ was ascertained by using various sulfhydryl blocking reagents. Membrane vesicles were incubated with p-CMBS or NEM prior to the addition of Cu"+ in order to block the available sulfhydryl groups; the transport of proline, glutamine, and glutamic acid in the presence of cupric chloride was then assayed. As shown in Fig. 2, sulfhydryl reagents inhibited the uptake of Cu"+-stimulated transport of proline (75%), glutamine (80%), and glutamic acid (85%). The inhibition by sulfhydryl reagents lowered the level of transport to about the level observed in the absence of substrate or Cu"+.

Effect of Cupric Ion on Sulfhydryl Residues - Studies were carried out to determine the total number of accessible sulfhydryl groups in the membrane vesicles in the presence and absence of Cu"+. The reaction mixture was the same as described for Fig. 1. The membrane vesicles were preincubated for 10 min with sulfhydryl blocking reagents, e.g. pCMBS (79 µM) and NEM (100 µM), prior to the addition of cupric chloride. O---O, amino acid transport in the absence of cupric ion; O---O, amino acid transport in the presence of 150 µM CuCl2; ×---×, amino acid transport in the presence of 150 µM CuCl2 and 100 µM NEM; O---O---O, amino acid transport in the presence of 150 µM CuCl2 and 79 µM p-CMBS. It is conceivable that o-phenanthroline may form a complex with Cu"+; however, studies have shown that the Cu"+-o-phenanthroline complex formation does not occur to any significant extent at pH 7.4 which was used in these studies. The Cu"+-o-phenanthroline complex requires a high pH and concentration of ClO"+ (3 mM). This concentration of Cu"+ is higher than that used in these studies.

Effect of Various Inhibitors on cupric ion-mediated uptake -
The reaction mixture was the same as described for Fig. 1. The assay mixture was incubated for 10 min with indicated inhibitors prior to the addition of 150 µM CuCl2. The uptake was initiated by the addition of labeled amino acids as described under "Experimental Procedures." The values represent the steady state of uptake of various amino acids. The accumulation of proline, glutamine, and glutamic acid in the absence of cupric ions was 68, 40, and 43 pmol/mg of protein, respectively.

| Inhibitors | Concentration | Steady state accumulation of amino acids (pmol/mg protein) |
|------------|---------------|----------------------------------------------------------|
|            |               | Proline | Glutamine | Glutamic acid |
| None       |               | 210     | 122      | 100          |
| Arsenate   | 50 mM         | 208     | 122      | 98           |
| Azide      | 100 mM        | 210     | 123      | 96           |
| n-CCP      | 100 µM        | 70      | 61       | 49           |
| Irradiation 360 nm | 210 | 125 | 97 |
| o-Phenanthroline | 5 mM | 60 | 52 | 40 |
| Atebrin    | 5 mM          | 65      | 55       | 42           |

Effect of Anaerobiosis on Transport - Studies with various respiratory inhibitors showed that the complete respiratory chain was not required for the cupric ion-stimulated uptake of amino acids. It was, therefore, of interest to determine whether oxygen itself was an obligatory requirement for the Cu"+ stimulated uptake. As shown in Fig. 1, the cupric mediated uptake of proline, glutamine, and glutamic acid was insensitive to anaerobiosis. It should be pointed out, however, that the presence of Cu"+ did inhibit succinate and NAD+-linked oxidation in these membrane vesicles.

Effect of Sulfhydryl Inhibitors - Cu"+ is a known oxidizing agent of the sulfhydryl groups of various proteins. The possibility that the oxidation of sulfhydryl groups of membrane proteins was associated with the stimulated uptake of proline, glutamine, and glutamic acid by Cu"+ was ascertained by using various sulfhydryl blocking reagents. Membrane vesicles were incubated with p-CMBS or NEM prior to the addition of Cu"+ in order to block the available sulfhydryl groups; the transport of proline, glutamine, and glutamic acid in the presence of cupric chloride was then assayed. As shown in Fig. 2, sulfhydryl reagents inhibited the uptake of Cu"+-stimulated transport of proline (75%), glutamine (80%), and glutamic acid (85%). The inhibition by sulfhydryl reagents lowered the level of transport to about the level observed in the absence of substrate or Cu"+.

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TABLE I - Sulphhydryl content of ETP membrane vesicles in presence and absence of cupric ions

| Membranes (mg of protein) | 6 mg of protein | Presence and absence of CuCl2, 150 µM | p-CMBS, 79 µM | NEM, 100 µM |
|--------------------------|----------------|---------------------------------------|---------------|-------------|
| Membranes (mg of protein) | 6 mg of protein | Presence and absence of CuCl2, 150 µM | p-CMBS, 79 µM | NEM, 100 µM |
| Membranes (mg of protein) | 6 mg of protein | Presence and absence of CuCl2, 150 µM | p-CMBS, 79 µM | NEM, 100 µM |
| Membranes (mg of protein) | 6 mg of protein | Presence and absence of CuCl2, 150 µM | p-CMBS, 79 µM | NEM, 100 µM |
| Membranes (mg of protein) | 6 mg of protein | Presence and absence of CuCl2, 150 µM | p-CMBS, 79 µM | NEM, 100 µM |

FIG. 2. Effect of sulfhydryl agents on cupric-mediated transport of proline, glutamine, and glutamic acid. The reaction system was the same as described for Fig. 1. The membrane vesicles were preincubated for 10 min with sulfhydryl blocking reagents, e.g. pCMBS (79 µM) and NEM (100 µM), prior to the addition of cupric chloride. O--O, amino acid transport in the absence of cupric ion; O--O, amino acid transport in the presence of 150 µM CuCl2; ×--×, amino acid transport in the presence of 150 µM CuCl2 and 100 µM NEM; O--O--O, amino acid transport in the presence of 150 µM CuCl2 and 79 µM p-CMBS.
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Fig. 3. Effect of GSH, dithiothreitol, cysteine, o-phenanthroline, and atebrin on Cu"+-mediated uptake of proline, glutamine, and glutamic acid. The reaction system was the same as described under "Experimental Procedures." Various reducing agents, e.g. glutathione (4 mM), dithiothreitol (4 mM), cysteine (4 mM), o-phenanthroline (5 mM), and atebrin (5 mM) were added at a steady state level of various amino acids as indicated by addition.

O—O, amino acid transport in the absence of cupric ions; O—O, amino acid transport in the presence of Cu"+; O—O, dithiothreitol; x—x, glutathione; O—O, cysteine; O—O, o-phenanthroline; O—O, atebrin.

Fig. 4. Kinetics of cupric ion-stimulated transport. Lineweaver-Burk plots of the cupric-mediated transport of proline, glutamine, and glutamic acid. The reaction mixture and assay procedures were similar to those of Fig. 1. The initial rates of reaction were taken at 30 s after the addition of Cu"+ ions.

Fig. 5. Lineweaver-Burk plot of the Na"+-dependent transport of proline mediated by Cu"+. The reaction mixture and assay procedures were similar to those of Fig. 1. The initial rates of reaction were taken at 30 s after the addition of Cu"+ ions.

### Table III

Q values for Cu"+-stimulated uptake of proline, glutamine, and glutamic acid

| Temperature | Proline | Glutamine | Glutamic acid |
|-------------|---------|-----------|---------------|
| 10-20       | 2.02    | 2.0       | 1.9           |
| 20-30       | 1.58    | 1.75      | 1.6           |
| 30-40       | 1.9     | 1.9       | 1.9           |

It was observed that the total number of DTNB-reactive sulfhydryl residues was reduced by 50% in the presence of Cu"+ (Table II) under both aerobic and anaerobic conditions. Moreover, the number of DTNB-reactive sulfhydryl groups was increased (35%) (Table II) when o-phenanthroline was added at the steady state level of accumulation of amino acids, which has been shown to result in the efflux of amino acids (Fig. 3).

Since the Cu"+-stimulated uptake of proline, glutamine, and glutamic acid in membrane vesicles was seen to be associated with the oxidation of sulfhydryl groups of carrier or membrane protein(s), it was of interest to determine the effect of sulfhydryl reducing agents. Accumulated amino acids...
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Kinetics of Cupric Ion-stimulated Transport — The concentration of Cu²⁺ ions necessary for maximal initial rate and the steady state levels of transport was found to be 150 μM. Higher concentrations of Cu²⁺ ions were found to inhibit amino acid uptake. Varying the concentration of Cu²⁺ did not affect the Kₘ value for proline, glutamine, and glutamic acid, but it did change the Vₘₐₓ values (Fig. 4 A to C). It is pertinent to mention that amino acids form a complex with Cu²⁺ (27) with a high stability constant (Cu²⁺-proline β₁ = 16.58; Cu²⁺-glutamine β₂ = 14.28; Cu²⁺-glutamic acid β₃ = 16.40), yet the Kₘ values for the uptake of these amino acids mediated by Cu²⁺ remained unaltered. This may suggest that the Cu²⁺-mediated transport of these amino acids may occur as the Cu²⁺-amino acids complex forms. Nevertheless, it should be noted that other amino acids with similar β constants, such as leucine 15.60, histidine 18.53, and lysine 13.90, are not transported in these vesicles by Cu²⁺. The Kₘ values of proline (4 μM), glutamine (3.85 μM), and glutamic acid (4.25 μM) were similar to the Kₘ values observed for these amino acids in the presence of succinate and NAD⁺-linked substrates (13, 14).

Requirement of Na⁺ for Proline Transport Mediated by Cu²⁺ — The uptake of proline, in contrast to glutamine and glutamic acid, in membrane vesicles mediated by succinate and NAD⁺-linked substrates has been shown to have an absolute requirement for Na⁺ (12). It was observed that Cu²⁺-mediated uptake of proline as compared to glutamine and glutamic acid also exhibited a requirement for Na⁺. Varying the concentration of Na⁺ did not affect the Kₘ value for proline (4.2 ± 0.2), but it did change the Vₘₐₓ value (Fig. 5).

Effect of Temperature on Cu²⁺-stimulated Transport — The rate of enzymatic reactions is characteristically dependent upon temperature. Hence, the rate and steady state levels of proline, glutamine, and glutamic acid were examined as a function of temperature. The studies were performed with membrane vesicles at 4°, 10°, 20°, 30°, 40°, and 50° in the presence of Cu²⁺. Both the rate of uptake and the steady state levels of transport increased from 0-40° (data not shown); above 40° transport activity was slightly decreased. The temperature coefficient Q₁₀ from 4-40° for the uptake of proline, glutamine, and glutamic acid mediated by Cu²⁺ was found to be in the range of 1.6 to 2.0, suggesting that the process is due to enzymatic catalysis (Table III).

Rate of Cupric Ion Reduction by Membrane Vesicles — Since the sulfhydryl contents of membrane proteins decreased upon the addition of Cu²⁺, it was of interest to determine whether there was an accumulation of cuprous ion during this oxida-

| Amino Acids               | Δ in Sulfhydryl Content | Cu²⁺ Content (nmol/mg protein) | Δ in Cu²⁺ Content | Cu²⁺ Content (nmol/mg protein) |
|--------------------------|-------------------------|-------------------------------|-------------------|-------------------------------|
| Membrane vesicles        | 4.15                    | 8.7                           | 4.55              | 8.7                           | 3.8                           | 4.9                           |
| Trichloroacetic acid-treated membrane vesicles | 7.75                    | 18.6                          | 10.85             | 14.4                          | 3.9                           | 10.5                          |
lated uptake of proline, glutamine, and glutamic acid in membrane vesicles or o-phenanthroline which affected the Cu”+ -stimulated uptake was inhibited when the membrane vesicles were preincubated with p-CMBS prior to the addition of Cu”+. The addition of cuprous ion. The amount of cuprous ions was determined with cupric ion. The uptake of amino acids mediated by Cu”+ ions in the absence of exogenously added substrates.

The uptake of proline, glutamine, and glutamic acid in the presence of 

Table V

Changes in internal and external pH and pH gradient of membrane vesicles during Cu”+-mediated transport of proline

The total volume of the reaction mixture was 2.0 ml containing 

The accumulation of these amino acids was an active process since there was a 6- to 10-fold concentration gradient established in these membrane vesicles. It is known that copper plays an important role in various hydroxylases (28) and forms metallocomplexes with amino acids. Therefore, the possibility of the uptake of proline, glutamine, and glutamic acid observed in membrane vesicles of M. phlei was not due to nonspecific complexes and was eliminated since the transport of other amino acids such as leucine, lysine, histidine, tryptophan, etc. does not occur in these membrane vesicles in the presence of Cu”+.

DISCUSSION

The uptake of proline, glutamine, and glutamic acid in the membrane vesicles of M. phlei was found to be stimulated by Cu”+ ions in the absence of exogenously added substrates. The accumulation of these amino acids was an active process since there was a 6- to 10-fold concentration gradient established in these membrane vesicles. It is known that copper plays an important role in various hydroxylases (28) and forms metallocomplexes with amino acids. Therefore, the possibility of the uptake of proline, glutamine, and glutamic acid observed in membrane vesicles of M. phlei was not due to nonspecific complexes and was eliminated since the transport of other amino acids such as leucine, lysine, histidine, tryptophan, etc. does not occur in these membrane vesicles in the presence of Cu”+. It has been shown previously (14) that membrane vesicles prepared by sonication of whole cells of M. phlei are incapable of accumulating amino acids other than proline, glutamine, and glutamic acid in the presence of succinate and NAD”-linked substrates, presumably, because of the loss of the carrier protein(s) during their preparation. Thenceforward, the Cu”+-mediated transport is apparently carrier-dependent and exhibits amino acid specificity with the membrane vesicles. However, it is not clear whether carrier or binding protein per se participates in the uptake process. It is pertinent to mention that Cu”+-mediated transport of proline in membrane vesicles of M. phlei exhibits an absolute requirement for Na” ion, as has been observed for substrate-linked proline transport in this system (12). In addition, membrane vesicles which are devoid of coupling factor-latent ATPase activity also exhibited the cupric-mediated stimulation of proline, glutamine, and glutamic acid transport, as has been observed with succinate and NAD”-linked substrates (14).

The Cu”+-stimulated uptake of proline, glutamine, and glutamic acid was insensitive to azide, m-CCP, arsenate, and anaerobiosis. The irradiation of M. phlei membrane vesicles at 360 nm which is known to inactivate naphthoquinone (MK-9 (II-H)) and a light-sensitive factor of the respiratory chain failed to affect Cu”+-mediated uptake of amino acids. However, o-phenanthroline and atebriim, which presumably block the electron flow at the flavoprotein(s) or non-heme iron level, inhibited the Cu”+-mediated uptake, and the addition of these inhibitors at the steady state level of accumulated amino acids elicited a rapid efflux.

Sulphydryl blocking agents, e.g. p-CMBS or NEM, inhibited the uptake of amino acids mediated by Cu”+. The oxidation of the sulphydryl groups of proteins by Cu”+ is well known (29); therefore, the effect elicited by p-CMBS and NEM appears to be due to the blockage of available oxidizable sulphydryl groups. The oxidation of the sulphydryl groups of membrane proteins in the presence of Cu”+ ions was confirmed by

| Addition          | Incubation pH | pH<sub>i</sub> | pH<sub>e</sub> | pH<sub>ΔpH</sub> | pH<sub>Gradient</sub> |
|-------------------|---------------|---------------|---------------|----------------|----------------------|
| None              | 7.36          | 7.25          | (7.29-7.23)   | 0.14           | 0.01 (0.01-0.00)     |
| Cu”+ (150 μM)     | 7.34          | 7.17          | (7.18-7.12)   | 0.17           | 0.71 (0.74-0.68)     |
| Cu”+ + proline (25 μM) | 7.31          | 7.24          | (7.29-7.21)   | 0.07           | 0.92 (0.93-0.92)     |
| Cu”+ + atebriim (750 μM) | 7.33          | 7.23          | (7.26-7.20)   | 0.07           | 0.96 (0.98-0.96)     |
| Cu”+ + p-CMBS (150 μM) | 7.34          | 7.21          | (7.25-7.19)   | 0.13           | 0.84 (0.83-0.85)     |
| Cu”+ + m-CCP (50 μM) | 7.30          | 7.21          | (7.24-7.20)   | 0.14           | 0.07 (0.09-0.06)     |
estimating the DTNB-reactive sulfhydryl groups. Furthermore, the addition of reducing agents such as glutathione, cysteine, and dithiothreitol at the steady state level of accumulated amino acids caused an efflux of these amino acids. The efflux of these amino acids was, thus, probably due to the reduction of Cu"+ to Cu+ by membrane vesicles. The rate of reduction of Cu"+ to Cu+ by membrane vesicles was observed. The rate of reduction of Cu"+ by membranes was faster than the rate of accumulation of amino acids. Moreover, reduction of Cu"+ to Cu+ was not observed in the presence of sulfhydryl inhibitors. The stoichiometry of 1:1 for the oxidation of available sulfhydryl residues in membrane to the reduction of Cu"+ suggests that the uptake of amino acids mediated by Cu"+ requires the oxidation of sulfhydryl groups in substrate level amounts rather than in catalytic amounts as shown in Equation 1.

The experiments cited that the disulfide state (%S) of the amino acid transport system appeared to be different. In addition, the active transport of proline may require an electrochemical gradient generated by sodium ions.

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