Biochimica et Biophysica Acta, 684 (1982) 21–26
Elsevier Biomedical Press

BBA 71004

TRANSPORT OF ASCORBATE INTO GUINEA PIG LIVER MITOCHONDRIA

OLE CHRISTIAN INGEBRETSEN [a] and PER T. NORMANN [b,*]

[a] *University of Bergen, Laboratory of Clinical Biochemistry N-5016 Haukeland Sykehus and* [b] *Department of Biochemistry, University of Bergen, Årstadveien 19, N-5000 Bergen (Norway)*

(Received August 3rd, 1981)

Key words: Diffusion; Ascorbate transport; Mitochondria; (Guinea pig liver)

The amount of ascorbate associated with guinea pig liver mitochondria was estimated by high-performance liquid chromatography. Incubation of mitochondria with ascorbate revealed a time-dependent and temperature-dependent accumulation of the vitamin. A steady-state level of ascorbate was obtained in the mitochondria after about 20 min of incubation at 37°C, whereas no accumulation was observed at 0°C. The matrix concentration of ascorbate was highly correlated to the concentration of ascorbate in the incubation medium. The initial rate of accumulation (about 7 pmol/mg protein per min at 10°C) was three orders of magnitude less than for compounds that are transported across the mitochondrial inner membrane by specific carriers. Experiments with the enzyme ascorbate oxidase demonstrated that the mitochondrial membrane is also permeable to dehydroascorbate, and that the accumulated dehydroascorbate is stable in the mitochondria. There was no effect of the energy state of the mitochondrial membrane on the initial transport rate of ascorbate. Electrostatic binding of ascorbate to the membrane was excluded from experiments performed in isosmotic potassium chloride medium. Diffusion of ascorbate across the mitochondrial inner membrane accounts for the experimental findings.

Introduction

Ascorbate is a cofactor in some enzyme reactions. Examples are the prolyl hydroxylase reaction [1,2], and the synthesis of noradrenaline in the chromaffin granules of the adrenal medulla [3]. Ascorbate is present in higher concentrations in the chromaffin granules than the K_m of ascorbate in the reaction catalyzed by dopamine-β-monooxygenase [3–5]. Ascorbate probably also functions as a scavenger of singlet oxygen in the cells [6], and is also involved in lipid peroxidation [7–9]. Recently, some apparently more complex effects of ascorbate have been described. Thus, it has been shown that the level of cytochrome *P*-450 in the microsomes of hepatocytes is influenced by the availability of ascorbate [10]. Moreover, advanced scurvy is associated with an increase in the weight of the adrenals, as well as an increase in the amount of cytochrome *P*-450, cholesterol side-chain cleavage activity and cortisol level in plasma [11]. Some of these enzyme systems are localized to mitochondria [11].

In order to evaluate the effects of ascorbate on mitochondrial enzyme systems, it is necessary to know the permeability of the mitochondrial inner membrane for this compound. In the present communication, the uptake of ascorbate into guinea

* Present address: National Institute of Forensic Toxicology, Sognsvannsveien 28, Oslo 3, Norway.

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; Bes, *N,N*-bis-(2-hydroxyethyl)-2-aminoethanesulphonic acid; HPLC, high-performance liquid chromatography.

0005-2736/82/0000–0000/$02.75

pig liver mitochondria has been studied. Moreover, experiments bearing on the permeability of mitochondria for the oxidized and reduced forms of the vitamin are presented.

In studies of ascorbate transport across biological membranes, it is essential to know the exact amount of the oxidized and reduced forms of the compound on both sides of the biological membrane throughout the experiment. In order to avoid speculations on possible isotope dilutions [12], and interferences with the assay as observed when indirect chemical methods are used [13], a direct chemical method for the quantitation of ascorbate is desirable. We have applied high-performance liquid chromatography (HPLC) as a direct estimation of the ascorbate concentration. The molar extinction coefficient of the oxidized and reduced forms of the vitamin are sufficiently different [13] to allow quantitation of the vitamin in its fully reduced form in the ultraviolet range.

Materials and Methods

Chemicals. Ascorbic acid was a product of Merck (Darmstadt, F.R.G.) and ascorbate oxidase and CCCP were supplied by Boehringer Mannheim GmbH (Mannheim, F.R.G.). Dithiothreitol and ATP were obtained form Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other reagents were of the highest purity commercially available.

Animals. Male guinea pigs (200–300 g) of the Pir/Srr/c strain were obtained from a local animal breeding laboratory.

Preparation of liver mitochondria. The mitochondria were isolated by differential centrifugation according to the procedure described in [14], except that EDTA and dithiothreitol were added to the buffer in order to stabilize ascorbate in the reduced form. The homogenization buffer contained 0.25 M sucrose, 5 mM Hepes (pH 7.2), 0.2 mM EDTA and 2 mM dithiothreitol. The final mitochondrial stock suspension (45–60 mg protein/ml) was kept on ice until the initiation of the incubation experiments.

Measurement of the uptake of ascorbate into mitochondria. Samples from the mitochondrial stock suspension were diluted to about 2–4 mg mitochondrial protein/ml in the standard incubation medium which contained 135 mM sucrose, 40 mM Bes (pH 7.0), 1 mM EDTA and 2 mM dithiothreitol. The temperature of the assay mixture was equilibrated in a water bath for 5 min at 0–37°C before ascorbate (stock solution adjusted to pH 7.0) was added to a final concentration of 3–12 mM. Samples (300 μl) were saved at appropriate times (see figures) and prepared for the analysis by HPLC.

Preparation of extracts of mitochondria for analysis by HPLC. The samples of the incubation mixture were transferred to precooled (0°C) tubes, in order to quench the membrane transport of ascorbate (see Results). The tubes were centrifuged for 2.5 min in an Eppendorf centrifuge, Model 3200, and subsequently washed in 300 μl of the incubation medium. The mitochondria were washed from one to four times in the incubation medium before addition of 300 μl of the protein-precipitating solution which contained 150 μl incubation medium and 150 μl ethanol (pH of the mixture adjusted to 4.0 with acetic acid). In some experiments the mitochondria were only pelleted (0 washing) see Fig. 2B.

Measurement of ascorbate by HPLC. A model 6000A pump from Waters Associated (Milford, MA, U.S.A.) was used in the experiments, and the eluate was monitored at 254 nm with the ultraviolet detector Spectromonitor 1203, Laboratory Data Control (Riviera Beach, FL, U.S.A.). The separation was achieved at room temperature on a micro-particulate, strong anion exchange column, Partisil 10 SAX (0.46 × 25 cm) with particle size 10 μm from Whatman, Inc. (Clifton, NJ, U.S.A.). A guard column (0.2 × 10 cm) was used in the experiments, packed with pellicular silica with particle size 37–44 μm (HC Pellosil, Whatman, U.S.A.). The mobile phase, 10 mM $(NH_4)_2HPO_4$, pH 4.0 and 2% (v/v) *n*-propanol, was a modification of the system used previously for the determination of ascorbate in chromaffin granules from bovine adrenal medulla [5]. The samples (20 μl) were injected into the chromatographic system with a Rheodyne injector, Model 7125 (Berkeley, CA, U.S.A.). A linear correlation was obtained between the injected amount of ascorbate and the peak height of ascorbate in the chromatogram.

Determination of the internal volume of the mitochondria. A suspension of mitochondria (2–3 mg protein/ml) in the incubation buffer, see above,

was incubated with ^3H_2O and [^{14}C]sucrose at 25°C for 5 min [15]. A magnetic bar was used in order to mix the suspension thoroughly. The suspension was then centrifuged in an Eppendorf centrifuge (model 3200) for 4 min at room temperature. The supernatant was saved and deproteinized with 10% (w/v) perchloric acid, and 100 μl of the deproteinized supernatant was counted for ^{14}C and ^{3}H in 5 ml Unisolve 100. Total water space (V_t μl) and sucrose accessible space (V_s μl) in the pellet were used to calculate the matrix space (V_m μl):

$$V_m = V_t - V_s = \frac{^3H_p}{[^3H_0]} - \frac{^{14}C_p}{[^{14}C_0]}$$

where ^3H_p and $^{14}C_p$ are the amount of the respective isotopes (cpm) in the pellet, and [$^3H_0$] and [$^{14}C_0$] are the concentration of the isotopes (cpm/μl) in the supernatant.

Polarographic oxygen measurement and protein determination. The functional integrity of the mitochondrial preparations was tested by measuring the respiratory control ratio with ADP, using succinate as the substrate. The oxygen consumption was measured with a Model 53 Biological Oxygen Monitor, Yellow Springs Instrument Co. (Yellow Springs, OH 45387, U.S.A.). Protein was determined by the method of Eggstein and Kreutz [16], and bovine serum albumin was used as standard.

Results

Determination of ascorbate in washed guinea pig liver mitochondria by HPLC

It is seen from Fig. 1 that ascorbate ($t_R = 4.8$ min) was separated from other ultraviolet absorbing compounds in the incubation mixture by the anionic exchange column. The purity and identity of the ascorbate peak were confirmed by addition of ascorbate oxidase (10 U) to 200 μl of a neutralized perchloric acid extract of mitochondria which had been incubated for 30 min with 6 mM ascorbate (Fig. 1B). After 20 min of incubation at 30°C, the ascorbic acid peak was absent from the chromatograms (data not shown). Ascorbate was preserved in the reduced form in the incubation medium by the presence of 2 mM dithiothreitol

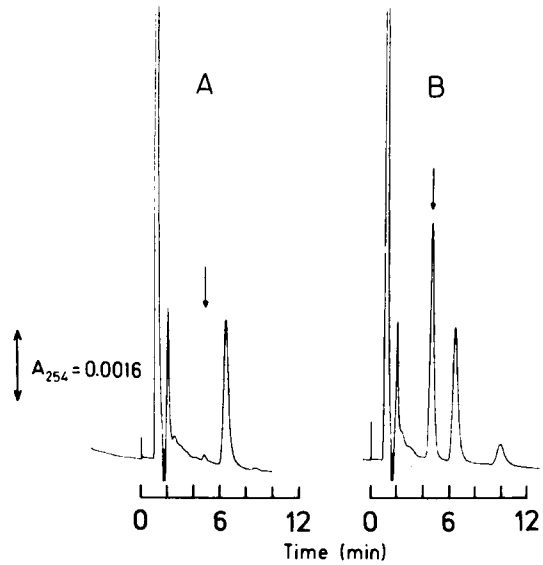

Fig. 1. HPLC elution pattern of an extract of mitochondria saved at the initiation of the incubation period at 37°C, i.e. before the addition of ascorbate to the incubation medium (A), and after incubation with 6 mM ascorbate for 30 min (B). The mitochondrial suspensions (300 μl) were washed once in the incubation medium before the protein-precipitating solution was added. Ascorbate has the retention time 4.8 min, see arrows. Full scale deflection in panels A and B is 0.016 absorbance units.

and 1 mM EDTA. Dithiothreitol had no effect on the respiratory control ratio (RC ratio). In typical preparations the resipiratory control ratios were from 4 to 5. The endogenous content of ascorbate in the mitochondrial preparations was below 0.2 nmol/mg protein (Figs. 1A and 2A) when the animals were supplied with only 0.1% (w/v) of ascorbate in the drinking water. After incubation of the mitochondria with 6 mM ascorbate for 30 min at 37°C, an increase in the ascorbate content of the washed mitochondria was readily observed (Figs. 1B and 2A).

Time-course of the accumulation of ascorbate in mitochondria

Mitochondrial suspensions were incubated in water baths at 0°C and 37°C for 5 min. Ascorbate was then added to a final concentration of 6 mM. The same amount of ascorbate is associated with mitochondria after one wash both at 0°C and 37°C immediately after addition of ascorbate to

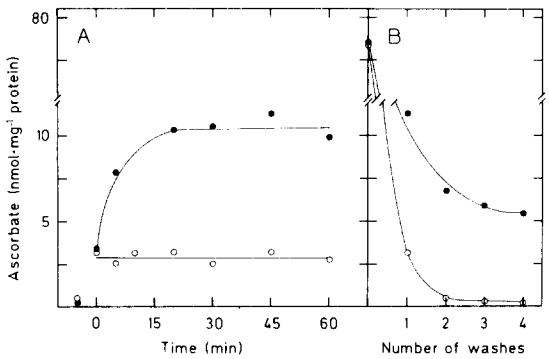

Fig. 2. (A) Effect of the incubation time and temperature on the amount of ascorbate associated with guinea pig liver mitochondria. The points at −5 min in the left side of the figure show the amount of ascorbate associated with the mitochondria at the initiation of the temperature equilibration period in a shaking water bath at 0°C (○) and 37°C (●). Zero time indicates the removal of the sample (300 μl) immediately after the addition of ascorbate (6 mM, final concentration). The mitochondria were washed once in the incubation medium before the protein-precipitating solution was added. The protein concentration was 3.3 mg/ml. (B) Effect of washing on the amount of ascorbate associated with the mitochondrial pellet. After incubation for 45 min (see panel A) the samples (300 μl) were either subjected to one centrifugation (0 washing), or washed for one to four times in 300 μl of the incubation medium before the protein-precipitating solution was added.

the mitochondrial suspension (0 min, Fig. 2A). Moreover, at 0°C this amount remains constant in the samples saved during the whole incubation period (up to 60 min), whereas at 37°C a time-dependent accumulation of ascorbate occurs until a steady-state level is reached after about 20 min of incubation (Fig. 2A).

Effect of washing on the amount of ascorbate associated with mitochondria

Mitochondrial suspensions incubated in the presence of 6 mM ascorbate for 45 min at 0°C and 37°C were used to study the effect of washing on the amount of ascorbate associated with the mitochondria. Fig. 2B shows that one centrifugation (0 washing) of the mitochondrial suspensions revealed a trapping of ascorbate in the medium of the pellets. However, further washing of the mitochondria which were incubated at 0°C resulted in a gradual decrease in the amount of ascorbate associated with the mitochondria, and after two washes, a concentration equal to that of the endogenous content of ascorbate was found. On the other hand, in the mitochondrial suspension incubated at 37°C, a higher amount of ascorbate was retained even after four washes (Fig. 2B). This experiment, together with the effect of high concentrations of salt in the medium, see below, suggests that the amount of ascorbate associated with the mitochondria after two washes is present in the mitochondrial matrix [17].

Effect of the medium concentration of ascorbate on the concentration of ascorbate in the mitochondria

For a number of experiments ($n = 4$) the water accessible space of the mitochondria was found to be (mean) 0.74 μl/mg protein (S.D. = 0.14). The mitochondria were incubated in the presence of 3, 6, and 12 mM of ascorbate for 30 min at 37°C. For each concentration of ascorbate, the mitochondria were washed four times, and the measured level of ascorbate was used to calculate the mitochondrial matrix concentration, which was found to be 2.5, 5.4, and 9.0 mM, respectively. An almost linear relationship was thus obtained between the medium concentration of ascorbate and the calculated matrix concentration, indicating that an equilibration of the medium concentration of ascorbate occurs across the membrane.

Effect of ascorbate oxidase on the amount of ascorbate recovered in the mitochondria

Ascorbate oxidase was used as a probe to determine the efflux of ascorbate from loaded mitochondria. The enzyme was added to the mitochondrial suspension after 30 min of incubation with 6 mM ascorbate and only 0.1 mM dithiothreitol at 37°C (Fig. 3). Otherwise, the medium composition was as stated above. The ascorbate loaded in the mitochondria was removed within 30 min of the subsequent incubation with ascorbate oxidase in the medium (Fig. 3). Also the ascorbate in the incubation medium was oxidized under these conditions (data not shown).

In order to determine whether dehydroascorbate is present in the mitochondrial matrix after incubation with ascorbate oxidase, the mitochondria were subsequently washed four times and precipitated in 6% (w/v) $HClO_4$. The supernatant was neutralized with potassium bicarbonate and treated with 5 mM dithiothreitol at pH 7.0 for

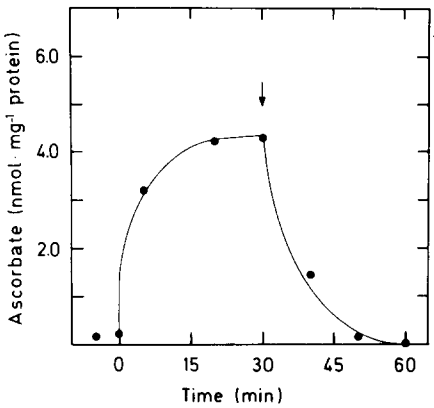

Fig. 3. Effect of ascorbate oxidase on the content of ascorbate in the mitochondria. The mitochondria were incubated with 6 mM ascorbate at 37°C for 30 min in the standard incubation medium except that 0.1 mM DTT was used. 20 U/ml ascorbate oxidase were then added to the mitochondrial suspension (see arrow). The amount of ascorbate associated with the mitochondria after four washes was plotted in the figure.

TABLE I

EFFECT OF THE MEDIUM COMPOSITION ON THE INITIAL RATE OF ACCUMULATION OF ASCORBATE IN MITOCHONDRIA

The mitochondria were incubated in the presence of 6 mM ascorbate at 25°C for 10 min, and washed four times before the analysis of ascorbate by HPLC.

Medium	Rate of ascorbate accumulation (nmol/mg protein per 10 min)
1. Standard incubation medium	0.37
2. Standard incubation medium +8 μM CCCP	0.36
3. Standard incubation medium +2.5 mM MgATP	0.39
4. Standard incubation medium +30 mM NH_4Cl +2.5 mM MgATP	0.37
5. KCl medium	0.37

15 min in order to reduce the dehydroascorbate present [18]. In parallel experiments ($n = 3$), about 90% of the expected amount of ascorbate were formed in the perchloric acid extracts, as compared with the amount of ascorbate (see Fig. 3) in the mitochondria before the addition of ascorbate oxidase.

Effects of the energy state of the mitochondrial membrane, the ionic strength of the medium, and the temperature on the accumulation of ascorbate in mitochondria

In control experiments the effects of CCCP (8 μM), MgATP (2.5 mM), and MgATP (2.5 mM) plus NH_4Cl (30 mM) were tested. No significant changes in the rate of the accumulation of ascorbate were observed in the presence of these reagents (Table I).

The standard incubation medium was compared with a medium in which 135 mM sucrose was replaced by 130 mM KCl. This experiment showed no differences in the time-dependent accumulation of ascorbate (Table I), and in the subsequent washing procedure (data not shown). Thus, electrostatic binding of ascorbate to the mitochondrial membrane is not a significant factor for retaining ascorbate in the mitochondrial preparation.

The rate of ascorbate accumulation was found to be 0.07, 0.37, and 3.50 nmol/mg protein per 10 min at 10, 25, and 37°C, respectively. The steady-state level of ascorbate accumulation was reached after 20 min of incubation at 37°C, whereas at 10 and 25°C the rate of accumulation was linear for at least 60 min. It is apparent that the increase in the rate of ascorbate accumulation per degree Celcius is higher above 20°C than below this temperature.

Discussion

The observations that chemical reactions in mitochondria are dependent on ascorbate, and that various membrane systems reveal different permeability for ascorbate [5,9,19–23], forwarded us to study the uptake of ascorbate in guinea pig liver mitochondria. The uptake experiments shown in Figs. 1–3 were performed at 37°C in order to determine a steady-state level for the accumulation of ascorbate in the mitochondria. This was necessary in order to establish the concentration gradients of ascorbate across the mitochondrial membrane. The steady-state level of ascorbate accumulation is not reached until after considerable longer incubation times at 25°C. However, it is a well known fact that the coupling of the mitochondria

detoriates rapidly (after about 10 min) at 37°C. The effect of the energy state of the mitochondrial membrane on ascorbate transport was therefore studied at 25°C, since the mitochondria at this temperature retain the coupling over a longer period of time. The 'energization' of the mitochondrial membrane did not influence the initial rate of ascorbate accumulation at 25°C, and the results obtained at 37°C presumably give a correct picture of the permeability characteristics of the mitochondrial membrane for ascorbate at this temperature.

The accumulation of ascorbate is time-dependent (Fig. 2A), and temperature-dependent, and proceeds until the concentration of ascorbate is almost the same in the matrix and incubation medium. The steady-state level of ascorbate accumulation is reached after 20 min of incubation at 37°C, irrespectively of whether the medium concentration of ascorbate is 3, 6, or 12 mM, i.e. the initial transport rate is dependent on the initial gradient of ascorbate across the membrane (data not included in the paper). The possibility of membrane binding of ascorbate by electrostatic interaction is less likely, due to the results of the experiments with a high concentration of KCl in the medium. Taken together, the data suggest that diffusion is the mechanism by which ascorbate is transported into the mitochondria.

The question remains to be settled whether the diffusion of ascorbate across the mitochondrial membrane occurs via a carrier mediated mechanism or not. Our data show that the ascorbate transport is a relatively slow process even at 37°C. On the other hand, it is often necessary to run metabolite transport experiments at low temperatures in order to measure the initial rate of uptake when a specific carrier is involved [17]. For instance, the tricarboxylate carrier of rat liver mitochondria has a V at 9°C of 22 nmol/min per mg protein (for review, see Ref. 24). At 10°C, the initial rate of ascorbate transport is about 7 pmol/min per mg protein, i.e. three orders of magnitude less. This suggest that a specific carrier is not involved in the ascorbate transport across the mitochondrial inner membrane. The same conclusion is also supported by experiments with isoascorbate. The time-course of accumulation of this compound is identical to that reported for ascorbate (data not shown).

As one would expect for a biological membrane, there is no linear correlation between the increase in temperature and the observed rate of ascorbate accumulation. This fits into the present knowledge of the effect of the temperature on the membrane fluidity [25].

References

1 Barnes, M.J. (1975) in 2nd Conference on Vitamin C (King, C.G. and Burns, J.J., eds.), pp. 264–277, New York Academy of Sciences, New York
2 Myllyla, R., Kuutti-Savolainen, E.-R. and Kivirikko, K.I. (1978) Biochem. Biophys. Res. Commun. 83, 441–448
3 Foldes, A., Jeffrey, P.L., Preston, B.N. and Austin, L. (1973) J. Neurochem. 20, 1431–1442
4 Terland, O. and Flatmark, T. (1975) FEBS Lett. 59, 52–56
5 Ingebretsen, O.C., Terland, O. and Flatmark, T. (1980) Biochim. Biophys. Acta 628, 182–189
6 Bodannes, R.S. and Chan, P.C. (1979) FEBS Lett. 105, 195–196
7 Ottelengi, A. (1959) Arch. Biochem. Biophys. 79, 355–363
8 Hunter, F.E., Jr., Scott, A., Hoffsten, P.E., Guerra, F., Weinstein, J., Schneider, A., Schutz, B., Fink, J., Ford, L. and Smith, E. (1964) J. Biol. Chem. 239, 604–613
9 Shimadu, O. and Yasuda, H. (1979) Biochim. Biophys. Acta 572, 531–536
10 Bissell, D.M. and Guzelian, P.S. (1979) Arch. Biochem. Biophys. 192, 569–576
11 Björkhem, I., Kallner, A. and Karlmar, K.-E. (1978) J. Lipid Res. 19, 695–704
12 Ussing, H.H. (1949) Physiol. Rev. 29, 127–155
13 Hewitt, E.J. and Dickes, G.J. (1961) Biochem. J. 78, 384–391
14 Romslo, I. and Flatmark, T. (1973) Biochim. Biophys. Acta 305, 29–40
15 Ingebretsen, O.C. and Flatmark, T. (1979) J. Biol. Chem. 254, 3833–3839
16 Eggstein, M. and Kreutz, F.H. (1955) Klin. Wochenschr. 33, 879–884
17 Palmieri, F. and Klingenberg, M. (1979) Methods Enzymol. 56, 279–301
18 Okamura, M. (1980) Clin. Chim. Acta 103, 259–268
19 Hornig, D., Weber, F. and Wiss, O. (1971) Clin. Chim. Acta 33, 187–196
20 Hornig, D., Weber, F. and Wiss, O. (1971) Clin. Chim. Acta 31, 25–35
21 Stankova, L., Demetrios, A. and Bigley, R.H. (1975) in 2nd Conference on Vitamin C (King, C.G. and Burns, J.J., eds.), pp. 238–252, New York Academy of Sciences, New York
22 Spector, R. and Greene, L.A. (1977) Brain Res. 136, 131–140
23 Tirrell, J.G. and Westhead, E.W. (1979) Neuroscience 4, 181–186
24 La Noue, K.F. and Schoolwerth, A.C. (1979) Annu. Rev. Biochem. 48, 871–922
25 Lee, M.P. and Gear, A.R.L. (1974) J. Biol. Chem. 249, 7541–7547