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HUMAN CELL DEHYDROASCORBATE REDUCTASE
KINETIC AND FUNCTIONAL PROPERTIES

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Summary

Dehydroascorbate reductase (glutathione dehydroascorbate oxidoreductase, EC 1.8.5 1) activity was examined in crude cytosol extracts of human cells. In blood neutrophil and lymphocyte extracts, the K_m at pH 6.85 for dehydroascorbate was 1.3 mM, and for reduced glutathione 3.8 mM Rates of dehydroascorbate uptake by intact human neutrophils, monocytes, lymphocytes and cultured fibroblasts were proportional to cytosol dehydroascorbate reductase activities.

Dehydroascorbate reduction during dehydroascorbate uptake by these cells may be entirely enzymatic.

Introduction

Dehydroascorbate, but not reduced ascorbate, is taken up from suspending media by human blood neutrophils. virtually all of the dehydroascorbate taken up is promptly reduced [1]. Dehydroascorbate reductase (glutathione: dehydroascorbate oxidoreductase, EC 1.8.5.1) catalyzes the reduction of dehydroascorbate to reduced ascorbate in plant [2] and animal [3—5] tissues. Since dehydroascorbate is readily reduced nonenzymatically by GSH, the function of dehydroascorbate reductase is problematic. Studies in glutathione reductase-deficient cells suggest that dehydroascorbate reduction is a necessary condition for dehydroascorbate transport across neutrophil membranes [6]

To explore the relationship between enzymatic and nonenzymatic dehydroascorbate reduction in cells, and between cellular dehydroascorbate uptake and reduction, we have determined some kinetic properties of crude cytosol dehy-

droascorbate reductase, and measured this activity along with dehydroascorbate uptake in human neutrophils, lymphocytes, monocytes and cultured fibroblasts

Experimental procedures

Venous blood and skin biopsies were obtained from healthy, informed and consenting human donors Neutrophils, 90 ± 5% pure, were separated from heparinized blood by Dextran (M_r 117 000, Sigma Chem. Co , St. Louis, MO) sedimentation followed by lysis of contaminating erythrocytes with ice-cold NH_4Cl, 0.87 g/100 ml. Lymphocytes and monocytes were separated by centrifugation over Ficoll-Hypaque (Ficoll-Paque, Pharmacia, Piscataway, NJ). After the monocytes had adhered to the surface of plastic petri dishes [7], the lymphocyte-containing supernatant was poured off. Adherent monocytes were washed three times, then removed from the plastic by incubation for 15 min at 26° C with 30 mM lidocaine-HCl (crystalline Xylocaine, kindly donated by Astra Pharmaceutical Prod., Inc., Worcester, MA) [8]. Skin biopsies were minced and explanted into 25 cm^2 tissue culture flasks containing 2 ml McCoy's 5a medium (Flow Laboratories, Rockville, MD) supplemented with heat-inactivated 20% (v/v) pooled human serum, 200 units/ml penicillin and 200 μg/ml streptomycin. The cells that grew from the explanted tissue were dissociated by incubation for 5 min with 0.05% trypsin-EDTA and subcultured in 75 cm^2 Falcon plastic tissue flasks containing growth medium with 10% (v/v) human serum. Culture medium was changed twice weekly. One-to-two week confluent cultures containing 4—6 · 10^6 fibroblasts per flask were used for assays.

The procedure for measuring dehydroascorbate uptake by suspended, intact cells was as described previously [9]. Briefly, 5 · 10^6 cells were incubated for 20 min at 37°C in 1 ml Ca^{2+}-free Krebs-Ringer phosphate buffer, pH 7.4, containing 5.5 mM glucose and 100 μg [^{14}C]dehydroascorbate, 0.02 μCi/μg, freshly prepared from L-[^{14}C]ascorbic acid (New England Nuclear, Boston, MA) and L-ascorbic acid (Sigma Chem. Co). Dehydroascorbate was prepared by bubbling Br_2 vapor through a freshly prepared solution of reduced ascorbate in 0.154 M NaCl, Br_2 was washed out with water-saturated N_2. Dehydroascorbate concentrations were measured colorimetrically, using the dinitrophenylhydrazine method of Roe et al. [10]. Uptake was stopped by placing reaction tubes in ice water. Cells were immediately washed three times with 4°C 0.154 M NaCl, then counted with a hemocytometer. Aquasol (New England Nuclear) was added to centrifuged cells and radioactivity was measured in a Packard TriCarb scintillation counter. For those assays made on adherent fibroblasts, culture medium was replaced with 9 ml Krebs-Ringer phosphate buffer containing 1.1 mg/ml glucose. After preincubation for 25 min at 37°C, 1 ml [^{14}C]-dehydroascorbate, 1 mg/ml, was added, and the incubation was continued for 20 min. The adherent cells were then washed three times with 20 ml 4°C 0.154 M NaCl, dissociated with trypsin-EDTA, washed once more, and counted as above. Measured colorimetrically by the method of Roe et al. [10], medium dehydroascorbate is quantitatively recovered as dehydroascorbate after 20 min incubation with cells, except for that fraction recovered as reduced ascorbate in cells, total recovery is 98.5 ± 1.1% [1]. Using reagent concentrations as in

the present study, colorimetrically-measured dehydroascorbate uptake and reduction are linear over the range $1-6 \cdot 10^6$ cells/ml reaction mixture [1]. Diketogulonate is detected only as a minor fraction of cellular total ascorbate, it does not change in amount during incubation [1]. Measured radiochromatographically after 20 min preincubation, 86—96% of the dehydroascorbate taken up by cells is in the form of reduced ascorbate [6]. This discrepancy between radiochromatographic and colorimetric measurements may reflect a fraction of dehydroascrobate which was reduced upon uptake by cells and subsequently oxidized. Radiochemical measurement of cellular dehydroascorbate uptake is 98.5 ± 3.6% efficient as compared to colorimetric measurement in duplicate samples ($n = 6$) The present study includes radiochemical measurements of cellular dehydroascorbate uptake. Reduction of dehydroascorbate upon uptake was assumed on the basis of previous measurements, it was not quantitated in this study.

Reduced glutathione and glutathione reductase activity were assayed according to Beutler [11]. Protein was measured by the method of Lowry et al. [12] using bovine serum albumin as a standard. Stimulated hexosemonophosphate shunt activity was measured continuously using the gas flow-ionization chamber method [1].

Cell extracts for enzyme activity measurements were prepared by homogenizing suspensions of $6-10 \cdot 10^7$ cells/ml in 0.154 M NaCl, for 5 min at 3500 rev./min in a glass-Teflon homogenizer held in ice, and centrifuging for 90 min at 4°C and 12 000 × g. The supernatants used for assaying dehydroascorbate reductase were dialyzed at 4°C for 20—24 h against 200 vol. 50 mM phosphate buffer, pH 6.85, in order to remove GSH. The resulting precipitate was separated by centrifugation at 12 000 × g, 4°C for 10 min, and discarded. The standard reaction mixture for the coupled dehydroascorbate reductase assay contained 50 mM phosphate buffer/0.27 mM EDTA/0.2 mM NADPH/2.0 mM GSH/ 2 I.U./ml glutathione reductase/2.0 mM dehydroascorbate/cell extract equivalent to $10-20 \cdot 10^6$ cells/ml; the final pH was 6.85. The reaction mixture without dehydroascorbate and cell extract was incubated for 5 min at 37°C. After addition of dehydroascorbate, A_{340} was recorded for approx. 1.5 min, as a control for reagent concentrations. Cell extract was then added, and A_{340} was recorded for another 1.5 min. The reaction mixture for direct assay contained the same components as the coupled assay except for NADPH and glutathione reductase; reduced ascorbate concentration was measured using the 2,6-dichloroindophenol technique of Wolf et al. [13] after 0, 3 and 6 min incubation at 37°C. In both assays, nonenzymatic reduction of dehydroascorbate by GSH was measured under identical conditions except that an equal volume of 0.154 M NaCl was substituted for cell extract. This measurement was subtracted from that obtained with cell extract to determine the net enzymatic rate of dehydroascorbate reduction.

Results

Dehydroascorbate reductase was measured both directly, as the rate of reduced ascorbate production (Eqn. 1), and in the coupled assay, as the rate of NADPH oxidation (Eqns. 1 and 2)

$$\text{dehydroascorbate} + 2\ \text{GSH} \xrightarrow{\text{dehydroascorbate reductase}} \text{reduced ascorbate} + \text{GSSG} \quad (1)$$

$$\text{GSSG} + \text{NADPH} + \text{H}^+ \xrightarrow{\text{GSSG reductase}} 2\ \text{GSH} + \text{NADP}^+ \quad (2)$$

The direct and coupled assays measure dehydroascorbate reductase activity with equal efficiency, with a measurement error of ±7.5%. This confirms that the oxidation of 1 mol NADPH in the coupled assay is equivalent to reduction of 1 mol dehydroascorbate (algebraic sum of Eqns. 1 + 2). Since the coupled reaction can be measured more quickly, is less subject to error and requires less enzyme, it was used for the other experiments described here.

The pH dependence of nonenzymatic dehydroascorbate reduction and of neutrophil extract activity are illustrated in Fig. 1. The pH optimum of the enzymatic reaction is close to 7.4. Since the rapidity of the nonenzymatic reaction increases measurement error in the higher pH range the standard assay was performed at pH 6.85.

Figs. 2 and 3 illustrate the effects of dehydroascorbate and GSH concentrations on neutrophil dehydroascorbate reductase activity At pH 6.85, the K_m for dehydroascorbate is 1.33 mM, for GSH it is 3.8 mM. Affinity of the enzyme for dehydroascorbate increases with increasing pH. At pH 7.25, the K_m for dehydroascorbate is 0.54 mM. Substrate inhibition is observed at dehydroascorbate concentrations above 2 mM. Lymphocyte dehydroascorbate reductase exhibits the same kinetic properties.

Table I shows rates of dehydroascorbate uptake by equal volumes of normal human cultured skin fibroblasts and blood neutrophils, lymphocytes and monocytes, along with cytosol GSH concentrations and glutathione reductase, stimulated hexosemonophosphate shunt and dehydroascorbate reductase activ-

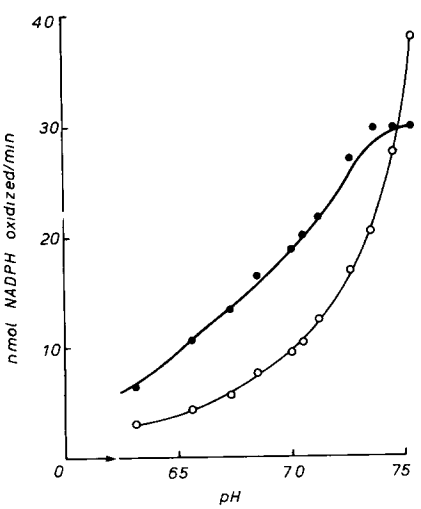


Fig 1 Enzymatic and nonenzymatic dehydroascorbate reduction pH dependence Dehydroascorbate reduction was measured by the standard coupled assay described in Experimental procedures, except that the composition of the phosphate buffer was varied to achieve final pH as indicated Enzymatic reduction represents activity in the presence of neutrophil extract equivalent to 6 10^6 cells in 1 ml of reaction mixture, less measured nonenzymatic activity ●———●, net enzymatic, ○———○, nonenzymatic

TABLE I

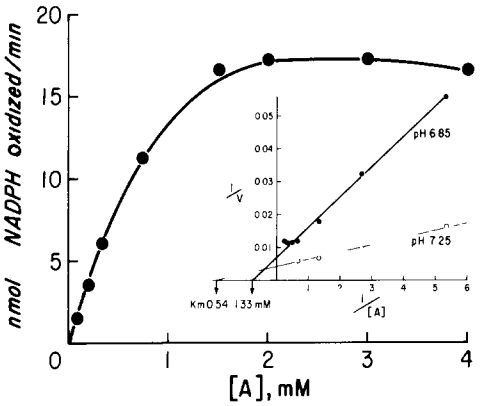
DEHYDROASCORBATE UPTAKE AND ITS POTENTIAL DETERMINANTS IN HUMAN BLOOD CELLS AND CULTURED FIBROBLASTS

HMS, hexosemonophosphate shunt

	Fibroblasts 10^7 cells = 0 1 ml	Lymphocytes 3 10^8 = 0 1 ml	Monocytes 10^8 = 0 1 ml	Neutrophils 10^8 = 0 1 ml
Dehydroascortate uptake nmol/min per 0 1 ml cells	0 48 ± 0 15	3 00 ± 0 90	8 05 ± 1 78	21 00 ± 2 00
HMS activity during dehydroascortate uptake * nmol NADPH oxid /min per 0 1 ml cells	0 57 ± 0 05	2 20 ± 0 42	4 05 ± 0 49	9 20 ± 0 69
HMS activity, methylene blue stim ** nmol NADPH oxid /min per 0 1 ml cells	4 28 ± 0 72	44 42 ± 4 12	15 30 ± 2 55	28 90 ± 2 77
GSH content nmol/0 1 ml cells	358 ± 159	678 ± 220	249 ± 128	172 ± 16
GSSG reductase nmol/min per mg protein	26 ± 5	64 ± 24	39 ± 14	51 ± 18
Dehydroascortate reductase nmol/min per mg protein	5 5 ± 2 3	17 0 ± 2 2	44 8 ± 6 3	119 0 ± 9 6

* Medium dehydroascrotate, 0 55 mM, glucose, 5 5 mM

** Methylene blue, 2 1 mM.



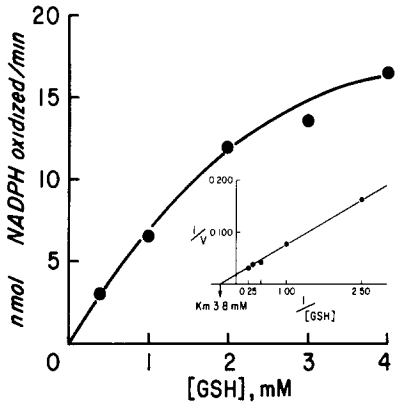


Fig 2 Neutrophil cytosol dehydroascorbate reductase activity vs dehydroascorbate concentration

Fig 3 Neutrophil cytosol dehydroascorbate reductase activity vs reduced glutathione concentration

ities In each cell type, both glutathione reductase activity and methylene blue-stimulated hexosemonophosphate shunt activity were greater than the measured rates of dehydroascorbate uptake Thus, it is unlikely that dehydroascorbate uptake as measured here could have been limited by GSH availability

Among cell types, dehydroascorbate uptake rates had no consistent relationship with GSH content, glutathione reductase activity or stimulated hexosemonophosphate shunt activity Fig 4, a plot of data from Table I, shows that rates of dehydroascorbate uptake for each cell type were directly proportional to measured cytosol dehydroascorbate reductase activities

To determine whether cell surface area or the dissociation procedure might affect the rate of dehydroascorbate transport across membranes, uptake was measured in four paired samples of cultured fibroblasts, one of each pair incubated with [^{14}C]dehydroascorbate while still adherent to the flask surface and the other incubated after harvesting with trypsin-EDTA. Both kinds of preparation took up dehydroascorbate at the same rate.

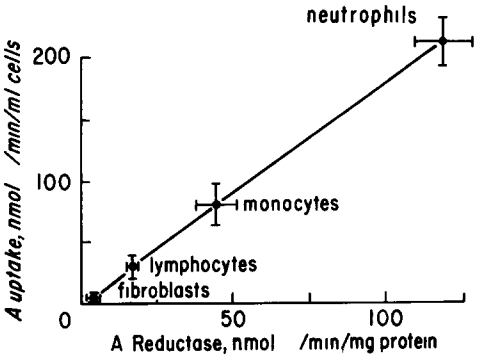


Fig 4. Cytosol dehydroascorbate reductase (A reductase) activities vs rates of dehydroascorbate uptake by intact human cells

Discussion

Plants contain both dehydroascorbate reductase and reduced ascorbate oxidase [14] activities. In the absence of evidence that mammalian tissues possess reduced ascorbate oxidase activity, it is reasonable to question the function of mammalian dehydroascorbate reductase. Dehydroascorbate constitutes 5—20% of total ascorbate in normal human plasma and leukocytes [15,16]. The plasma dehydroascorbate fraction may be considerably higher in certain disease states [15]. Although enzymatic dehydroascorbate production has not been demonstrated in intact mammalian tissues, it may occur, for example in those situations where reduced ascorbate appears to protect *p*-hydroxyphenylpyruvic acid oxidase from substrate inhibition [17,18] and during norepinephrine biosynthesis catalyzed by dopamine β-hydroxylase [19]. Reduced ascorbate autoxidation catalyzed by transition metal ions and reactions of reduced ascorbate with oxidants and free radicals are other potential sources of tissue dehydroascorbate. Cellular total and reduced ascorbate are depleted during the enhanced production of oxidants and free radicals which accompanies phagocytosis, at the same time, cell dehydroascorbate increases [20]. One function of dehydroascorbate reductase may be to regenerate tissue reduced ascorbate which has been enzymatically or nonenzymatically oxidized. Its efficiency may determine the capacity of cellular reduced ascorbate to serve as an enzyme protector and substrate, and as a part of mechanisms which inactivate potentially injurious oxidants and free radicals.

Dehydroascorbate taken up by intact neutrophils from suspending solution is virtually completely reduced when measured immediately after uptake [1]. Dehydroascorbate uptake is restricted by the rate of GSH regeneration in glutathione reductase-deficient human neutrophils [6] Thus, at least in neutrophils, dehydroascorbate reduction is required for dehydroascorbate uptake. The present study shows that the rate of dehydroascorbate uptake among four normal human cell types correlates with cytosol dehydroascorbate reductase activity, it does not correlate with GSH content or with the activities of GSH-regenerating enzymes. These observations suggest that the dehydroascorbate reduction which occurs during dehydroascorbate uptake by intact cells depends on dehydroascorbate reductase activity.

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