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Purification and Characterization of a Glutathione Dependent Dehydroascorbate Reductase from Human Erythrocytes

Dian Peng Xu, Michael P. Washburn, Guo Ping Sun, and William W. Wells[1]

Department of Biochemistry, Michigan State University, East Lansing, Michigan 48824

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A GSH-dependent dehydroascorbate reductase (EC 1.8.5.1) was purified to homogeneity from human erythrocytes. The enzyme was a monomer of 32 kDa and was purified 133-fold from a crude DEAE-Sepharose fraction with a 25% yield. The reduced protein had a pI of 5.1 as judged by isoelectric focusing. Kinetic analysis gave a k_{cat} of 316 min^{-1}, a K_m of 0.21 mM for DHA with a k_{cat}/K_m of 2.47×10^4 M^{-1} sec^{-1}, and a K_m of 3.5 mM for GSH with a k_{cat}/K_m of 1.51×10^3 M^{-1} sec^{-1}. This is the second DHA reductase (after thioltransferase) isolated from human erythrocytes, but unlike thioltransferase, it has no thiol-disulfide oxidoreductase activity.

Ascorbic acid (AAHH) is an essential enzyme cofactor and an antioxidant in both plant and animal tissues (1,2). AAHH is easily oxidized by one-electron reactions, e.g., as a scavenger of $O_2\cdot^-$, to form the AAHH free radical (AAH·), which rapidly (1×10^5 M^{-1} s^{-1}) disproportionates to AAHH and dehydro-L-ascorbic acid (DHA) (3). Given the importance of AAHH in cellular functions, and the potentially cytotoxic action of DHA (4,5), it is of current biological and medical interest to determine the factors and pathways in cellular ascorbate recycling from DHA. The reduction of DHA by glutathione (GSH) is a well-documented chemical reaction (6). In a study by Christine, et al. (7), evidence was obtained that an enzyme or enzymes other than glutathione disulfide (GSSG) reductase were involved in the GSH dependent reduction of DHA in human erythrocytes. In 1990, it was shown that two purified mammalian proteins, thioltransferase, (also known as glutaredoxin), and protein disulfide isomerase (PDI), had DHA reductase activity (8). Thioltransferase was later isolated from human erythrocytes by Mieyal et al. (9) confirming the existence of a known DHA reductase in human erythrocytes. Recently, Maellaro et al. (10) purified and characterized a GSH-dependent DHA reductase from rat liver that had no thiol-disulfide oxidoreductase activity. In the same laboratory, a NADPH-dependent DHA reductase activity was also detected in rat liver and was identified as 3α-hydroxysteroid dehydrogenase (11). The discovery in rat liver of a 31 kDa GSH-dependent DHA reductase raised the question whether the activity in human erythrocytes, first noted by Christine et al. (7), could be accounted for solely by thioltransferase. Accordingly, we examined human erythrocytes for such activity and purified to homogeneity a 32 kDa protein with DHA reductase activity. In this communication, we report its purification, some physical properties, and its DHA reductase kinetic properties.

MATERIALS AND METHODS

Outdated human erythrocytes were kindly provided by the American Red Cross, Great Lakes Region, Lansing, MI. DEAE Sepharose, CM Sepharose, Sephacryl S-200, Ampholine (pH 3.5–10.0) and polybuffer 74 and PBE 94 were purchased from Pharmacia Biotech. Bio-Gel HT hydroxylapatite was from Bio-Rad Laboratories. DL-histidine, ascorbic acid, glutathione disulfide reductase, β-mercaptoethanol, ammonium sulfate, hydroxyethyl disulfide and phenylmethylsulfonylfluoride (PMSF) were products of Sigma Chemical Co. Potassium phosphate, EDTA, and NaCl were purchased from J. T. Baker. MES, DTT and glutathione were from Boehringer Mannheim Laboratories, and bromine was purchased from Aldrich Chemical Co.

Dehydroascorbate reductase assay. For the enzyme purification, DHA reductase activity was assayed essentially as

[1] To whom correspondence should be addressed. Fax: (517) 353-9334. E-mail: wellsw@pilot.msu.edu.

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described previously (12). Briefly, the mixture contained 100 mM potassium phosphate, pH 7.0, 1 mM EDTA, 1 mM GSH, 0.4 mM DHA and various amounts of enzyme in a volume of 500 μl. The rate of ascorbic acid formation was followed by measuring the absorbance increase at 265 nm and 30°C. The extinction coefficient of ascorbate at 265 nm is 14,700 M^{-1} cm^{-1} (13). A blank without enzyme was run simultaneously for each analysis.

Protein determination. Protein was analyzed by the method of Brown et al. (14) using bovine serum albumin as a standard.

Enzyme kinetics. Kinetic analysis of the human erythrocyte DHA reductase was conducted according to the procedure described for thioltransferase and PDI (12) except that the pH was 7.2. The studies were carried out in a volume of 0.5 ml and the presence of 2.3 μg enzyme. The assay consisted of 100 mM potassium phosphate, pH 7.2, GSH, enzyme, and DHA. The reaction was initiated upon the addition of DHA and was followed at 265 nm at 30°C for 3 min in a Gilford Response II UV/Vis spectrophotometer. A blank without enzyme was run simultaneously for each assay and the difference gave activity in nmoles/min. For determination of the Km for DHA, [GHS] was held at 3.0 mM and [DHA] varied, and for determination of the Km for GSH, [DHA] was held at 1.5 mM and the [GSH] varied.

Thioltransferase activity assay. The enzyme activity was assayed as described previously (15). Briefly, the reaction mixture contained 0.5 mM GSH, 1.4 units of glutathione disulfide reductase, 2.5 mM S-sulfocysteine, 0.35 mM NADPH, 0.137 M sodium phosphate buffer, pH 7.5 and the enzyme activity was monitored spectrophotometrically at 340 nm and 30°C. One unit of thioltransferase activity is defined as that amount of enzyme catalyzing the formation of 1 μmol of GSSG/min under standard conditions. A control reaction (without enzyme) was monitored simultaneously with each group of enzyme samples.

Enzyme purification. All steps in the purification were carried out at 4°C. Four units of outdated human red blood cells (250 ml packed cells/unit) were washed two times in 3 volumes of cold 0.15 M NaCl and centrifuged for 10 min at 2,000 × g. The supernatant was discarded, and the cells were lysed with 3 volumes of 10 mM potassium phosphate, pH 7.8 containing 1 mM EDTA, 5 mM β-mercaptoethanol and 0.5 mM PMSF. The suspension was stirred at 4°C for 3 hr, and the lysate was centrifuged at 20,000 × g for 20 min. The supernatant solution was removed and adjusted to pH 7.8 before application to a DEAE-Sepharose column (5 × 10 cm) equilibrated with 10 mM potassium phosphate, pH 7.8, 1 mM EDTA, and 1 mM β-mercaptoethanol. The column was washed with the equilibration buffer and the enzyme was eluted with a linear salt gradient consisting of 250 ml of equilibration buffer and 250 ml of 0.25 M NaCl in equilibration buffer. Fractions containing DHA reductase activity were pooled.

Ammonium sulfate fractionation and CM-Sepharose chromatography. The enzyme pool was precipitated by ammonium sulfate in the fractions between 50–75% saturation. The precipitate was dissolved in 10 mM MES, pH 5.5, 1 mM EDTA and 1 mM β-mercaptoethanol. The sample was dialyzed twice against 1 liter of 10 mM MES, pH 5.5, 1 mM EDTA and 1 mM β-mercaptoethanol. The dialysate was centrifuged at 20,000 × g for 20 min to remove the precipitate and was applied to CM-Sepharose (2.5 × 16 cm) equilibrated with the dialyzing buffer. The column was washed with the same buffer and the enzyme activity was eluted with a linear salt gradient of 250 ml each of 10 mM potassium phosphate, pH 6.5, 1 mM EDTA, 1 mM β-mercaptoethanol and 0.35 M NaCl in the same buffer. Complete elution of enzyme was terminated by the addition of 150 ml of the latter solution. The fractions containing enzyme activity were pooled.

Bio-Gel HT hydroxylapatite. The pooled sample was dialyzed overnight against 2 × 1 liter of 10 mM potassium phosphate, pH 6.8, 1 mM EDTA, and 1 mM β-mercaptoethanol. The dialysate was centrifuged at 20,000 × g for 20 min to remove percipitated protein. The supernatant fluid was applied to a Bio-Gel HT hydroxylapatite column (2.5 × 6 cm) which was equilibrated with 10 mM potassium phosphate, pH 6.8, 1 mM EDTA, 1 mM β-mercaptoethanol. The column was washed with the equilibrating buffer. The enzyme activity was eluted with 45 mM potassium phosphate, pH 6.8, 1 mM EDTA, 1 mM β-mercaptoethanol. The fractions containing enzyme activity were pooled and dialyzed against 25 mM DL-histidine, pH 6.2, 1 mM β-mercaptethanol.

Chromatofocusing. The dialyzed sample was applied to a column (1 × 22 cm) of PBE 94 which was equilibrated with 25 mM DL-histidine, pH 6.2, 1 mM β-mercaptoethanol. The column was washed with 2 column volumes of equilibrating buffer. The enzyme was eluted with polybuffer 74, pH 4.0, using a dilution factor of 9 from the commercial stock solution. Two peaks of DHA reductase activity were detected. The earlier peak was homogeneous but the second peak contained a low molecular weight contaminant. The second peak was further purified to homogeneity by Sephacryl S-200 gel filtration (2.5 × 90 cm) using 10 mM potassium phosphate, 150 mM NaCl, pH 6.8 as equilibration and separation buffer. The fractions containing DHA reductase activity were pooled and concentrated using a Centriprep 3 concentrator.

SDS-polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis was conducted as described by Laemmli (16). The stacking and separating gels (0.75 mm) had polyacrylamide concentrations of 6 and 12%, respectively. The gels were run at a constant voltage of 100V for stacking gel and 200V for separating gel in a Bio-Rad minigel apparatus and the gel was stained with Coomassie Brilliant Blue R-250.

Isoelectric focusing. Purified human 32 kDa DHA reductase was subjected to isoelectric focusing analysis following the procedure of Robertson et al. (17), and using isoelectric focusing standards from Serva Feinbiochemica.

RESULTS AND DISCUSSION

Purification of the enzyme. Dehydroascorbate reductase from 4 × 250 ml units of packed human erythrocytes was purified 133 fold with a yield of 25% calculated from the starting DEAE Seph-

arose chromatography fraction (Table I). Most hemoglobin was removed by passing the equilibrated lysate through the DEAE Sepharose column. Purification was especially effective as a result of the hydroxylapatite and chromatofocusing steps. The enzyme was judged to be homogeneous based on the analysis of the final preparation by SDS-PAGE (Fig. 1) and isoelectric focusing (Fig. 2). The subunit molecular weight was estimated to be 32,000 by comparison with molecular weight standards on SDS-PAGE. The enzyme was likely a monomeric protein based on gel filtration chromatography (data not shown). The purified protein remained relatively stable in the reduced form since treatment with 3 mM dithiothreitol for 10 min at room temperature did not shift the pI (5.1) of the purified protein (Fig. 2.; lane C vs. lane A). Moreover, treatment of the reduced protein with 3 mM hydroxyethyl disulfide for 10 min at room temperature caused a shift of pI for only a minor fraction of the total signifying only a partial oxidation to the disulfide under conditions which previously oxidized all the active center thiols for pig liver thioltransferase (18).

Kinetic parameters of human erythrocyte DHA reductase. Kinetic analysis of the purified human erythrocyte DHA reductase at pH 7.2 was conducted to compare directly with the conditions used by Maellaro et al. (9), and because the intracellular pH of erythrocytes is approximately 7.25 (19). Standard kinetic parameters are compared in Table II with those of the rat liver cytosolic enzyme using data reported by the authors (9). The k_{cat} of the rat liver DHA reductase (140 min^{-1}) and for DHA, the k_{cat}/K_m (9.52×10^3 $M^{-1}sec^{-1}$) was somewhat lower in each case than the corresponding values for human DHA reductases reported here. In addition, the DHA reductase kinetic parameters of pig liver thioltransferase and bovine liver PDI (12) are provided for comparison although the assays were performed at pH 6.9. The human DHA reductase has a k_{cat} slightly lower than that of pig liver thioltransferase (316 vs 374 min^{-1}). However, its efficiency, k_{cat}/K_m, based on the K_m for DHA, was nearly identical (2.47×10^4 vs 2.43×10^4 $M^{-1}sec^{-1}$).

Thioltransferase activity. The purified human erythrocyte DHA reductase was assayed for thioltransferase activity using S-sulfocytsteine and GSH as cosubstrates. No activity above the control reaction in the absence of enzyme was detected (data not shown). Other thiols such as L-cysteine and cysteamine were tested for the GSH dependent DHA reductase activity, but only GSH was effective under the conditions of the standard assay procedure (data not shown).

The early studies of Christine et al. (11) showed that human erythrocytes have the ability to regenerate AA from DHA by enzyme catalysis. In the present study, we have purified to homogeneity what is now the second known DHA reductase from human erythrocytes and characterized its molecular weight and kinetic properties. In the purification protocol of the present work, direct DHA reductase activity was monitored during each step. The only other DHA reductase peak to be observed during the isolation was shown to be thioltransferase as previously reported for this source (3).

Although thioltransferase has both thiol-disulfide oxidoreductase and DHA reductase activities,

TABLE I
Purification of Human Erythrocyte[a] DHA Reductase

Purification step	Protein (mg)	Total DHA reductase activity (nmol/min)	Specific activity (nmol/min/mg)	Yield (%)	Purification (fold)
DEAE Sepharose	564	7520	13.4	100	1
Ammonium sulfate fractionation and CM–Sepharose	154	3650	23.6	48	1.8
Bio-Gel HT hydroxylapatite	5.3	2380	465.3	32	34.3
PBE 94 chromatofocusing	1.08	1920	1770	25	133

[a] From 4 units (1 liter) of packed human erythrocytes.

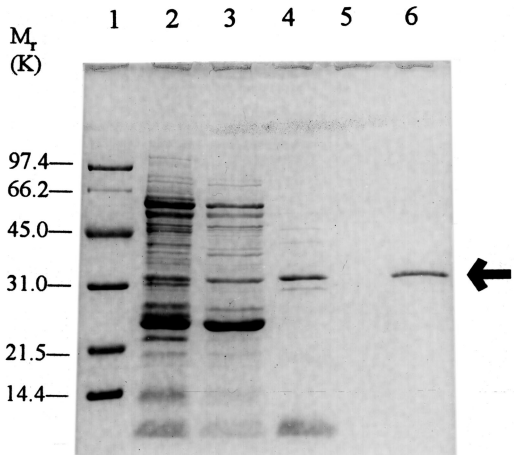

FIG. 1. SDS-PAGE (12%) analysis of human erythrocyte DHA reductase from various stages of purification. Lane 1, Bio-Rad low molecular weight standards; lane 2, 21 μg of DEAE Sepharose fraction; lane 3, 12 μg of ammonium sulfate fractionation and CM-Sepharose fraction; lane 4, 1.6 μg of Bio-Gel HT fraction; lane 5, a blank; and lane 6, 0.5 μg of PBE 94 chromatofocusing fraction. The gel was stained with Coomassie Brilliant Blue R-250.

the new human erythrocyte DHA reductase does not have thiol-disulfide oxidoreductase activity. In addition, the latter protein once reduced by beta-mercaptoethanol is relatively stable against oxidation by hydroxyethyl disulfide, but loses activity on storage. When stored at 4°C in polybuffer 74 at pH 6.8, 55% of the activity was lost after 3 weeks. This raises intriguing speculation regarding

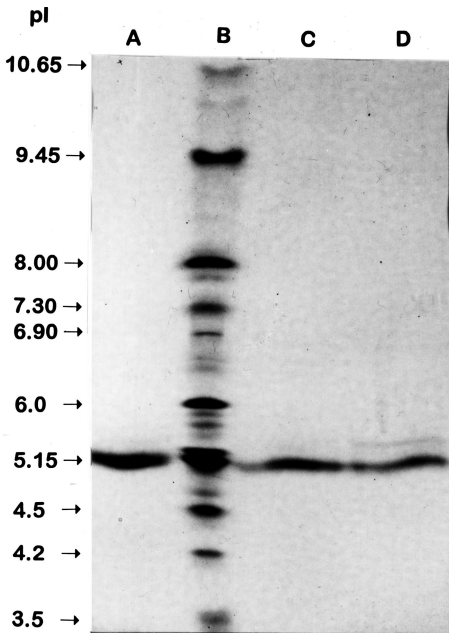

FIG. 2. Vertical slab isoelectric focusing of purified human erythrocyte dehydroascorbic acid reductase. Lane A, 5.5 μg of PBE 94 chromatofocusing fraction; lane B, 5 μl of Serva pI marker mixture, pH 3–10; lane C, 5.5 μg of human dehydroascorbic acid reductase treated with 3 mM dithiothreitol, 10 mM sodium phosphate, pH 6.8, 150 mM NaCl, for 10 min at room temperature; lane D, 5.5 μg of human dehydroascorbic acid reductase treated with 3 mM hydroxyethyl disulfide, 10 mM sodium phosphate, pH 6.8, 150 mM NaCl, for 10 min at room temperature. The gel was stained with Coomassie Brilliant Blue R-250.

TABLE II
Kinetic Parameters for Mammalian Dehydroascorbate Reductases

Parameter	Human RBC DHA reductase[a]	Rat liver DHA reductase[b]	Pig liver thioltransferase[c]	Bovine protein disulfide isomerase[c]
$k_{cat}(min^{-1})$[d]	316 ± 1	140 ± 40	374 ± 20	16 ± 1
K_m(app) (mM)[e]	DHA 0.21 ± 0.06	0.25 ± 0.06	0.26 ± 0.09	2.8 ± 0.4
	GSH 3.5 ± 0.3	2.8 ± 0.6	3.5 ± 0.3	2.9 ± 0.4
$k_{cat}/K_m(M^{-1}sec^{-1})$				
DHA	$2.47 \pm 0.64 \times 10^4$	$9.52 \pm 3.64 \times 10^3$	$2.43 \pm 0.85 \times 10^4$	93 ± 14
GSH	$1.51 \pm 0.11 \times 10^3$	$0.83 \pm 0.30 \times 10^3$	$1.81 \pm 0.2 \times 10^3$	91 ± 14

[a] Human erythrocyte DHA reductase from this study.
[b] Taken from (9), pH 7.2.
[c] Taken from (12), pH 6.9.
[d] k_{cat} values were calculated by dividing V_{max}(app) by the molar concentration of the enzymes.
[e] K_m(app) values were calculated by nonlinear least-square fit to the velocity versus substrate concentration data using the PSI-Plot 3.5 software.

the active center structure of the new DHA reductase. More extensive investigation of the protein structure is now underway and should enhance our understanding of the reaction mechanism in comparison with that of thioltransferase.

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REFERENCES

1. Englard, S., and Seifter, S. (1986) *Annu. Rev. Nutr.* **6,** 365–406.
2. Padh, H. (1991) *Nutr. Rev.* **49,** 65–70.
3. Bielski, B. H. J. (1982) *in* Ascorbic Acid: Chemistry, Metabolism, and Uses (Seib, P. A. and Tolbert, B. M., Eds.), pp. 81–100, *Am. Chem. Soc.*, Washington, DC.
4. Patterson, J. W. (1950) *J. Biol. Chem.* **183,** 81–88.
5. Patterson, J. W. (1951) *Am. J. Physiol.* **165,** 61–65.
6. Borsook, H., Davenport, H. W., Jeffreys, C. E. P., and Warner, R. C. (1937) *J. Biol. Chem.* **117,** 237–279.
7. Christine, L., Thomas, G., Iggo, B., Brownie, A. C., and Stewart, C. P. (1956) *Clin. Chim. Acta* **1,** 557–569.
8. Wells, W. W., Xu, D. P., Yang, Y., and Rocque, P. A. (1990) *J. Biol. Chem.* **265,** 15361–15364.
9. Mieyal, J. J., Starke, D. W., Garvina, S. A., Cothey, C., and Chung, J. S. (1991) *Biochemistry* **30,** 6088–6097.
10. Maellaro, E., Del Bello, B., Sugherini, L., Santucci, A., Comporti, M., and Casini, A. (1994) *Biochem. J.* **301,** 471–476.
11. DelBello, B., Maellaro, E., Sugherini, L., Santucci, A., Comporti, M., and Casini, A. F. (1994) *Biochem. J.* **304,** 385–390.
12. Wells, W. W., Xu, D. P., and Washburn, M. P. (1995) *Methods Enzymol.* **252,** 30–38.
13. Daglish, C. (1951) *Biochem. J.* **49,** 635–639.
14. Brown, R. E., Jarvis, K. L., and Hyland, K. J. (1989) *Anal. Biochem.* **180,** 136–139.
15. Gan, Z.-R., and Wells, W. W. (1986) *J. Biol. Chem.* **261,** 996–1001.
16. Laemmli, U. K. (1970) *Nature* **227,** 680–685.
17. Robertson, E. F., Dannelly, H. K., Malloy, P. J., and Reeves, H. C. (1987) *Anal. Biochem.* **167,** 290–294.
18. Gan, Z-R., and Wells, W. W. (1987) *Anal. Biochem.* **162,** 265–273.
19. Roos, A., and Boron, W. F. (1981) *Physiol. Rev.* **61,** 296–434.