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Molecular Cloning and Functional Expression of Rat Liver Glutathione-dependent Dehydroascorbate Reductase*

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We have isolated a cDNA clone for a novel glutathione-dependent dehydroascorbate reductase from a rat liver cDNA library in λgt11 by immunoscreening. The authenticity of the clone was confirmed as follows: first, the antibody that had been purified through affinity for the protein expressed by the cloned λgt11 phage recognized only the enzyme in a crude extract from rat liver; and second, two internal amino acid sequences of purified enzyme were identified in the protein sequence predicted from the cDNA. The predicted protein consists of 213 amino acids with a molecular weight of 24,929, which is smaller by ~3,000 than the value obtained by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. This discrepancy of the molecular weight was explained by post-translational modification because the recombinant protein expressed by a mammalian system (Chinese hamster ovary cells) was of the same size as rat liver enzyme but larger than the protein expressed by a bacterial system (*Escherichia coli*). Chinese hamster ovary cells, originally devoid of glutathione-dependent dehydroascorbate reductase activity, was made to elicit the enzyme activity (1.5 nmol/min/mg of cytosolic protein) by expression of the recombinant protein. Additionally, the cells expressing the enzyme were found to accumulate 1.7 times as much ascorbate as the parental cells after incubation with dehydroascorbate. This result points to the importance of the dehydroascorbic acid reductase in maintaining a high concentration of ascorbate in the cell.

L-Ascorbic acid (AA)[1] acts as an important cofactor in various enzymatic reactions and also as an effective antioxidant in scavenging reactive oxygen species *in vivo*. These physiological functions of AA are associated with its univalent or divalent oxidation. The univalent oxidation of AA leads to the formation of monodehydroascorbate that is converted to the divalent oxidation product dehydroascorbic acid (DHA) through spontaneous disproportionation or further oxidation. Because DHA is unstable at physiological pH and temperature (1), regeneration of AA from DHA could be a beneficial process even for many organisms that can synthesize AA themselves. Especially for humans and primates that cannot synthesize it, dietary intake of AA is the only way to supply this vitamin; therefore, a system for regeneration of AA from its oxidized forms would be important for the cell to maintain a normal cellular level of AA.

Many reactions potentially contributing to the regeneration of AA in animal cells have been reported. Monodehydroascorbate is reduced to AA by an NADH-dependent enzymatic reaction occurring on subcellular membranes of mitochondria (2) and microsomes (3, 4). As for the conversion of DHA to AA, nonenzymatic reduction by GSH has been suggested for a long time (5). However, because of the slowness of the reaction, much attention has been directed to enzyme-catalyzed reduction of DHA in recent years. Wells *et al*. (6) reported that porcine liver thioltransferase (glutaredoxin) and bovine thymus and human placenta protein disulfide isomerase catalyze the reduction of DHA to AA using GSH as a hydrogen donor. Park and Levine (7) also reported that the same enzymatic activity purified from human neutrophils was attributable to glutaredoxin. Besides these enzymes belonging to a family of thiol-disulfide oxidoreductase, 3α-hydroxysteroid dehydrogenase (8) and thioredoxin reductase (9), both from rat liver, have recently been demonstrated to catalyze the same reaction using NADPH as a hydrogen donor. The reduction of DHA to AA by all of these enzymes appears to take place as a secondary reaction because of their broad substrate specificity. In addition to these enzymes, Maellaro *et al*. (10) reported a novel enzyme that catalyzes GSH-dependent reduction of DHA in rat liver. The enzyme shows no thiol-disulfide oxidoreductase activity and has enzymatic properties different from any of the above-mentioned enzymes. More recently, Xu *et al*. (11) have also indicated the occurrence of GSH-dependent DHA reductase with no thiol-disulfide oxidoreductase activity in human erythrocytes. To characterize this kind of DHA reductase at the molecular level, we have decided to clone a cDNA for the former enzyme. A study of expression of an obtained cDNA in Chinese hamster ovary (CHO) cells and in *Escherichia coli* revealed that the molecular weight of the primary translation product is increased by certain modification in the former cells. It was also shown that accumulation of AA is increased in the CHO cells that had been made to express DHA reductase, indicating the importance of this enzyme in maintaining a high intracellular concentration of AA.

EXPERIMENTAL PROCEDURES

Cloning of a cDNA for Rat Liver GSH-dependent DHA Reductase—A rat liver cDNA library in λgt11 (Stratagene, La Jolla, CA) was screened with antiserum against rat DHA reductase. The antiserum used was the same that had been raised in a rabbit in our previous study (12). After three rounds of screening, positive clones were isolated. The insert in one of the positive clones was excised from the phage DNA and subcloned into pUC19. The resulting plasmids were analyzed by diges-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AB008807.

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[1] The abbreviations used are: AA, L-ascorbic acid; DHA, dehydroascorbic acid; bp, base pair; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; HPLC, high-performance liquid chromatography; CHO, Chinese hamster ovary.

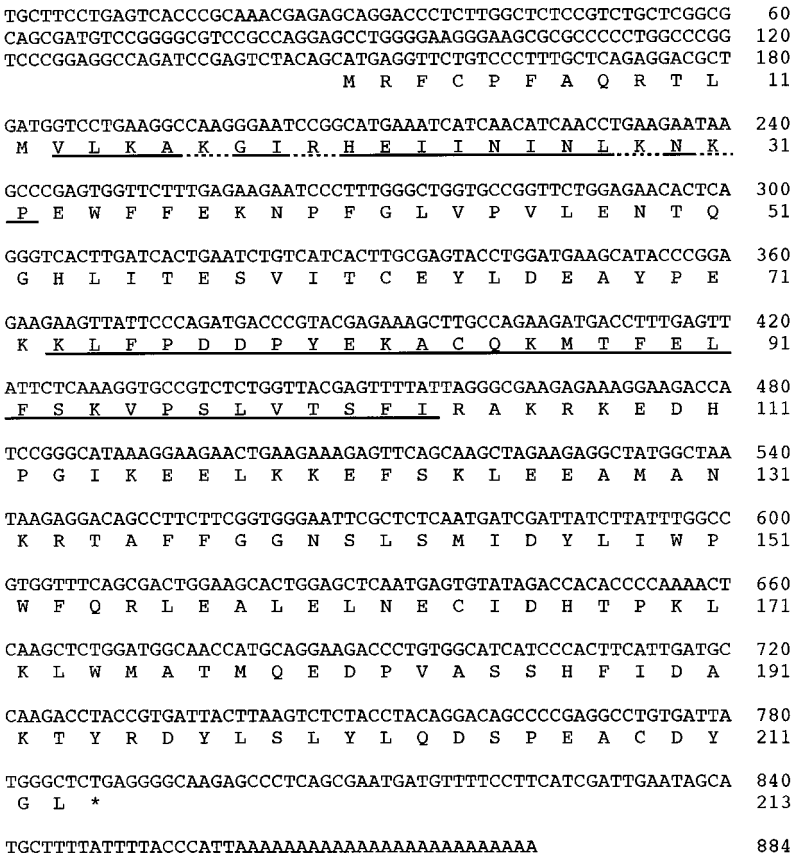

FIG. 1. **Nucleotide sequence and deduced amino acid sequence of rat liver GSH-dependent DHA reductase.** The nucleotides are numbered on the *right* from the first base of the cDNA. The deduced amino acid sequence is shown below the nucleotide sequence in *single-letter code*. The *upper underlined* peptide sequence indicates the sequence previously determined by Maellaro *et al*. (10); the *lower underlined* sequence is the one determined in this study. The former sequence does not completely match the deduced sequence; the unmatched amino acids are underlined with a *broken line*.

tion with restriction enzymes and then sequenced completely on both strands by the dideoxy chain termination method using an automatic DNA sequencer (373A, Applied Biosystems, Foster City, CA).

Analysis of the 5′ End of cDNA—5′-Rapid amplification of cDNA ends was performed with 5′-RACE System for Rapid Amplification of cDNA Ends, version 2.0 (Life Technologies, Inc.), according to the manufacturer's instructions. Total RNA was isolated from rat liver by the method of Chomczynski and Sacchi (13). The 5′ end portion of DHA reductase-encoding cDNA was synthesized from the RNA using a primer located at nucleotides 330–352 of the cDNA sequence. The subsequent polymerase chain reaction was carried out using an anchor primer and a primer (corresponding to nucleotides 289–312) which is located 5′ to the primer used for the cDNA synthesis, and the resulting product was subcloned into the pGEM-T vector (Promega, Madison, WI).

Purification of Antibody Specific for Protein Expressed by a λgt11 Clone—Synthesis of the recombinant protein in *E. coli* cells harboring a positive λgt11 clone was induced by isopropyl-1-thio-β-D-galactopyranoside, and proteins in the phage plaques were immobilized on nitrocellulose membranes. The resulting membranes were used to purify the antibody that was specific for the recombinant protein expressed by the λgt11 clone, as described by Weinberger *et al*. (14).

Protein Digestion—GSH-dependent DHA reductase was purified from rat liver as described previously (12). The DHA reductase was partially digested by *Achromobacter* lysyl endopeptidase (Wako Chemicals, Osaka, Japan) at 37 °C for 1 h. The proteolytic fragments generated were separated by SDS-polyacrylamide gel electrophoresis (15% gels) after treatment with 2-mercaptoethanol according to the method of Laemmli (15) and were blotted electrophoretically onto an Immobilon membrane (Millipore, Bedford, MA). The membrane was washed extensively with water, stained with 0.06% Coomassie Blue R-250 in 50% methanol for 5 min, and destained with 30% (v/v) methanol/7% (v/v) acetic acid for 10 min. The portions of the membrane stained for the fragments of interest were excised with a razor blade, and the N-terminal sequence was determined using a gas phase protein sequencer (model 492, Applied Biosystems).

Mass Spectrometry—The purified preparation of DHA reductase was dialyzed against distilled water and was mixed with a sinnapinic acid matrix solution. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry was performed in positive linear mode with pulsed ion extraction on a MALDI-TOF mass spectrometer (Reflex II, Bruker Japan Co. Tsukuba, Japan).

Deglycosylation—Chemical deglycosylation of the purified DHA reductase was performed using trifluoromethane sulfonic acid as described previously (16). Briefly, 10 μg of the dry sample was resuspended in 10 μl of anisol (Sigma). After 90 μl of trifluoromethane sulfonic acid was added, the mixture was incubated at 4 °C for 2 h. The sample was precipitated with trichloroacetic acid and washed five times with ice-cold diethyl ether. The precipitate was vacuum dried and analyzed by immunoblot analysis according to the procedure described below.

Expression of Recombinant DHA Reductase in E. coli—To amplify the enzyme-coding region of the cDNA by polymerase chain reaction, we used an oligonucleotide (5′-CCGAGTCTACCATATGAGGTTCTG-3′) containing an *Nde*I site followed by nucleotides 136–159 of the DHA reductase cDNA as an upstream primer and an oligonucleotide (5′-CTGAGGGATCCTGCCCCTCAGAGC-3′) containing a *Bam*HI site followed by nucleotides 784–807 of the same cDNA as a downstream primer. The polymerase chain reaction was performed in 50-μl reaction mixtures containing a 1.0 μM concentration of each of the primers, 1.25 units of Ex *Taq* DNA polymerase (Takara, Kyoto, Japan), 200 μM dNTPs, and 1 × Ex *Taq* buffer supplied by the manufacturer (Takara). The polymerase chain reaction cycle consisted of 30 cycles at 94 °C for 1 min, 55 °C for 30 s, and 72 °C for 1 min. The amplified DNA fragment was isolated and subcloned into pGEM-T and then into the *Nde*I-*Bam*HI site of pET-3a (Novagen, Madison, WI). The sequence of the insert region in the pGEM-T vector was verified by DNA sequencing. *E. coli* cells of the strain BL21(DE3)pLysS (Novagen) were transformed with the resulting plasmid, pET/DHAR, and grown in Luria-Bertani medium supplemented with 50 μg/ml ampicillin at 37 °C. When the culture reached an absorbance of 0.6 at 600 nm, isopropyl-1-thio-β-D-galactopyranoside was added to a concentration of 400 μM, and the cells were grown further at 37 °C for 4 h and harvested by centrifugation, suspended in 50 mM potassium phosphate buffer (pH 7.0), and disrupted by sonication for preparation of cell lysate.

Construction of Mammalian Expression Vector—To amplify the enzyme-coding region of the cDNA by polymerase chain reaction, we used an oligonucleotide (5′-GTTCTAGAGGCCAGATCCGAGTC-3′) containing an *Xba*I site followed by nucleotides 120–142 of the DHA reductase cDNA as an upstream primer and an oligonucleotide (5′-CTGAGGGC-

CCTTGCCCCTCAGAGC-3′) containing an *Apa*I site followed by nucleotides 784–807 of the same cDNA as a downstream primer. The polymerase chain reaction was performed under the same conditions as described above except that the temperature of the annealing step was 60 °C. The amplified DNA fragment was isolated and subcloned into pGEM-T and then into the *Xba*I-*Apa*I site of pRc/CMV. The resulting plasmid, designated pRc/DHAR, is driven by the cytomegalovirus promoter.

Cell Culture and Transfection—CHO cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 50 units/ml penicillin, and 50 μg/ml streptomycin. Exponentially growing cells (1×10^7 cells/ml) were electroporated with 10 μg of pRc/DHAR at 200 V and 950 microfarads with a time constant of 30–40 ms using an electroporation apparatus (Gene Pulser II). The transfected cells were subsequently selected in the same medium containing 800 μg/ml G418 by exchanging the medium every 3 days for 2 weeks, and individual G418-resistant colonies were isolated with cloning cylinders. Thereafter, the cloned cells were maintained in the medium containing 400 μg/ml G418.

Immunoblot Analysis—Proteins were separated on a 12.5% polyacrylamide gel by SDS-polyacrylamide gel electrophoresis and transblotted onto a nitrocellulose membrane with the transfer buffer of Towbin *et al*. (17) at 15 V for 1 h in a semidry electroblot apparatus (Taitec, Saitama, Japan). The membrane was incubated with rabbit anti-DHA reductase antibody and then visualized with horseradish peroxidase-conjugated goat anti-rabbit IgG (Cappel, Organon Pharmaceuticals) as secondary antibody and 4-methoxy-1-naphthol (Aldrich) as a chromogenic substrate.

DHA Reductase Activity—Cells were suspended in ice-cold 100 mM potassium phosphate buffer (pH 7.2) containing 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 50 μg/ml leupeptin, 50 μg/ml aprotinin, and 50 μg/ml pepstatin A and sonicated meticulously in an ice water bath by three 2-s bursts with 30-s cooling intervals. The resulting cell lysate was centrifuged at 12,000 × *g* at 4 °C for 10 min. Then, GSH-dependent DHA reductase activity in the supernatant obtained was measured as described previously (10). Briefly, the formation of AA was followed by an increase in absorbance at 265 nm ($\epsilon = 14{,}700\ M^{-1}\ cm^{-1}$) when the supernatant (40 μg of protein) was added to a mixture (0.2 ml) containing 100 mM potassium phosphate buffer (pH 7.2), 0.25 mM DHA, and 1 mM GSH.

Northern Analysis—Total RNA was isolated from various organs by the method of Chomczynski and Sacchi (13). The RNA (5 μg) was separated by electrophoresis through a 1.2% agarose-glyoxal gel, blotted onto GeneScreen Plus membrane (Dupont NEN), and hybridized with a ^{32}P-labeled DHA reductase cDNA prepared by random priming. Hybridization was carried out in 5 × saline/sodium phosphate/EDTA, 1% SDS, 1 × Denhardt's solution, and 100 μg/ml denatured salmon sperm at 60 °C overnight. Finally, the membrane was washed at 60 °C for 1 h in 0.1 × saline/sodium phosphate/EDTA and 0.1% SDS and exposed to x-ray film with an intensifying screen at −80 °C. For standardization of the amount of RNA in each lane, after hybridization with DHA reductase cDNA, the blots were stripped by incubation in 0.01 × saline/sodium phosphate/EDTA and 0.01% SDS at 95 °C for 1 h and reprobed with mouse β-actin cDNA (18) that had been labeled with ^{32}P.

Accumulation of Ascorbic Acid—Cells were seeded in T-25 tissue culture flasks and grown until semiconfluency. After the culture medium was removed, the cells were once rinsed with incubation buffer (10 mM Hepes, pH 7.4, 150 mM NaCl, 5 mM KCl, 1.9 mM KH_2PO_4, 1.1 mM Na_2HPO_4, 5.5 mM glucose, 0.3 mM $MgSO_4$, 1 mM $MgCl_2$, and 1.5 mM $CaCl_2$) and incubated in 5 ml of the same fresh buffer at 30 °C for various times after addition of 150 μM DHA that had been prepared immediately before use by bromine oxidation of AA. The AA in the cells was determined by HPLC after conversion to its dinitrophenyl hydrazine derivative as described by Kodaka *et al*. (19). Briefly, collected cells were sonicated in 3% metaphosphoric acid, and the supernatant was collected after centrifugation. Ten microliters of 0.2% dichlorophenol indophenol, 150 μl of 2% thiourea, and 30 μl of 2% dinitrophenylhydrazine and 9 N sulfuric acid were successively added to 165 μl of the supernatant, and the resulting mixture was incubated at 50 °C for 90 min. Then, the reaction product was extracted into 200 μl of ethyl acetate. The sample was subjected to HPLC on a Shodex SIL-5B silica column (4.6 × 250 mm; Showadenko, Tokyo, Japan) with a mobile phase of *n*-hexane/ethyl acetate/acetic acid (3.5:5.5:1) at a flow rate of 1.0 ml/min, and the detection was carried out spectrophotometrically at 495 nm. For this analysis a LaChrom HPLC apparatus (Hitachi, Tokyo, Japan) was used. A total amount of vitamin C (a sum of AA and DHA) is determined by this procedure. By skipping the addition of dichlorophenol indophenol, one can measure the amount of DHA only, and the amount of AA is obtained as the difference between the two measurements.

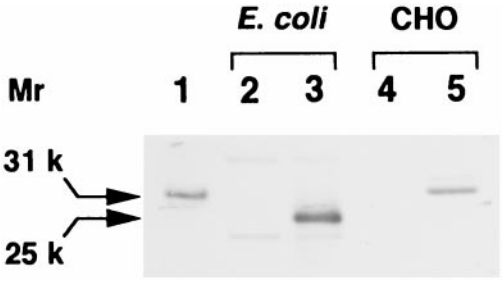

FIG. 2. **Immunoblot analysis of extracts from *E. coli* and CHO cells expressing GSH-dependent DHA reductase.** Recombinant proteins were expressed in *E. coli* BL21(DE3)pLysS and CHO cells and analyzed by immunoblot analysis with anti-DHA reductase antibody, as detailed under "Experimental Procedures." The samples used were purified DHA reductase from rat liver (1 μg of protein, *lane 1*), lysates (5 μg of protein) from *E. coli* harboring pET-3a (*lane 2*) and pET/DHAR (*lane 3*), and cell extracts (50 μg of protein) from nontransfected (*lane 4*) and pRc/DHAR-transfected (*lane 5*) CHO cells.

RESULTS AND DISCUSSION

Cloning of Rat DHA Reductase cDNA—To isolate a cDNA encoding rat liver DHA reductase, we screened a rat liver cDNA library in λgt 11 using antiserum directed against the enzyme as a probe. Four positive plaques were obtained from ~1×10^6 phages from the library. The cDNA inserts in these clones were almost the same in length (~850 bp), and their restriction patterns were the same in fragment sizes (data not shown); however, their first nucleotides at 5′ ends were different from each other, indicating that all the clones were independent ones. The cDNA of the longest clone was 847 bp in length, and a 5′-rapid amplification of cDNA ends experiment led us to isolate a cDNA with an additional 37-bp sequence at its 5′ end. As shown in Fig. 1, the determined cDNA sequence, 884 bp in length, consists of a 5′ untranslated region of 148 bp, a 3′ untranslated region of 97 bp, and an open reading frame of 639 bp that encoded a polypeptide of 213 amino acids with a predicted molecular weight of 24,929. There is no standard polyadenylation sequence in the vicinity of the poly(A) tail. The start of the coding sequence should be the first ATG, because

FIG. 3. **Alignment of amino acid sequence of rat liver GSH-dependent DHA reductase and its homologs.** The sequences compared are rat DHA reductase and hypothetical proteins from mouse (GenBank accession number U80819) and human (GenBank accession number U90313) lymphoma cells. A gap introduced to optimize the alignment is indicated by a *hyphen*. Amino acids identical to those of rat DHA reductase are indicated by *dots*. Amino acids conserved in all proteins are *boxed*.

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rat     MRFCPFAQRTLMVLKAKGIRHEIININLKNKPEWFFEKNPFGLVPVLENDQGHLITESVI  60
mouse   ......|.|..|..........V.............|...L........S...V.....  60
human   ......E..RL.........V.............K...|........S..Q..Y..A.  60

rat     TCEYLDEAYPEKKLFPDDPYEKAQQKMTFELFSKVPSLVTSFIRAKRKEDHPGIKEELKK 120
mouse   ..........|...|.....K..R...L.S.....P.IA..V.S.....S.NLR.A.EN 120
human   .........G...L.....|...|...II.|.....|..G...|.SQN...YA.L..|.FR. 120

rat     EFSKLEEAMANKRTAFFGGNSLSMIDYLIWPWFQRLEALELNECIDHTPKLKLWMAIMQE 180
mouse   ..K....G.D.YK-S.L..D.P..V...T.....|.....K..LA.........A..Q 179
human   ..T....VLT..K.T.|...|..I...|....B....MK..|..V.........A.K. 180

rat     DPVASSHFIDAKTYRDYLSLYLQDSPEACDYGL                           213
mouse   ..|...K...|...E..N.....|.........                           212
human   ..IV.ALLTSE.DWQGF.E....N.........                           213
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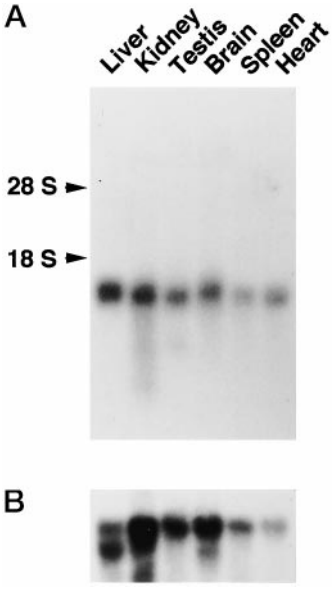

FIG. 4. **Northern blot analysis for GSH-dependent DHA reductase mRNA in various rat organs.** *A*, total RNA (5 μg) was electrophoresed and blotted onto a GeneScreen Plus membrane. The membrane was hybridized with the ^{32}P-labeled DHA reductase cDNA as a probe. The positions of 18 S rRNA (1869 nucleotides) and 28 S rRNA (4712 nucleotides) are shown as size markers. *B*, the membrane was stripped of the probe and rehybridized with ^{32}P-labeled mouse β-actin cDNA as a probe. The experimental details are described under "Experimental Procedures."

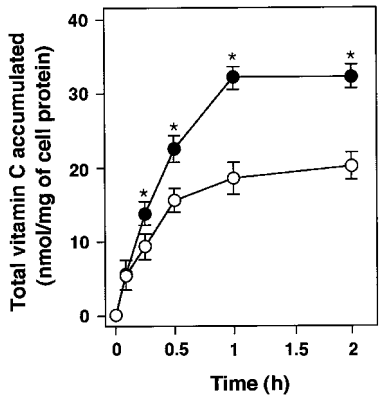

FIG. 5. **Time course of total vitamin C (AA plus DHA) accumulation in CHO cells expressing GSH-dependent DHA reductase and in the parental CHO cells.** pRc/DHAR-transfected (*closed circles*) and nontransfected (*open circles*) CHO cells were incubated with 150 μM DHA at 30 °C for indicated times, and the amounts of total vitamin C (AA plus DHA) in the cells were determined by HPLC, as detailed under "Experimental Procedures." Data are expressed as means ± S.D. of three independent experiments. *, significantly ($p < 0.05$) different from measurements at respective times in nontransfected cells by Student's *t* test.

an in-frame stop codon (TGA) is located upstream of it (at nucleotides 8–10 of the cDNA). The sequence (ACAGCATGAG) surrounding the ATG essentially conforms to a consensus sequence for the translation initiation site (20).

The molecular weight calculated from the deduced amino acid sequence (24,929) is considerably smaller than the value (31,000) obtained previously by SDS-polyacrylamide gel electrophoresis for purified DHA reductase (10). To determine the actual molecular weight of the enzyme, we carried out MALDI-TOF mass analysis and found a peak at m/z = 27,920 in positive ion mode (data not shown). The result indicated that the molecular weight of the enzyme estimated by SDS-polyacrylamide gel electrophoresis is anomalously larger than that obtained by MALDI-TOF mass spectrometry. It is noted that the molecular weight of the deduced amino acid sequence is still smaller (by ~3,000) than the actual one.

Because of the definitive discrepancy of the molecular weights, we carefully investigated the authenticity of the isolated clone. First, a possibility was excluded that the antibody reacting with the recombinant protein expressed by the cloned λgt11 was an autoantibody that was fortuitously present in the antiserum used. The antibody reacting with the recombinant protein was selected from the antiserum with the protein of plaques formed by the cloned λgt11, as described by Weinberger *et al*. (14), and used for immunoblotting of a crude extract from rat liver tissue. This analysis showed only a single band at the same position as that of rat liver DHA reductase (data not shown), indicating that the antibody reacted only with the reductase protein in the liver. Second, the authenticity of the cDNA clone was further confirmed by sequence analysis of purified DHA reductase. The sequences of two peptide fragments produced by partial digestion of the enzyme with *Achromobacter* lysyl endopeptidase were found to be identical to the deduced amino acids at positions 73–99 and 83–103, respectively (Fig. 1). A peptide sequence determined previously for this enzyme by Maellaro *et al*. (10) also agreed, albeit not completely, with the deduced amino acids at positions 13–32. Thus, it is clear that the obtained cDNA clone does encode GSH-dependent DHA reductase.

We hypothesized that the above-mentioned discrepancy of the molecular weight is attributable to post-translational modification of the enzyme. To test this hypothesis, we prepared bacterial and eukaryotic expression plasmids, pET/DHAR and pRc/DHAR, respectively, containing the cloned cDNA and used them to express recombinant protein in *E. coli* and CHO cells. Immunoblot analysis disclosed that the recombinant protein produced in CHO cells had the same molecular weight as that of purified DHA reductase (Fig. 2). On the other hand, the molecular weight of the recombinant protein produced in *E. coli* cells was smaller and comparable to the value predicted from the cDNA sequence (Fig. 2). These results clearly indicate that the increase in molecular weight observed for the protein produced in the mammalian system is caused by a certain post-translational modification that does not take place in the bacterial system. In the deduced amino acid sequence, no potential N-glycosylation site (Asn-Xaa-Ser/Thr) is present. Moreover, trifluoromethane sulfonic acid treatment, which removes both N- and O-linked carbohydrates, did not reduce the molecular weight of purified DHA reductase (data not shown), excluding the possibility of glycosylation as a means of the modification. The means of the modification remains to be elucidated. Computer-assisted comparison of the deduced amino acid sequence of rat DHA reductase with sequences in the database revealed that the enzyme had 81.2 and 76.5% amino acid sequence identity with hypothetical proteins from mouse and human lymphoma cells,[2] respectively (Fig. 3). Because the homologies are relatively low for the phylogenetically near species, whether they have GSH-dependent DHA reductase is an interesting point. On the other hand, there is no significant sequence homology with either glutaredoxin or protein disulfide isomerase.

Organ Distribution of GSH-dependent DHA Reductase mRNA—Various rat organs, including liver, kidney, testis, brain, spleen, and heart, were analyzed for DHA reductase mRNA by Northern blot analysis (Fig. 4). A single major band was observed at ~0.9 kilobase for all the organs examined, and

[2] The database information used is: GenBank accession no. U80819, hypothetical protein from mouse lymphoma cells; and GenBank accession no. U90313, hypothetical protein from human lymphoma cells.

this result agreed with the broad distribution of the DHA reductase among organs, as previously revealed by activity measurement and immunoblot analysis (12).

Functional Analysis of Recombinant DHA Reductase—Next, we examined whether the recombinant protein expressed with the cloned cDNA was catalytically active. Because CHO cells had no detectable level of GSH-dependent DHA reductase activity, we used this cell line for this test. An appreciable degree of the enzyme activity was observed in the cytosolic fraction from CHO cells that had been stably transfected with the expression vector pRc/DHAR. The activity was 1.5 nmol/min/mg of protein, a value similar to that in the cytosol from rat liver (10, 12).

Furthermore, to test a physiological function of the enzyme expressed, we measured the level of AA accumulated in cells that had been incubated with DHA, because this could be a cumulative parameter that would reflect DHA reductase expression. DHA has been recognized as a source of AA because it is imported into cells more readily than AA via glucose transporters (21–23), and it is reasonable to consider that the more DHA reductase is present in the cells, the faster the DHA imported into cells is reduced to AA. In fact, the rate of DHA accumulation by various kinds of cells was reported to increase in proportion to the activity of DHA reductase in their cytosol (24); and human neutrophils (7) and erythrocytes (11, 25), both of which contain high concentrations of AA, have recently been shown to possess DHA reductases such as glutaredoxin and GSH-dependent DHA reductase.

pRc/DHAR-transfected CHO cells expressing the recombinant DHA reductase and the nontransfected CHO cells both contained no appreciable amount of AA when cultured in the usual medium, which is deficient in vitamin C. When these cells were incubated with 150 μM DHA, they accumulated total vitamin C (AA plus DHA) to steady-state levels within 1 h. The cells expressing the recombinant enzyme accumulated 32.2 nmol of total vitamin C/mg of cell protein, whereas the nontransfected cells accumulated 18.6 nmol of total vitamin C/mg of cell protein; thus the DHA reductase-expressing cells accumulated 1.7 times the amount of total vitamin C compared with nontransfected cells (Fig. 5). More than 95% of the accumulated vitamin C was found to be in the reduced form. Because the nontransfected CHO cells contained no protein immunoreactive to anti-DHA reductase antibody (Fig. 2), it should be that the increase in the accumulation of AA is effected by the DHA reductase expressed by pRc/DHAR; thus the present study points to the importance of DHA reductase in maintaining a high concentration of AA in cells.

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