Purification, cloning and expression of dehydroascorbic acid-reducing activity from human neutrophils: identification as glutaredoxin

Jae B. PARK and Mark LEVINE*

Laboratory of Cell Biology and Genetics, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892, U.S.A.

Dehydroascorbic acid-reducing activity in normal human neutrophil lysates was characterized and identified by activity-based purification and measurement of newly synthesized ascorbate by HPLC. The initial reducing activity was non-dialysable and could not be accounted for by the activity of glutathione as a reducing agent. The reducing activity was purified to homogeneity as an 11 kDa protein. The protein had a specific activity of 3 μmol/min per mg of protein and was glutathione dependent. Kinetic experiments showed that the protein had a K_m for glutathione of 2.0 mM and a K_m for dehydroascorbic acid of 250 μM. Dehydroascorbic acid reduction by the purified protein was pH dependent and was maximal at pH 7.5. Peptide fragments from the purified protein were analysed for amino acid sequence and the protein was identified as glutaredoxin. By using degenerate oligonucleotides based on the amino acid sequence, glutaredoxin was cloned from a human neutrophil library. Expressed purified glutaredoxin displayed reducing activity and kinetics that were indistinguishable from those of native purified enzyme. Several approaches indicated that glutaredoxin was responsible for the most of the protein-mediated dehydroascorbic acid reduction in lysates. From protein purification data, glutaredoxin was responsible for at least 47% of the initial reducing activity. Dehydroascorbic acid reduction was at least 5-fold greater in neutrophil lysates than in myeloid tumour cell lysates, and glutaredoxin was detected in normal neutrophil lysates but not in myeloid tumour cell lysates by Western blotting. Glutaredoxin inhibitors inhibited dehydroascorbic acid reduction in neutrophil lysates as much as 80%. These findings indicate that glutaredoxin plays a major role in dehydroascorbic acid reduction in normal human neutrophil lysates, and represent the first identification of dehydroascorbic acid reductase in human tissue by activity-based purification.

INTRODUCTION

Ascorbic acid is accumulated in human neutrophils by two distinct mechanisms. The first involves sodium-dependent ascorbate transport against a concentration gradient [1–4]. The protein responsible for ascorbate transport remains to be isolated [5,6].

The second mechanism for ascorbate accumulation in neutrophils is ascorbate recycling [2,7]. Oxidants from activated neutrophils oxidize extracellular ascorbate to dehydroascorbic acid [2,7]. In contrast with ascorbate, dehydroascorbic acid is transported intracellularly by glucose transporters [6,8,9]. The rate of dehydroascorbic acid transport is at least 10 times that of ascorbate transport [2]. Once inside cells, dehydroascorbic acid is immediately reduced to ascorbate [2,7,10]. Ascorbate recycling results in rapid increases in intracellular ascorbate at the same time that protection is needed from oxidants generated by the neutrophil respiratory burst [2,6,7].

Ascorbate recycling is dependent on rapid intracellular reduction of dehydroascorbic acid. The mechanism of dehydroascorbic acid reduction in neutrophils was uncertain [2,11–14]. We recently reported that neutrophil lysates reduced dehydroascorbic acid and that reduction was dependent on glutathione and NADPH [2]. In the present study we characterized the reducing activity in neutrophil lysates as protein-dependent; the responsible protein was isolated, sequenced, identified as glutaredoxin, cloned and expressed.

EXPERIMENTAL

Materials

Ascorbic acid, EDTA, Hepes, Tris/HCl, dithiothreitol and Histopaque 1083 (Ficoll, Type 400 and Hypaque) were purchased from Sigma. Dextran T-500, Red-Sepharose and Superdex 75 HR were obtained from Pharmacia LKB Biotechnology Inc.

Assays

Dehydroascorbic acid-reducing activity

Reducing activity was determined in 25 μl of 100 mM Tris/HCl buffer, pH 7.5, containing (final concentrations) 0.8 mM reduced glutathione, 0.4 mM NADPH, 400 μM dehydroascorbic acid and appropriate amounts of enzyme. The reaction was initiated by adding dehydroascorbic acid, allowed to proceed for 3 min at room temperature, and terminated by the addition of 35 μl of 90% (v/v) methanol containing 1 mM EDTA. The final mixture was centrifuged for 10 min at 14000 ***g*** at 4 °C and the supernatant immediately analysed by HPLC. A chemical reaction was simultaneously measured under identical conditions but without enzyme. Where indicated, chemical activity was subtracted from total reducing activity to yield enzymic activity. The amount of reduced dehydroascorbic acid was determined as ascorbic acid by HPLC with coulometric electrochemical detection as described previously [15]. Dehydroascorbic acid was always freshly prepared immediately before experiments [7].

Protein determination

Protein was measured by the bicinchoninic acid protein assay (Pierce Chemical Co.) [16]. BSA was used as the protein standard.

Isolation of neutrophils

Neutrophil-enriched blood was collected by apheresis from healthy human donors with an automated cell-separator apheresis instrument (Fenwal CS-3000, Baxter Healthcare) [17]. Neutrophils were isolated as described [1,18].

* To whom correspondence should be addressed.

Purification of dehydroascorbic acid-reducing activity

Purification steps were performed at 4 °C unless otherwise indicated. Isolated cells [$(2–3) \times 10^9$] were suspended in 10 ml of 50 mM Hepes buffer, pH 7.5, containing 2 mM PMSF, 5 μg/ml aprotinin and 1 μg/ml leupeptin. Cells were lysed in a cavitator (bomb) at a pressure of approx. 3.45 MPa for 20 min. Cavitated cells were centrifuged at 50000 ***g*** for 30 min. The supernatant was treated with 30–60 % (w/v) ammonium sulphate. The precipitate was collected by centrifugation at 5000 ***g*** for 30 min at 4 °C, resuspended in 10 ml of 50 mM Tris/HCl, pH 7.5, then dialysed overnight in 1 litre of 50 mM Tris/HCl, pH 7.5. For initial characterization of reducing activity, samples were dialysed after cavitation and centrifugation at 50000 ***g*** without ammonium sulphate precipitation.

The dialysate was applied to a Red-Sepharose affinity column (1 cm × 10 cm) that had been equilibrated with 50 mM Tris/HCl, pH 7.5. The column was washed with 100 ml of equilibration buffer, and proteins were eluted with a linear NaCl gradient 0–2 M in 0.5 litre of equilibration buffer, flow rate 2 ml/min. Dehydroascorbic acid-reducing activity was eluted with 0.8–1.0 M NaCl. Fractions with activity were pooled and concentrated to 0.7 ml in a centrifugal concentrator (Amicon).

The concentrated sample was analysed by gel filtration at a flow rate of 0.5 ml/min on a Superdex 75 HR column (1.0 cm × 50 cm) equilibrated with 50 mM Tris/HCl, pH 7.5. Dehydroascorbic acid-reducing activity was eluted as a low-molecular-mass species that required glutathione but no longer required NADPH for activity. The eluted protein was concentrated by centrifugal ultrafiltration (Amicon) and centrifuged at 10000 ***g*** for 10 min. The supernatant was injected into an HPLC system connected with a C_{18} column (4.6 mm × 250 mm, Vydac) that had been equilibrated with 10 mM sodium phosphate, pH 7.0. The proteins were eluted with a linear gradient of 0–50 % acetonitrile in the same buffer for 50 min at a flow rate of 1 ml/min. Fractions of 1 ml were collected and acetonitrile was removed by evaporation. A single peak with a retention time of 41 min exhibited dehydroascorbic acid-reducing activity. SDS/PAGE was used to confirm homogeneity of the HPLC peak.

Silver staining

Silver staining was performed with commercially available materials (Bio-Rad).

Sequencing of isolated protein

Approx. 20 μg of the homogeneous protein was dissolved in 100 μl of denaturing buffer, pH 8.3, containing 6 M guanidine/HCl, 100 mM Tris/acetate and 1 mM EDTA. The sample mixture was incubated at 37 °C for 60 min. Dithiothreitol solution (10 μl; 200 mM) was added and the sample purged with nitrogen gas. Iodoacetate solution (15 μl; 15 mM) was added and the sample purged again with nitrogen gas. This *S*-carboxymethylated protein was dissolved in 100 μl of 100 mM NH_4HCO_3, pH 8.5, and the dissolved sample was digested with trypsin (50:1, substrate/trypsin) for 2 h. The trypsin-digested sample was fractionated by HPLC (Waters 626) connected to a C_{18} reverse-phase column (Vydac). The resulting peptide fragments were analysed by laser desorption mass spectroscopy. Suitable peptide fragments were sequenced (Applied Biosystems Sequencer) [19].

Immunoblotting

SDS/PAGE analyses were performed with the Laemmli system [20]. Immunoblotting (Western blotting) was performed as described [21] using blotting modules (NOVEX). Antibody to purified dehydroascorbic acid-reducing activity (glutaredoxin) was raised in rabbits. Rabbit antiserum was used as the primary antibody, and peroxidase-conjugated goat anti-rabbit IgG serum was used to reveal the protein band [22].

DNA cloning and sequencing

Poly(A)$^+$ RNA was isolated from 2×10^8 human neutrophils with an oligo(dT) column as described [23]. Isolated poly(A)$^+$ RNA was used to construct a cDNA neutrophil library in Uni-Zap vector (Stratagene).

Human neutrophil glutaredoxin was amplified by PCR (Perkin-Elmer). Forward and reverse primer sets were synthesized (DNA Synthesizer model 380 B, Applied Biosystems). The primers were designed for accurate expression and manipulation of cloned protein. Each set contained nucleotide sequences complementary to possible codon combinations for the selected amino acid sequence. Forward primers corresponded to amino acids 1–7 of human thioltransferase [24] and were composed of sequences 5′-CATATGGC(ACGT)CA(AG)GA(AG)TT(TC)G-T(ACGT)AA(CT)-3′. Three nucleotides, CAT, were present at the 5′ end to generate an *Nde*I site. Reverse primers corresponded to amino acids 100–106 of glutaredoxin plus the termination codon TAA and were composed of the sequences 5′-TTA-(CT)TG(TGC)AG(AGC)GC(TGC)CC(AGT)AT(TC)TG-(TC)TT-3′. PCR was performed with these primer sets and the human neutrophil library as template.

Amplified DNA was subjected to electrophoresis on a 1 % (w/v) agarose gel. DNA fragments of approx. 300 bp were isolated from the gel and cloned into TA-vector (Invitrogen). The nucleotide sequences of cloned DNA fragments were determined by double-strand sequencing (Sequenase, United States Biochemical).

Expression and purification of recombinant human glutaredoxin

The TA-plasmid containing the human glutaredoxin gene was double digested with *Nde*I and *Eco*RI and the digested DNA fragment was subjected to electrophoresis on a 1 % agarose gel. The gene isolated from the gel was ligated to the expression vector pET (Novagene), which had been previously digested with *Nde*I and *Eco*RI. Expression vector with ligated glutaredoxin was transformed into host bacteria *Escherichia coli* BL21 (LyS). Bacteria containing the expression plasmid (human glutaredoxin–pET) were cultured overnight at 37 °C in Luria–Bertani medium supplemented with ampicillin (100 μg/ml), transferred to fresh medium, and further cultured with vigorous shaking. When culture D_{600} was approx. 0.7, isopropyl-β-D-thiogalactoside was added to a final concentration of 0.5 mM. After 4 h of induction, bacteria were collected by centrifugation and the pellet was frozen at −70 °C.

Purification of the expressed protein was performed at 4 °C unless otherwise indicated. Approx. 2 g of frozen bacteria were resuspended in 20 ml of 50 mM Tris/HCl, pH 7.8, containing 1 mM PMSF, 5 μg/ml aprotinin and 1 μg/ml leupeptin. Bacteria were disrupted by sonication (Kontes) and cell debris was sedimented by centrifugation at 23000 ***g*** (4 °C) for 20 min. The resulting supernatant was incubated for 10 min in a water bath at 75 °C. Denatured proteins were removed by centrifugation at 23000 ***g*** (4 °C) for 20 min, and the supernatant was dialysed overnight in 10 mM phosphate buffer, pH 6.5. The dialysate was applied to carboxymethyl-Sepharose (1.0 cm × 10 cm) equilibrated with 10 mM phosphate buffer, pH 6.5. The column was washed with 50 ml of 10 mM phosphate buffer, pH 7.5. Bound proteins were eluted with a linear NaCl gradient of

0–0.5 M in 200 ml of 10 mM phosphate buffer, pH 7.5, at a flow rate of 2 ml/min. Fractions with dehydroascorbic acid-reducing activity were pooled and concentrated with a centrifugal concentrator (Centricon). The concentrated sample was applied to a Superdex 75 HR column (Pharmacia) equilibrated with 50 mM Tris/HCl, pH 7.5, at a flow rate of 0.7 ml/min. Eluted protein containing reducing activity was homogeneous by SDS/PAGE and was stored at −70 °C until used.

RESULTS

Characterization of dehydroascorbic acid-reducing activity in neutrophil lysates

Normal human neutrophils were disrupted by nitrogen cavitation and tested for dehydroascorbic acid-reducing activity by measuring ascorbate formation from added dehydroascorbic acid (Table 1). Undialysed lysates had background ascorbate from endogenous ascorbate in neutrophils, as expected [1]. Lysates were dialysed to decrease background (Table 1). The amount of reducing activity was similar in dialysed and undialysed samples. Both dialysed and undialysed lysates had maximal dehydroascorbic acid reduction with glutathione and NADPH, and chemical reduction by these agents alone could not account for the results. Protein-mediated reduction was 10–20-fold more than glutathione-mediated reduction at glutathione concentrations as high as 8 mM (results not shown). Neither NADPH nor NADH alone had any effect on reduction, and reduction with NADH plus glutathione was identical with reduction with glutathione alone (results not shown). Reducing activity was similar in lysates compared with the equivalent number of intact cells, and more than 90% of activity was cytosolic and not membrane-bound [2,7] (Table 1) (results not shown). Reducing activity in crude neutrophil lysates was linearly dependent on time and lysate amount (Figure 1). The results imply that the reducing activity is protein-mediated because activity was non-dialysable and could not be accounted for by glutathione and/or NADPH.

Isolation and characterization of dehydroascorbic acid-reducing activity

Reducing activity was purified from neutrophils that had been cavitated and dialysed. The activity was isolated to homogeneity by ammonium sulphate precipitation, application to a Red-Sepharose affinity column, gel filtration and HPLC (Table 2). Activity that eluted at the gel filtration step and thereafter no longer was augmented by NADPH. The glutathione requirement persisted, and the activity was heat stable (results not shown). On analysis by HPLC, reducing activity corresponded to only one HPLC peak. The peak contained only one protein that was homogeneous by SDS/PAGE, with detection by Coomassie Blue and silver staining. The molecular mass was approx. 11 kDa (Figures 2A and 2B). The specific activity of the purified protein was 3 μmol/min per mg of protein (Table 2).

Polyclonal antibody was prepared by injecting purified dehydroascorbic acid-reducing activity into rabbits. Protein

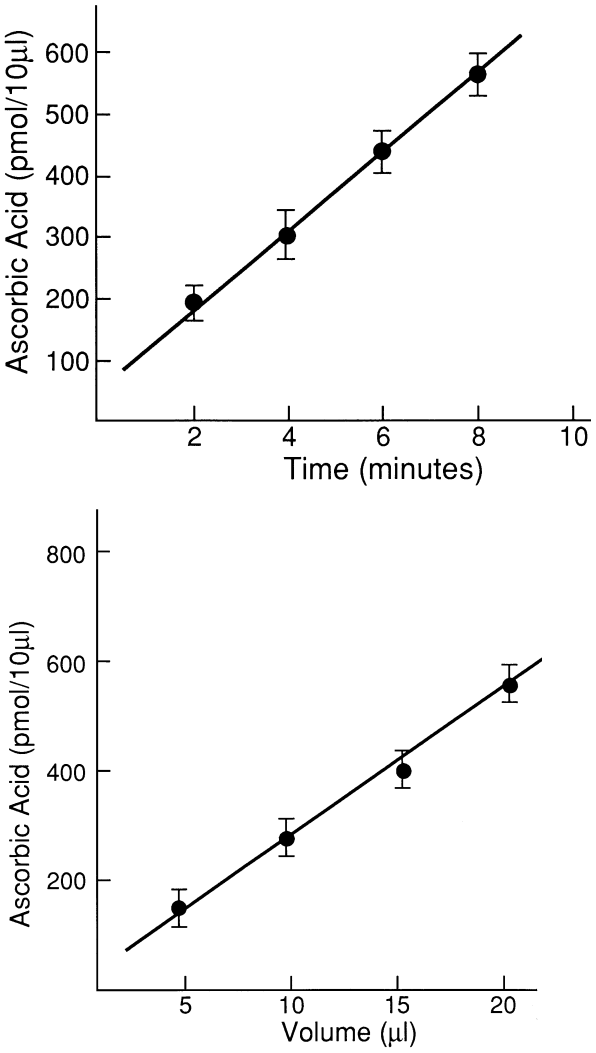


Figure 1 Characteristics of dehydroascorbic acid-reducing activity in neutrophil lysates

Effect of incubation time (top panel) and lysate amount (bottom panel) on dehydroascorbic acid-reducing activity. Cell concentration in neutrophil sample lysates was 2×10^8 cells per ml. Reducing activity was measured as described in Table 1 and represented pmol of ascorbate per 10 μl of final volume. Reaction concentrations were: NADPH, 0.4 mM; glutathione, 0.8 mM; dehydroascorbic acid, 0.4 mM. Lysates were not dialysed. Chemical activity was subtracted from total reducing activity to yield protein-mediated activity (see Table 1 and the Experimental section). Results represent means ± S.D. for at least three samples; S.D. is not shown when smaller than point size.

Table 1 Dehydroascorbic acid-reducing activity of neutrophils: activity in lysates and dialysates

Dehydroascorbic acid-reducing activity was measured under different conditions with the following additions as indicated: neutrophil fractions (F), 10 μl (2×10^8 homogenized cells per ml); reduced glutathione (G), 2 μl of 10 mM solution, reaction concentration 0.8 mM; NADPH (N), 1 μl of 10 mM solution, reaction concentration 0.4 mM; dehydroascorbic acid (D), 2 μl of 5 mM solution, reaction concentration 0.4 mM; and Tris/HCl buffer (B), pH 7.5, to bring the reaction volume to 25 μl. After 3 min, 35 μl of 90% (v/v) methanol/1 mM EDTA was added to stop the reaction, final volume 60 μl. Values represent pmol of ascorbic acid in 10 μl final volume as determined by HPLC with coulometric electrochemical detection, and are means ± S.D. for three samples. Lysate preparation, dialysis conditions and additional assay details are described in the Experimental section.

	Lysate (pmol per 10 μl)	Dialysate (pmol per 10 μl)
F+D+B	128±8	13±3
F+N+D+B	120±9	12±1
F+G+D+B	250±12	100±12
F+G+N+D+B	300±15	200±18
G+N+D+B	10±1	10±1
F+B	80±6	10±2

Table 2 Purification of dehydroascorbic acid-reducing activity from neutrophils

The concentration of neutrophils in lysates was (2–3) $\times 10^8$/ml. Total units of activity represent μmol of ascorbate formed per min. See the Experimental section for details. Adjusted activity reflects contribution of the NADPH-dependent fraction to reducing activity at the gel filtration step and protein loss at the C_{18} HPLC step (see text for details). Glutathione reaction concentration was 0.8 mM, NADPH reaction concentration was 0.4 mM and dehydroascorbic acid reaction concentration was 0.4 mM. Chemical activity was subtracted from total reducing activity to yield protein-mediated activity (see Table 1 and the Experimental section).

	Protein (mg)	Total units of activity (nmol/min)	Specific activity (nmol/min per mg)	Adjusted activity (nmol/min)
Lysate	450	4000	9	4000
Ammonium sulphate	140	3500	25	3500
Red-Sepharose	30	2700	90	2700
Gel filtration	1	600	600	2400
C_{18}	0.02	60	3000	1920

samples from different steps of the purification procedure were analysed by Coomassie staining and by Western blotting (Figures 2A and 2C). These results confirm that the reducing activity is present at all phases of the purification from normal neutrophils as predicted.

Biochemical properties of the purified reducing activity were characterized. For glutathione the apparent K_m was 2.0 mM and V_{max} was 5 nmol/min per μg of protein (Figure 3, top panel). For dehydroascorbic acid the apparent K_m was 250 μM and V_{max} was 6 nmol/min per μg of protein (Figure 3, middle panel). The protein showed maximal activity at pH 7.5 and above (Figure 3, bottom panel). The purified protein had no requirement for NADPH (results not shown).

Identification of dehydroascorbic acid-reducing activity

To identify the activity, isolated protein was subjected to trypsin digestion. Two isolated peptide fragments were sequenced by Edman degradation [19]. A search of the Swiss Protein Database revealed that the sequence of the first peptide was completely identical with human glutaredoxin residues 14–26, and the sequence of the second peptide was 94% similar to human glutaredoxin residues 77–93 (Figure 4) [24]. All other peptide fragments prepared for sequencing could be matched to human glutaredoxin sequences based on the molecular mass of the fragments analysed by laser desorption mass spectroscopy compared with predicted glutaredoxin sequences (results not shown). These results indicate that glutaredoxin is responsible for at least part of the dehydroascorbic acid-reducing activity in human neutrophils.

Cloning and expression of human glutaredoxin

Cloning of glutaredoxin from neutrophils and characterization of recombinant protein activity could provide further evidence for glutaredoxin's role in dehydroascorbic acid reduction. By using a neutrophil library, degenerate oligonucleotide primers based on the amino acid sequence, and PCR, glutaredoxin was cloned and sequenced (Figure 5). Degenerate oligonucleotides were used because human cDNA was not available at the time that this work was begun. The nucleotide sequence for glutaredoxin reported here is not completely identical with a recently reported human sequence [25]. There were differences at codons 225, 285 and 286, and a change from Val-96 to Leu in the previously predicted amino acid sequence [25].

Recombinant human neutrophil glutaredoxin was expressed in *E. coli* BL21 containing pET–glutaredoxin plasmid. Expressed recombinant glutaredoxin was purified and detected by Coomassie staining and by Western blotting (Figures 6A and 6B). The specific activity of purified glutaredoxin for dehydroascorbic acid reduction was 3 μmol/min per mg of protein, similar to that of native glutaredoxin (Table 2). The kinetic properties of recombinant glutaredoxin were virtually indistinguishable from those of the native enzyme (Table 3). Maximal recombinant enzyme activity was observed at pH 7.5 (results not shown), similar to findings with native enzyme (Figure 3, bottom panel). These data indicate that expressed and native glutaredoxin are essentially identical with respect to dehydroascorbic acid reduction and immunological detection.

Role of glutaredoxin in dehydroascorbic acid reduction in neutrophil lysates

It was possible that other proteins that used NADPH were involved in dehydroascorbic acid reduction. This is because the initial reducing activity in neutrophil lysates was dependent on

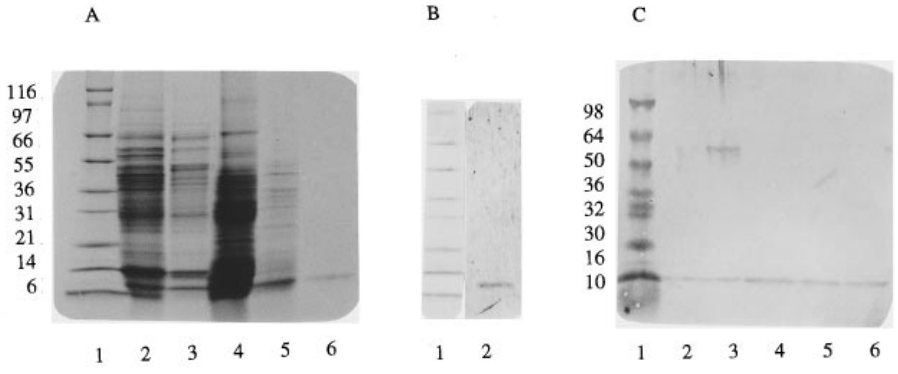
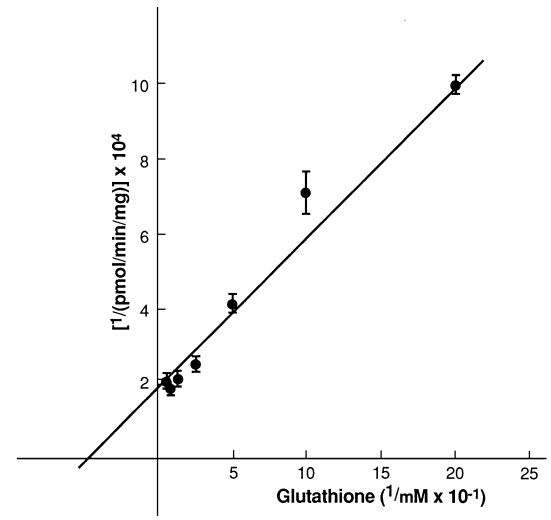
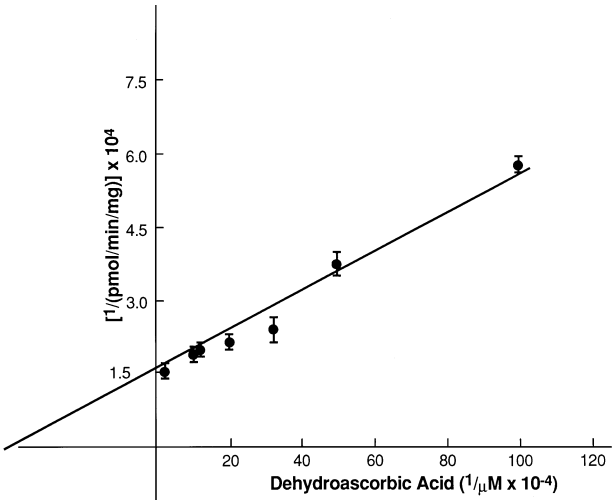


Figure 2 Gel electrophoresis and Western blotting of purified dehydroascorbic acid-reducing activity

(**A**) SDS/PAGE of isolated activity and Coomassie Blue staining. Lane 1, marker proteins (kDa); lane 2, lysate, 40 μg of protein; lane 3, 30–60% ammonium sulphate, 40 μg of protein; lane 4, Red-Sepharose, 40 μg of protein; lane 5, gel filtration, 20 μg of protein; lane 6, 0.7 μg of purified protein. (**B**) SDS/PAGE (4–20% gel) of isolated activity and silver staining. Lane 1, marker proteins; lane 2, purified protein. (**C**) Immunoblotting analysis with anti-dehydroascorbic acid-reducing activity serum. Lanes and conditions are as described in (**A**).





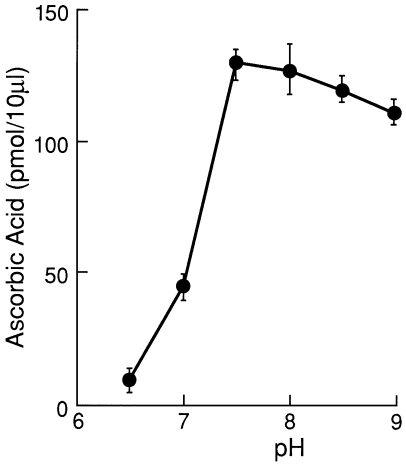


Figure 3 Characteristics of purified dehydroascorbic acid-reducing activity

Top and middle panels: Lineweaver–Burk displays of isolated purified dehydroascorbic acid-reducing activity for reduced glutathione (top panel) and dehydroascorbic acid (middle panel). Assay conditions were as described in Table 1 and in the Experimental section. Activity was determined as pmol of ascorbate per min per μg of protein. Results represent means $\pm$ S.D. for at least three samples; S.D. is not shown when smaller than point size. Chemical activity was subtracted from total reducing activity to yield protein-mediated activity (see Table 1 and the Experimental section). Dehydroascorbic acid concentration was 400 μM in the top panel and glutathione concentration was 4 mM in the middle panel. Bottom panel: effect of pH on isolated dehydroascorbic acid-reducing activity. Activity represents pmol of ascorbate per 10 μl of final volume as described in Table 1. The following buffers (100 mM) were used: Mes (pH 6.5–7.5); Tris/HCl (pH 7.5–8.5); phosphate (pH 8.5–9.5). Chemical activity was subtracted from total reducing activity to yield protein-mediated activity (see Table 1 and the Experimental section). Dehydroascorbic acid concentration was 0.4 mM and glutathione concentration was 0.8 mM.

```
1                                                    30
                          V V V F I K P T C P Y C R
Ac-A Q E F V N C K I Q P G K V V V F I K P T C P Y C R R A Q E · · · · · ·

71                                                                  105
            D C I G G C S D L V X L Q Q S G E
R V F I G K D C I G G C S D L V S L Q Q S G E L L T R L K Q I G A L Q
```

Figure 4 Sequence comparison between peptides of trypsin-digested dehydroascorbic acid-reducing activity and human thioltransferase (glutaredoxin)

The two underlined fragments were compared. The upper sequences were determined from amino acid sequencing of pure dehydroascorbic acid-reducing activity, and the lower sequences are those of human thioltransferase (glutaredoxin) [24].

```
ATGGCTCAAGAGTTTGTGAACTGCAAAATCCAGCCTGGGAAGGTGGTTGTGTTCATCAAG (60)
METAlaGlnGluPheValAsnCysLysIleGlnProGlyLysValValValPheIleLys (20)

CCCACCTGCCCGTACTGCAGGAGGGCCCAAGAGATCCTCAGTCAATTGCCCATCAAACAA (120)
ProThrCysProTyrCysArgArgAlaGlnGluIleLeuSerGlnLeuProIleLysGln (40)

GGGCTTCTGGAATTTGTCGATATCACAGCCACCAACCACACTAACGAGATTCAAGATTAT (180)
GlyLeuLeuGluPheValAspIleThrAlaThrAsnHisThrAsnGluIleGlnAspTyr (60)

TTGCAACAGCTCACGGGAGCAAGAACGGTGCCTCGAGTCTTTATTGGTAAAGATTGTATA (240)
LeuGlnGlnLeuThrGlyAlaArgThrValProArgValPheIleGlyLysAspCysIle (80)

GGCGGATGCAGTGATCTAGTCTCTTTGCAACAGAGTGGGGAACTGCTGACGCGGCTAAAG (300)
GlyGlyCysSerAspLeuValSerLeuGlnGlnSerGlyGluLeuLeuThrArgLeuLys (100)

CAGATTGGAGCTCTGCAGTAA (321)
GlnIleGlyAlaLeuGln--- (106)
```

Figure 5 Nucleotide sequence and deduced amino acid sequence of human neutrophil glutaredoxin

The nucleotides are numbered 5′ to 3′ (upper numbers in parentheses at the right) and amino acids are numbered from N-terminus to C-terminus (lower numbers in parentheses at the right).

glutathione and NADPH for maximal activity (Table 1), and the NADPH requirement was lost after gel filtration (see Table 2). There are two general possibilities to explain the NADPH effect on dehydroascorbic acid reduction: NADPH could be required either for a completely separate reduction pathway or as part of the same pathway.

We investigated the relationship between glutaredoxin and the requirement for NADPH. NADPH had no effect on purified glutaredoxin in the presence or absence of glutathione (results not shown). Because the NADPH effect was present in crude lysates and was lost after gel filtration, purified glutaredoxin was tested for reducing activity with other fractions from gel filtration; one fraction restored the NADPH requirement. On addition

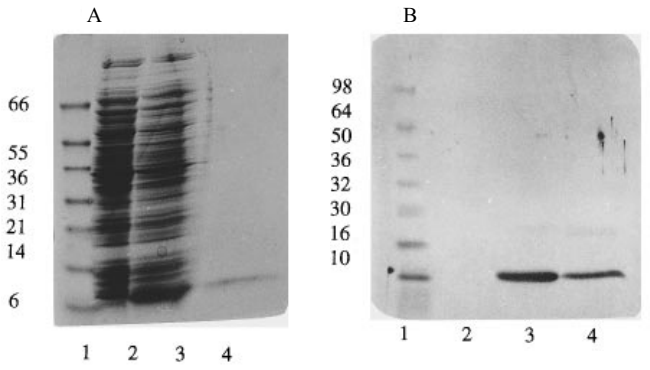


Figure 6 SDS/PAGE and immunoblotting analyses of recombinant glutaredoxin

(**A**) SDS/PAGE (4–20% gels) and Coomassie Blue staining of recombinant glutaredoxin. Lane 1, marker proteins (kDa); Lane 2, control BL21(LyS)/pET after 4 h induction, 50 μg of protein; lane 3, BL21(LyS)/pET–human glutaredoxin after 4 h induction, 50 μg of protein; lane 4, purified recombinant human neutrophil glutaredoxin, 1 μg of protein. (**B**) Immunoblotting analysis of recombinant glutaredoxin with anti-dehydroascorbic acid-reducing activity serum. Lanes and conditions are the same as in (**A**).

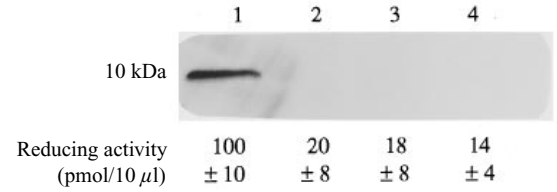


Figure 7 Dehydroascorbic acid-reducing activity in neutrophils and myeloid cell lines

(Upper part) Glutaredoxin detection by immunoblotting with anti-dehydroascorbic acid-reducing activity serum in normal neutrophils (lane 1), U937 cells (lane 2), HL60 cells (lane 3), and PLB cells (lane 4). (Lower part) Dehydroascorbic acid-reducing activity (pmol per 3 min per 10 μl) in the cells from the upper part. Dehydroascorbic acid reduction was determined in dialysed cell lysates in the presence of 0.8 mM glutathione and 0.4 mM dehydroascorbic acid. The protein concentration of each lysate was 10 mg/ml. Reducing activity was measured as described in Table 1. Chemical activity was subtracted from total reducing activity to yield protein-mediated activity (see Table 1 and the Experimental section). Values are means ± S.D. for at least three samples.

of this fraction, NADPH, purified glutaredoxin and glutathione, reduction of dehydroascorbic acid was 3–5-fold greater than with glutaredoxin and glutathione. These findings recapitulate those with neutrophil lysates (Table 1). When glutaredoxin was omitted, only background chemical reduction occurred; the NADPH-requiring fraction did not reduce dehydroascorbic acid unless glutaredoxin was also present. With the use of glutaredoxin to assay NADPH-mediated reducing activity, partial purification revealed that the active fraction contained 6-phosphogluconate dehydrogenase and glutathione reductase activities (results not shown; J. B. Park and M. Levine, unpublished work). All of these results indicate that proteins using NADPH augment glutaredoxin activity but have no independent reducing activity of their own: glutaredoxin must be present for reducing activity.

It was investigated whether glutaredoxin is a major mediator of dehydroascorbic acid reduction. First, the contribution of glutaredoxin to dehydroascorbic acid reduction was estimated from activity-based protein purification (Table 2). Initial reducing activity was diminished by one-third at the end of the Red-Sepharose step. After gel filtration, activity decreased approx. 4-fold (Table 2, column 2). At this step NADPH-requiring activity was lost, but one fraction contained the NADPH-requiring activity as described above. When this fraction was combined with the active reducing fraction, reducing activity was 2400 nmol/min (Table 2, column 4). At the C_{18} HPLC step, approx. 90% of the reducing activity was lost (Table 2, column 2). Nearly all of this loss could be accounted for by irreversible binding of glutaredoxin to the column or by glutaredoxin oxidation (results not shown). Adjusted activity for column losses and the contribution of the NADPH fraction shows that at least 47% of the reducing activity is glutaredoxin dependent (Table 2, column 4). These results were obtained during protein purification, with a goal of protein purity at the expense of lost activity, and therefore underestimate the contribution of glutaredoxin to total dehydroascorbic acid-reducing activity.

Normal human neutrophils reduce dehydroascorbic acid 5–10-fold faster than human myeloid tumour cells [2,7]. One possible explanation for this finding is that myeloid tumour cell lines lack glutaredoxin. Therefore another means of investigating the role of glutaredoxin in dehydroascorbic acid reduction was to measure dehydroascorbic acid-reducing activity in normal neutrophil and myeloid tumour line lysates, and to determine by Western blotting whether glutaredoxin was present in the same lysates. Dehydroascorbic acid-reducing activity was highest in neutrophil lysates and decreased approx. 80% in myeloid tumour line lysates (Figure 7, lower part). Western blotting revealed that glutaredoxin was present in neutrophil lysates but was not detected in the tumour line lysates (Figure 7, upper part). These results suggest that glutaredoxin plays a major role in dehydroascorbic acid reduction in neutrophil lysates.

Glutaredoxin inhibitors [26,27] were used as the third means of investigating the contribution of glutaredoxin to dehydroascorbic acid-reducing activity in neutrophil lysates. Neutrophil lysates were preincubated with inhibitors, with subsequent addition of glutathione plus NADPH or glutathione alone (Table 4). All inhibitors inhibited at least 46% of the dehydroascorbic acid-reducing activity under both conditions. Iodoacetamide was the most effective inhibitor. These results could not be explained by

Table 3 Kinetic analyses of dehydroascorbic acid reduction by native and recombinant human neutrophil glutaredoxins

Glutathione and dehydroascorbic acid concentrations were as described in Figure 3 (top and middle panels). The experiments were repeated three times with similar results.

	Glutathione		Dehydroascorbic acid	
	K_m (mM)	V_{max} (μmol/min per mg)	K_m (μM)	V_{max} (μmol/min per mg)
Native glutaredoxin	2.0	5.0	250	6.0
Recombinant glutaredoxin	2.0	5.5	200	6.0

Table 4 Inhibitors of dehydroascorbic acid reduction in neutrophil lysates

Dehydroascorbic acid reduction was determined in dialysed cell lysates. Inhibitors (1 mM) were preincubated with lysates for 30 min before the addition of 0.8 mM glutathione, 0.4 mM dehydroascorbic acid and 0.4 mM NADPH as indicated. Reducing activity (pmol of ascorbate per 3 min per 10 μl) was measured as described in Table 1. Chemical activity was subtracted from total reducing activity to yield protein-mediated activity (see Table 1 and the Experimental section). Values are means ± S.D. for at least three samples, with the percentage inhibition given in parentheses.

	Inhibition (%)	
	Glutathione + NADPH	Glutathione
Control	1730 ± 98 (0)	800 ± 79 (0)
Iodoacetamide	346 ± 40 (80)	110 ± 15 (86)
Mefenamic acid	865 ± 50 (50)	300 ± 20 (65)
Piroxicam	950 ± 45 (46)	250 ± 15 (70)

the effects of iodoacetamide on glutathione because iodoacetamide did not change glutathione concentrations (results not shown). In the presence of NADPH the reducing activity was higher, consistent with earlier findings (Table 1). Nevertheless, the percentage inhibition of reducing activity was similar in the presence or absence of NADPH. These findings provide additional evidence that glutaredoxin is a major mediator of dehydroascorbic acid reduction in neutrophil lysates and that glutaredoxin is required for NADPH to increase dehydroascorbic acid reduction.

DISCUSSION

The results in this paper describe the first activity-based purification and identification of dehydroascorbic acid-reducing activity as glutaredoxin in human neutrophil lysates and provide new evidence for the function of glutaredoxin in humans [28,29]. The data indicated that reduction was protein-mediated, because reduction by dialysed and non-dialysed neutrophil lysates was similar. Protein-mediated reduction was 10–20-fold greater than chemical reduction by glutathione. Using activity-based purification and amino acid sequencing we identified that at least part of the protein-mediated reducing activity was glutaredoxin. This conclusion was strengthened by cloning glutaredoxin from a human neutrophil cDNA library and expressing the cloned protein. The properties of the cloned protein for dehydroascorbic acid reduction were nearly identical with those of native glutaredoxin. Isolation of glutaredoxin based on its activity, and cloning and expression of glutaredoxin from the same cell type used for protein isolation, provide evidence that glutaredoxin plays at least a partial role in dehydroascorbic acid reduction in human neutrophil lysates.

Additional experiments indicated that glutaredoxin was a major participant in the process of dehydroascorbic acid reduction. Results from protein purification suggest that glutaredoxin was responsible for approx. 50% of initial dehydroascorbic acid reduction, and these results underestimate the contribution of glutaredoxin. Results from neutrophil and myelocyte tumour cell lysates showed that protein-mediated reduction was more than 5-fold higher in neutrophil lysates. Corresponding Western blots indicated that glutaredoxin was detectable in neutrophils but not in tumour cells. Dehydroascorbic acid reduction in neutrophil lysates was inhibited by at least 46% by glutaredoxin inhibitors [25,27], and inhibition occurred in the presence or absence of NADPH. The contribution of glutaredoxin to overall reduction is even greater than these results indicate because they were not adjusted to account for protein dilution during lysate preparation.

The effects of NADPH provide further evidence that glutaredoxin is responsible for most of the dehydroascorbic acid reduction in neutrophil lysates. NADPH was required with glutathione for maximal reducing activity in neutrophil lysates. During activity-based purification, the NADPH-requiring activity was lost after gel filtration and was recovered in a fraction from gel filtration. For the fraction to show activity, glutaredoxin was absolutely necessary. Without glutaredoxin the NADPH-requiring fraction had no effect on dehydroascorbic acid reduction. Inhibition of dehydroascorbic acid reduction by inhibitors was similar in the presence or absence of NADPH, again suggesting that glutaredoxin is necessary for NADPH to increase reduction.

These results suggest that glutaredoxin mediates most dehydroascorbic acid reduction. We also found that 6-phosphogluconate dehydrogenase activity and glutathione reductase activity were present in the NADPH-requiring fraction from gel filtration. A model is shown in Figure 8 that accounts for all of the experimental observations and places glutaredoxin in a framework of dehydroasorbic acid reduction. Glutaredoxin is the final protein in the reduction pathway. NADPH formed by 6-phosphogluconate dehydrogenase activity could be used to reduce glutathione disulphide. Glutathione could then be available for glutaredoxin. NADPH could increase glutaredoxin activity by increasing glutathione, but reduction does not occur without glutaredoxin. Experiments are in progress to identify conclusively the protein or proteins responsible for increasing

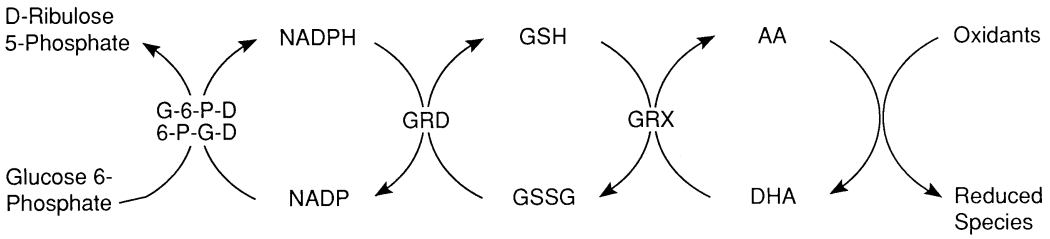


Figure 8 Dehydroascorbic acid reduction in human neutrophils: a model

NADPH is generated from glucose by glucose 6-phosphate dehydrogenase (G-6-P-D) and 6-phosphogluconate dehydrogenase (6-P-G-D). NADPH is used by glutathione reductase (GRD) to regenerate glutathione (GSH) from glutathione disulphide (GSSG). Glutathione is essential for glutaredoxin (GRX) reduction of dehydroascorbic acid (DHA) to ascorbate (AA). NADPH can increase glutaredoxin activity via glutathione reductase, but NADPH is not essential for dehydroascorbic acid reduction. However, if glutaredoxin is absent, dehydroascorbic acid reduction will not occur. See text for additional details.

glutaredoxin activity, and the mechanism of interaction with glutaredoxin.

The model predicts that the NADPH-requiring fraction in neutrophil lysates serves to maintain glutathione and to prevent formation of the oxidized species glutathione disulphide. As expected, NADPH was no longer required for dehydroascorbic acid reduction in neutrophil lysates at glutathione concentrations above 5 mM (results not shown).

The results in this paper indicate the importance of activity-based purification and specific assays for ascorbate and dehydroascorbic acid. Isolated glutaredoxin or protein disulphide isomerase will reduce dehydroascorbic acid with glutathione [13]. However, there was no evidence that protein disulphide isomerase was involved in reduction in neutrophil lysates. Protein-dependent glutathione-mediated reducing activity never segregated into fractions with different molecular masses, and the protein requirement for glutathione was isolated distinctly as one protein. At several steps, protein reducing activity was not consistent with the molecular mass of protein disulphide isomerase. Other investigators isolated a new 31 kDa reducing protein distinct from glutaredoxin and protein disulphide isomerase [30]. However, the assay used to measure reducing activity was not specific. Without clear results for ascorbate formation, the role of this protein in dehydroascorbic acid reduction is uncertain. The experiments here avoid these problems by using activity-based purification, specific assays for ascorbate and dehydroascorbic acid, and freshly prepared pure substrate. Activity-based purification provides new information that glutaredoxin plays a major role in dehydroascorbic acid reduction in neutrophil lysates. Nevertheless it remains possible that protein disulphide isomerase, the 31 kDa reducing protein, or other proteins reduce dehydroascorbic acid in other tissues.

Dehydroascorbic acid reduction in neutrophil lysates was primarily protein-mediated and not mediated by glutathione. It was suggested that unpurified enzymic reducing activities from other tissues were not as effective as glutathione-mediated reduction [14,31–33]. However, the specific activity of purified neutrophil glutaredoxin (Table 2) was as much as 1000 times greater than the other unpurified enzymic reducing activities [14,31–33]. Although dehydroascorbic acid-reducing activity was accounted for by glutathione alone in several mammalian systems [14,34–36], these findings can be questioned. The tissues chosen for study might not have been ideally enriched in putative enzymic activity. Homogenization of tissue leads to many-fold dilution of intracellular contents. An enzymic process that could occur in an intracellular volume could be masked by dilution of more than 10-fold, intra-assay pH changes and a concurrent non-enzymic chemical contribution, especially with glutathione concentrations not less than 5 mM [11,14,37]. Nevertheless the contribution of glutathione-mediated compared with protein-mediated reduction could vary in different tissues, and chemical reduction by glutathione could play a major role in some tissues. It is also possible that intracellular dehydroascorbic acid could be reduced chemically by other reducing agents, but their identities are obscure.

The distribution of glutaredoxin in many tissues in comparison with dehydroascorbic acid reduction is unknown, and should be investigated. It will be worth while to study further the contribution of glutaredoxin to reduction reactions in normal and tumour cells, and to characterize the regulation of glutaredoxin under different conditions.

Received 31 July 1995/17 November 1995; accepted 8 January 1996

We thank the Department of Transfusion Medicine, NIH Clinical Center, for performing aphereses; the many patients who served as neutrophil donors; Dr. Ken Williams and the W.M. Keck Foundation for performing the sequence analyses; Dr. J. Noel and Hazelton Laboratories for assisting in the preparation of anti-glutaredoxin antibodies; Dr. S. Rhee for providing the HPLC system used in glutaredoxin purification; and Dr. S. Chanock for providing myeloid tumour cells.

REFERENCES

1 Washko, P. W., Rotrosen, D. and Levine, M. (1989) J. Biol. Chem. **264**, 18996–19002
2 Welch, R. W., Wang, Y., Crossman, Jr., A., Park, J. B., Kirk, K. L. and Levine, M. (1995) J. Biol. Chem. **270**, 12584–12592
3 Welch, R. W., Bergsten, P., Bulter, J. DeB. and Levine, M. (1993) Biochem. J. **294**, 505–510
4 Lam, K.-W., Yu, H.-S., Blickman, R. D. and Lin, T. (1993) Ophthalmol. Res. **25**, 100–107
5 Dyer, D. L., Kanai, Y., Hediger, M. A., Rubin, S. A. and Said, H. M. (1994) Am. J. Physiol. **267**, C301–C306
6 Levine, M., Dhariwal, K.R, Wang, Y. H., Park, J. and Welch, R. (1994) in Natural Antioxidants in Human Health and Disease (Frei, B., ed.), pp. 469–488, Academic Press, San Diego
7 Washko, P. W., Wang, Y. and Levine, M. (1993) J. Biol. Chem. **268**, 15531–15535
8 Bigley, R., Wirth, M., Layman, D. and Stankova, L. (1983) Diabetes **32**, 545–548
9 Stankova, L., Bigley, R. and Ingermann, R. L. (1991) Gen. Pharmacol. **22**, 903–905
10 Hendry, J. M., Easson, L. H. and Owen, J. A. (1964) Clin. Chim. Acta. **9**, 498–499
11 Bigley, R., Riddle, M., Layman, D. and Stankova, L. (1981) Biochim. Biophys. Acta **659**, 15–22
12 Stahl, R. L., Liebes, L. F. and Silber, R. (1985) Biochim. Biophys. Acta **839**, 119–121
13 Wells, W. W., Xu, D. P., Yang, Y. and Rocque, P. A. (1990) J. Biol. Chem. **265**, 15361–15664
14 Winkler, B. S., Orselli, S. M. and Rex, T. S. (1994) Free Radicals Biol. Med. **17**, 333–349
15 Washko, P. W., Hartzell, W. O. and Levine, M. (1989) Anal. Biochem. **181**, 276–282
16 Smith, P., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Probenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. and Klenk, D. K. (1985) Anal. Biochem. **150**, 76–85
17 Klein, H. (1994) in Scientific Basis of Transfusion Medicine (Anderson, K. C. and Ness, P. M., eds.), pp.781–795, Saunders and Co., Philadelphia
18 Boyum, A. (1968) Scand. J. Clin. Lab. Invest. Suppl. **21**, 77–89
19 Edman, P. (1949) Arch. Biochem. Biophys. **22**, 475–480
20 Laemmli, U. K. (1970) Nature (London) **227**, 680–685
21 Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 4350–4354
22 Hsu, S. M., Raine, L. and Fanger, H. (1981) J. Histochem. Cytochem. **29**, 577–580
23 Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. and Rutter, W. J. (1979) Biochemistry **18**, 5294–5299
24 Papov, V. V., Gravina, S. A., Mieyal, J. J. and Biemann, K. (1994) Protein Sci. **3**, 428–434
25 Fernando, M. R., Sumimoto, H., Nanri, H., Kawabata, S., Iwanaga, S., Minakami, S., Fukumaki, Y. and Takeshige, K. (1994) Biochim. Biophys. Acta **1218**, 229–231
26 Mizoguchi, T., Nishinaka, T., Uchida, G., Mizuta, J., Uchida, H., Terada, T. and Toya, H. (1993) Biol. Pharm. Bull. **16**, 840–842
27 Gan, Z. R. and Wells, W. W. (1987) J. Biol. Chem. **262**, 6704–6707
28 Wells, W. W., Yang, Y. and Deits, T. L. (1993) Adv. Enzymol. **66**, 149–201
29 Holmgren, A. (1989) J. Biol. Chem. **264**, 13963–13966
30 Maellaro, E., Bello, B. D., Sugherini, L., Santucci, A., Comporti, M. and Casini, A. F. (1994) Biochem. J. **301**, 471–476
31 Bode, A. M., Vanderpoos, S. S., Carlson, E. C., Meyer, D. A. and Rose, R. C. (1991) Invest. Ophthalmol. Vis. Sci. **32**, 2266–2271
32 Bode, A. M., Green, E., Yavarow, C. R., Wheeldon, S. L. and Rose, R. C. (1993) Curr. Eye Res. **12**, 593–601
33 Bode, A. M., Yavarow, C. R., Fry, D. A. and Vargas, T. (1993) Biochem. Biophys. Res. Commun. **191**, 1347–1353
34 Melhorn, R. J. (1991) J. Biol. Chem. **266**, 2724–2731
35 Coassin, M., Tomasi, A., Vannini, V. and Ursino, F. (1991) Arch. Biochem. Biophys. **290**, 458–462
36 Winkler, B. S. (1992) Biochim. Biophys. Acta **1117**, 287–290
37 Borsook, H., Davenport, H. W., Jeffres, C. E. P. and Warner, R. C. (1937) J. Biol. Chem. **117**, 237–279