Ascorbate Free Radical Reductases and Diaphorases in Soluble Fractions of the Human Lens

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Major and minor ascorbate free radical (AFR) reductases, with diaphorase activity, and three other diaphorases were separated from the human lens soluble fraction by DEAE-cellulose ion-exchange column chromatography. They were characterized for adsorptivity to ion-exchange and 5'AMP-Sepharose 4B affinity columns, kinetic properties, and substrate specificity. The latter diaphorases were closely correlated with NADH-cytochrome b5 reductase. The major and minor AFR reductases were regarded as a major diaphorase group different from two ubiquitous diaphorases, i.e., NADH-cytochrome b5 reductase and DT-diaphorase. A major AFR reductase was partially purified approximately 50 fold over the lens soluble fraction by ion-exchange, affinity, and gel filtration (Sephacryl S-200 HR) column chromatography. From the partially purified enzyme, 2 bands, one sharp and one diffuse, were obtained by native polyacrylamide gel electrophoresis. Two proteins, of 20 and 24 kDa, were identified in the active enzyme bands by SDS-polyacrylamide gel electrophoresis. This suggests that the 20 and/or 24 kDa proteins may be components of the major AFR reductase.

(Key Words: human lens, soluble fraction, ascorbate free radical reductase, diaphorase, NADH-cytochrome b5 reductase)

INTRODUCTION

The lenses of humans and some animals contain ascorbate at high concentrations, in excess of 1 mM (10). It is known that ascorbate can reduce oxidants and free radicals very effectively in vitro (6), and protect the rat lens from oxidants in organ culture (27). In addition, Ogino et al. (23) reported that vitamin C-deficient guinea pigs develop cataracts following injection of various quinoids derived from endogenous and exogenous compounds. However, oxidation products of ascorbate are cataractogenic due to crosslinking and browning of lens protein (21). In previous papers (1-3), we have shown that in the human lens, and in other tissues as well, ascorbate is maintained in the reduced state by ascorbate free radical (AFR) reductase (NADH:AFR oxidoreductase; EC 1.6.5.4), and its decline in activity is closely correlated with lens protein aggregation in senile cataractogenesis and aging. Therefore, it seems likely that AFR reductase is needed for ascorbate to protect the lens from oxidants and free radicals.

Recently, we (4) performed a partial purification of a major soluble AFR reductase from the cortex of transparent and immature senile cataractous human lenses with a two-step DEAE-cellulose ion-exchange and 5'AMP-Sepharose 4B affinity column chromatography. We found that AFR reductase also exhibits NADH-dependent diaphorase activity using 2,6-dichlorophenolindophenol (DCPIP) and ferricyanide as electron acceptors. Approximately 70-90% of the AFR and DCPIP reductase activities and 50-60% of the ferricyanide reductase activity in the human lens are extracted in the soluble fraction (3, 5). Equal to the ascorbate-AFR reductase system, it may be important to investigate the diaphorases in the lens, since the enzymes can reduce directly oxidants and free radicals with NADH or NADPH. Rao et al. (24) reported that ζ-crystallin, a major lens protein in the guinea pig, possesses NADPH: quinone oxidoreductase activity. A mutation in the gene for this protein is associated with an autosomal dominant con-
genital cataract, and the mutant ζ-crystallin appears to lack this quinone reductase activity.

The present paper reports the characterization of the above AFR reductases and diaphorases, and the greater purification of a major AFR reductase in the human lens soluble fraction.

MATERIALS AND METHODS

Materials and Chemicals
The soluble fraction in the human lens cortex was prepared from apparently transparent lenses (luxated by trauma, etc.) and immature senile cataractous lenses obtained after intracapsular lens extraction in the ophthalmology operating rooms of the Tokai University Hospital and Isehara Kyodo Hospital, as described previously (4).

DEAE-cellulose (DE52) was obtained from Whatman Chemical Separation Ltd. (Kent, England), and 5′AMP-Sepharose 4B and Sephacryl S-200 HR from Pharmacia LKB Biotechnology (Uppsala, Sweden). Ascorbate oxidase (EC 1.1.3.3, Curcurbita species) was purchased from Boehringer Mannheim GmbH (Mannheim, Germany); NADH (disodium salt) from Wako Pure Chemical Industries (Osaka, Japan); and NADPH from Oriental Yeast Company (Tokyo, Japan). Other reagents and chemicals used were of the purest grade available from commercial sources.

Column Chromatography
All chromatography was carried out at 0–4°C. The soluble fraction of the lens was subjected to DEAE-cellulose ion-exchange column chromatography as reported previously (4). Five diaphorase fractions, I to V (shown in Fig. 1), were collected and used in the following experiments.

The major and minor AFR reductase fractions (diaphorase I and II) were applied to 5′AMP-Sepharose 4B affinity column chromatography as previously described (4). The column was then eluted with 10 mM K-phosphate, pH 6.1 and 0.1 M K-phosphate, pH 7.2, in stepwise fashion. Diaphorase III and IV fractions were similarly applied to 5′AMP-Sepharose 4B affinity columns, except we used 0.1 M K-phosphate, pH 7.2 and 0.2 mM NADH in 25 mM K-phosphate, pH 7.2 as the first and second elution buffers, respectively.

The collected 0.1 M phosphate fractions of the major AFR reductase were concentrated to about 2.5 ml by ultrafiltration. KCl was then added to a final concentration of 0.1 M, and the sample fractionated on a Sephacryl S-200 HR column (2.6 cm in diameter × 32 cm in length), eluted with 0.1 M KCl and 10 mM K-phosphate, pH 7.2, at a flow rate of about 60 ml/hr. The eluates of the active enzyme were pooled, concentrated, and then electrophoresed.

Electrophoresis
All electrophoreses were run at room temperature. Native polyacrylamide slab gel electrophoresis of the major AFR reductase, isolated by the above three steps of column chromatography, was performed with a 10% gel using the method of Davis (8). After the electrophoresis, the gel was stained for diaphorase activity by the method of Kaplan and Beutler (13) using NADH as electron donor and DCPIP plus thiazolyl blue tetrazolium as electron acceptor. The gels were stained for protein with Coomassie Brilliant Blue (CBB) R-250 (26) (Quick-CBB: Wako Pure Chemical Industries, Ltd., Osaka, Japan), or for greater sensitivity, by silver staining (22) (Silver Stain II Kit Wako: Wako Pure Chemical Industries, Ltd., Osaka, Japan). From two CBB-stained bands of the enzyme on the gel, protein was extracted in the sample buffer of SDS-gel electrophoresis by the centrifugal homogenization procedures of Kobayashi et al. (16).

SDS-polyacrylamide slab gel (10%) electrophoresis of the extracted enzyme preparations was carried out according to the method of Laemmli (17), and the gel was visualized by silver staining. Approximate molecular weights were estimated by comparing the relative mobility with those of the standard protein controls (bovine serum albumin, 66; ovalbumin, 45; trypsinogen, 24; and β-lactoglobulin, 18.4 kDa).

Assays of Enzyme Activities
AFR and ferricyanide reductase activities were spectrophotometrically determined by measuring the oxidation rate of 100 or 200 μM NADH at 340 nm in the presence of AFR (4.5 μM) generated by 1 mM ascorbate plus 0.12 unit/ml ascorbate oxidase and of 150 μM ferri-
Lens Ascorbate Radical Reductases and Diaphorases

Fig. 1 Separation of AFR reductases and diaphorases in the human lens soluble fraction by DEAE-cellulose ion-exchange column chromatography. The eluates were assayed for activities of AFR (A and B) (○), DCPIP (A) (●), and ferricyanide (B) (△) reductases. Absorbances at 280 nm (---) and KCl concentrations (---) measured are also indicated.

cyanide, respectively. DCPIP reductase activity was assayed by measuring the reduction rate of 40 μM DCPIP at 600 nm in the presence of 200 μM NADH. In some experiments, the concentrations of NADH, ascorbate oxidase, and ferricyanide were changed, and NADH was replaced by NADPH. The details of those reductase activity assays and the method of estimation of AFR concentrations in the ascorbate plus ascorbate oxidase system have been reported (1, 4). Protein was determined by the dye-binding method of Bradford (7) using bovine serum albumin as the standard.

RESULTS

Ion-Exchange and Affinity Column Chromatography of AFR Reductases and Diaphorases

Five diaphorase fractions were separated by DEAE-cellulose ion-exchange column chromatography of the lens soluble fraction (Fig. 1).
The major and minor AFR reductases (diaphorase I and II), and diaphorase III and IV were then applied to 5'AMP-Sepharose 4B affinity columns. In the affinity chromatography of the major AFR reductase, most (78%) of the reductase activity was eluted as a sharp peak by 0.1 M K-phosphate, pH 7.2 (Table 1). The affinity of the minor AFR reductase for 5'AMP-Sepharose 4B was less than that of the major, and 82% of the activity was washed out at 10 mM phosphate, pH 6.1, and only 18% eluted by 0.1 M phosphate, pH 7.2 (Table 1). In contrast, diaphorases III and IV had much greater affinity for the Sepharose than the major and minor AFR reductases, and about 70% of the diaphorase activities were eluted with the buffer containing 0.2 mM NADH (Table 2).

**Purification of the Major AFR Reductase**

The 0.1 M phosphate fraction of the major AFR reductase recovered by affinity chromatography was then subjected to gel filtration on a Sephacryl S-200 HR column, and a single peak of AFR reductase activity was obtained (Fig. 2A). During the above three passages on the ion-exchange, affinity, and gel filtration columns, the specific activity of AFR reductase was increased to about 50 times that of the original soluble fraction. Diaphorase activity also increased in a similar manner, although the activity ratio of diaphorase to AFR reductase gradually decreased slightly (Table 3). Finally, two bands, one sharp and one diffuse and stained for diaphorase activity and protein, were isolated from the active enzyme fractions of the gel filtration by native polyacrylamide gel electrophoresis (Fig. 2B). The sharp band appeared to have greater diaphorase activity. In SDS-gel electrophoresis, however, two proteins (20 and 24 kDa) were commonly identified to both the enzyme bands (Fig. 2C).

**Kinetic Properties of the AFR Reductases and Diaphorases**

The apparent K_m values (15 and 7 μM) of the major and minor AFR reductases for AFR did not differ much (Table 4). Such similarity between the two AFR reductases was also observed in the ratio of diaphorase activity to AFR reductase activity (Table 3), and in kinetic constants and NADH specificity of their ferricyanide reductase activities (Table 5). The K_m values of ferricyanide reductase activity for NADH and ferricyanide were a little less for the major and minor AFR reductases than in the diaphorases III, IV and V (Table 5). The NADPH/NADH ratios of ferricyanide reduc-

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**Table 1** Percentage of AFR reductase activity eluted by 10mM phosphate, pH 6.1, and 0.1 M phosphate, pH 7.2, by 5'AMP-Sepharose 4B affinity column chromatography.

<table>
<thead>
<tr>
<th>Percentage of AFR reductase activity</th>
<th>10 mM phosphate fraction</th>
<th>0.1 M phosphate fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Major AFR reductase (Diaphorase I)</td>
<td>22</td>
<td>78</td>
</tr>
<tr>
<td>Minor AFR reductase (Diaphorase II)</td>
<td>82</td>
<td>18</td>
</tr>
</tbody>
</table>

**Table 2** Percentage of ferricyanide reductase activity eluted by 0.1 M phosphate, pH 7.2, and 0.2 mM NADH in 25 mM phosphate, pH 7.2, by 5'AMP-Sepharose 4B affinity column chromatography.

<table>
<thead>
<tr>
<th>Percentage of K_3Fe(CN)_6 reductase activity</th>
<th>0.1 M phosphate fraction</th>
<th>0.2 mM NADH fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diaphorase III</td>
<td>38</td>
<td>62</td>
</tr>
<tr>
<td>Diaphorase IV</td>
<td>28</td>
<td>72</td>
</tr>
</tbody>
</table>
Fig. 2  Gel filtration on a Sephacryl S-200 HR column, native polyacrylamide gel electrophoresis, and SDS-polyacrylamide gel electrophoresis of the major AFR reductase from the human lens soluble fraction. (A) 0.1 M phosphate fraction of the reductase (125 µg protein), from the 5'AMP-Sepharose 4B affinity column, was subjected to gel filtration. The activities of AFR reductase and absorbance at 280 nm are indicated by (○) and (---), respectively. (B) An enzyme fraction (marked by a bar) from the above gel filtration was applied to native slab gel electrophoresis (about 0.5 µg protein/each loading). Left: staining for diaphorase activity. Indicated (by stars) are a weakly stained diffuse band and a more deeply stained band. Right: silver staining for protein. The same two bands were also stained by CBB. (C) SDS-slab gel electrophoresis of the enzyme preparations extracted from the CBB-stained bands, as described in (B). The gels were visualized with silver staining.
Table 3  Diaphorase activities of the major and minor AFR reductases from the human lens soluble fraction.

<table>
<thead>
<tr>
<th>Specific reductase activity (nmol min(^{-1}) mg protein(^{-1}))</th>
<th>Activity ratio</th>
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<tbody>
<tr>
<td>AFR</td>
<td>DCPIP</td>
</tr>
<tr>
<td>Soluble protein fraction</td>
<td>4.15</td>
</tr>
<tr>
<td>Major AFR reductase</td>
<td></td>
</tr>
<tr>
<td>Isolation step</td>
<td></td>
</tr>
<tr>
<td>1. DEAE-cellulose</td>
<td></td>
</tr>
<tr>
<td>Non-adsorbed fraction (Fractions No.13-19)</td>
<td>31.8</td>
</tr>
<tr>
<td>2. 5'AMP-Sepharose 4B</td>
<td></td>
</tr>
<tr>
<td>0.1 M phosphate fraction (Fractions No.24 &amp; 25)</td>
<td>84.2</td>
</tr>
<tr>
<td>3. Sephacryl S-200 HR</td>
<td></td>
</tr>
<tr>
<td>(Fractions No.43-46)</td>
<td>191</td>
</tr>
<tr>
<td>Minor AFR reductase</td>
<td></td>
</tr>
<tr>
<td>Isolation step</td>
<td></td>
</tr>
<tr>
<td>1. DEAE-cellulose</td>
<td></td>
</tr>
<tr>
<td>Adsorbed fraction (Fractions No.39-44)</td>
<td>5.48</td>
</tr>
</tbody>
</table>

N. D.: Not determined

Table 4  Kinetic constants of the major and minor AFR reductases in the human lens soluble fraction.

<table>
<thead>
<tr>
<th>Apparent K(_m) for AFR (µM)</th>
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<tbody>
<tr>
<td>Soluble protein fraction</td>
</tr>
<tr>
<td>Major AFR reductase (Diaphorase I)</td>
</tr>
<tr>
<td>Minor AFR reductase (Diaphorase II)</td>
</tr>
</tbody>
</table>

The major AFR reductase was isolated by DEAE-cellulose and 5'AMP-Sepharose 4B column chromatography, and the minor one was separated by ion-exchange column chromatography. The K\(_m\) values for AFR were determined from Lineweaver-Burk plots based on the activities at 200 µM NADH. K\(_m\) values of the reductases for NADH could not be determined accurately by our conventional absorption spectrophotometer, since those were too small (less than 2 µM).

tase activities of the major AFR reductase, minor AFR reductase, and diaphorase III were greater than those of the diaphorases IV and V (Table 5). The higher ratio of diaphorase III might depend to some extent on contamination by the minor AFR reductase.

**DISCUSSION**

The present study shows that the major and minor AFR reductases (diaphorases I and II) isolated from the human lens soluble fraction possess distinctive kinetic properties, and electron acceptor and donor specificities, different from the lens diaphorases III–V (Tables 3–5). Various compounds, including quinones, are known to cause cataracts in humans and animals (9). Oxidants and free radicals may be photochemically and metabolically generated from such compounds, with a loss of antioxidation agents of ascorbate (1–3), glutathione (9), and diaphorase redox systems in the lens, and so be involved in the formation of cataracts.
Table 5 Kinetic constants and NADH specificity of ferricyanide reductase activities of the diaphorases in the human lens soluble fraction.

<table>
<thead>
<tr>
<th>Diaphorase</th>
<th>Apparent $K_m$ for NADH (μM)</th>
<th>Apparent $K_m$ for $K_3Fe(CN)₆$ (μM)</th>
<th>Activity ratio NADPH/NADH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diaphorase I (Major AFR reductase)</td>
<td>2.5</td>
<td>2.1</td>
<td>0.37</td>
</tr>
<tr>
<td>Diaphorase II (Minor AFR reductase)</td>
<td>3.6</td>
<td>2.5</td>
<td>0.29</td>
</tr>
<tr>
<td>Diaphorase III</td>
<td>5.0</td>
<td>10.0</td>
<td>0.25</td>
</tr>
<tr>
<td>Diaphorase IV</td>
<td>8.3</td>
<td>14.7</td>
<td>0.01</td>
</tr>
<tr>
<td>Diaphorase V</td>
<td>9.7</td>
<td>17.9</td>
<td>0.03</td>
</tr>
</tbody>
</table>

The diaphorase fractions were separated by DEAE-cellulose ion-exchange column chromatography. The $K_m$ values for NADH and ferricyanide were determined from Lineweaver-Burk plots based on the activities at 150 μM ferricyanide and 100 μM NADH, respectively. The activity ratios of NADPH to NADH were calculated from the means of two measurements.

It has been reported that a congenital cataract in guinea pig is associated with a mutation of a particular enzyme/crystallin, ζ-crystallin/NADPH-quinone reductase (24). However, the diaphorases of the human lens have been little studied until now. AFR reductase is recognized from this study as a major enzyme among the human lens diaphorases, since 80 and 50% of DCPIP and ferricyanide reductase activities in the lens soluble fraction originate from the AFR reductase fractions (Fig. 1). Our latest study (Matsukura, Bando and Obazawa, unpublished observations) has shown that, in senile cataractous lenses, there is a close correlation between lens protein aggregation and a decrease in ferricyanide reductase activity as observed in AFR reductase activity.

NADH-cytochrome $b_5$ reductase (NADH:cytochrome $b_5$ oxidoreductase, EC 1.6.2.2) is known to be widely distributed in the organelles and cytosol of various animal tissues (18), and to catalyze in vitro the one-electron reduction of foreign compounds and AFR (11, 12). The enzyme is highly specific for NADH, and reacts with ferricyanide much better than with cytochrome $b_5$ or DCPIP (14, 20). The isolation behaviors (15, 28, 29) and substrate specificity of the soluble enzyme are comparable to those of diaphorases III–V from the human lens soluble fraction (Fig. 1, and Tables 2 and 5). The lens diaphorases III–V are closely correlated with NADH-cytochrome $b_5$ reductase, but the lens AFR reductases (diaphorases I and II) differ from NADH-cytochrome $b_5$ reductase itself. The 20 and/or 24 kDa proteins are supposed to be components of the major lens AFR reductase (Fig. 2), whereas soluble NADH-cytochrome $b_5$ reductase is reported to be a monomer of a 32 kDa protein (14).

DT-diaphorase (NAD(P)H: quinone oxidoreductase, EC 1.6.99.2), a ubiquitous enzyme in the cytosol of different tissues, catalyzes the two-electron reduction of various dyes and quinones (19). Russell et al. (25) have shown that DT-diaphorase activity is induced in a lens epithelial cell line from a transgenic mouse by incubation with naphthoquinone (a cataractogenenic compound). However, the reduction of AFR by DT-diaphorase has not been reported (19), and DCPIP reductase activities of the major and minor lens AFR reductases were not inhibited by 10 and 100 μM dicoumarol, a specific inhibitor of DT-diaphorase (Bando and Obazawa, unpublished observations).

From the above, it is speculated that the human lens requires not only the ascorbate and glutathione redox systems but also a diaphorase system made up of AFR reductase, NADH-cytochrome $b_5$ reductase, and similar enzymes in order to reduce cataractogenetic oxidants and free radicals. It seems likely that AFR reductase, a chief diaphorase in the lens, plays an important role in both ascorbate regeneration and diaphorase activity. Most of the oxidants and free radicals may be effectively reduced by the ascorbate-AFR reductase system, but some compounds may have to be more quickly scavenged by the diaphorase activity of AFR reductase or NADH-
cytochrome b₅ reductase. The glutathione redox system specifically detoxifies peroxides (9). Therefore, the human lens is protected from a wide variety of oxidants and free radicals.

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