Spectral characterization and chemical modification of FMN-containing ascorbyl free-radical reductase from *Pleurotus ostreatus*

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Ascorbyl free-radical reductase was purified 1143-fold with an overall yield of 9.9% from the cytosolic fraction of *Pleurotus ostreatus*. The native enzyme had a molecular mass of 127 kDa and SDS/PAGE revealed that the enzyme consists of two subunits, each with a molecular mass of 62 kDa. The enzyme utilized only NADH as an electron donor. The enzyme was highly specific for ascorbyl free radical as an electron acceptor and capable of catalysing the reduction of ferricyanide and 2,6-dichloroindophenol as artificial electron acceptors. The apparent K_m values of the enzyme towards NADH and ascorbyl free radical were 35 ± 0.22 and 2.1 ± 0.03 μ M, respectively. The catalytic mechanism of this enzyme is of Ping Pong type. The enzyme contained FMN as a prosthetic group and showed the characteristic absorption spectrum ascribed to the charge-transfer interaction of thiolate anion with FMN. The enzyme contained

eight cysteine residues per monomer and was inactivated more rapidly by mercurials than by thiol-alkylating reagents. Kinetic analysis of the inactivation process revealed that the enzyme had 1 mol of thiol group/mol of subunit in the active site with a pK_a of 6.9. The modification of the thiol group of the enzyme caused the loss of charge-transfer absorbance centred at 540 nm and blocked the electron-transfer process from NADH to FMN. The modification of lysine, arginine and histidine residues led to the loss of its activity. Unlike the active enzyme, the fluorescence quenching of NADH was not observed in the lysine-modified enzyme, which implies that lysine residues can participate in the interaction with NADH.

Key words: active site, charge-transfer interaction, thiol group.

INTRODUCTION

L-Ascorbic acid (ASC) is a ubiquitous and essential substance in the defence against oxidative stress and as a cofactor in many enzyme systems [1]. ASC undergoes continuous oxidation to dehydro-L-ascorbic acid (DHA) by a successive reversible one-electron-transfer process with a free-radical intermediate. Thus the ascorbate redox system consists of ASC, ascorbyl free radical (AFR) and DHA. In the biological system, the relative steady-state level of the ascorbate redox system seems to be maintained by certain enzymes. Glutathione-dependent dehydroascorbate oxidoreductase (EC 1.8.5.1) has been supposed to be responsible for the reduction of dehydroascorbate and NAD(P)H:AFR oxidoreductase (EC 1.6.5.4) for the reduction of AFR to ASC [2]. The oxidation of ascorbate, on the other hand, seems to be mediated by copper-containing L-ascorbate oxidase (EC 1.10.3.3) in plants [3] and by dopamine β -hydroxylase (EC 1.14.17.1) [4] or ceruloplasmin [5] in animal tissues.

Regeneration of ascorbate is indispensable in view of the physiological importance of ascorbate and the lowered nutrient requirement. AFR reductase is an essential enzyme in maintaining the ASC system in the reduced state. Because of the higher oxidation–reduction potential of the AFR/ASC pair (+320 mV) compared with the DHA/ASC pair (+60 mV) at pH 7 [6], the regeneration of ascorbate from AFR is thermodynamically favourable. Therefore, whenever ASC plays its role of electron donor, its oxidation is prevalently restricted to the first step, leading to the formation of AFR, and the production of DHA decreases.

AFR reductase appears to be widespread in various plants, animals, algae and protists compared with DHA reductase [2]. In plants, the activity of AFR reductase is found in chloroplasts, mitochondria and microsomes as well as in the cytosol. The

enzymes purified from cucumber [7] and soya bean root nodule [8] are FAD-containing monomers with molecular masses of 47 and 39 kDa, respectively. In animals, the enzyme activity is present in microsomes and mitochondria, and seems to be associated with cytochrome b_5 and NADH–cytochrome b_5 reductase [9]. However, the characteristics of the enzyme have not been reported yet. In fungi, some characteristics of AFR reductase were reported only for *Neurospora crassa* [10].

Compared with many reports about the ascorbate redox system well documented in plants, the role of the ascorbate system in micro-organisms has remained elusive. For that reason, we have chosen the white rot fungus, *Pleurotus ostreatus*, in an attempt to explore the physiological role of the ascorbate redox system in the differentiation of fungi. We recently purified a novel haem-containing and H_2O_2 -producing ascorbate oxidase [11], which may produce AFR as a reaction intermediate. Also, glutathione-dependent DHA reductase has been characterized as a monomer with a molecular mass of 86 kDa [12]. In the present study, we report the molecular properties of *P. ostreatus* AFR reductase, the third component of the ascorbate redox system. Our analysis reveals that *P. ostreatus* AFR reductase is a homodimeric flavoprotein containing FMN and has novel spectral and structural features distinguished from the other previously reported AFR reductases.

MATERIALS AND METHODS

Materials

ASC was purchased from Merck; *Cucurbita* ascorbate oxidase was from Roche Molecular Biochemicals; dithiothreitol, NADH, Reactive Red 120–agarose CL-4B, Sephadex G-15 and Tris were from Sigma; Mono-Q 5/5 and Superdex 200 HR 10/30 were

Abbreviations used: AFR, ascorbyl free radical; ASC, L-ascorbic acid; CMB, *p*-chloromercuribenzoate; DHA, dehydro-L-ascorbic acid.

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from Amersham Pharmacia Biotech; and molecular-mass markers were from Bio-Rad.

Enzyme assay

The activity of AFR reductase was measured spectrophotometrically by the decrease in absorbance at 340 nm due to the oxidation of NADH. AFR was generated by adding ascorbate oxidase to the ASC solution. In this mixed solution, AFR was produced in steady state for a few min. The concentration of AFR was determined from the absorbance at 360 nm using a molar absorption coefficient of $3.3 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ [13]. The reaction mixture contained 0.1 mM NADH, 1 mM ASC, 0.1 unit of ascorbate oxidase in 0.1 M Tris/HCl buffer, pH 7.4, and an aliquot of the enzyme in a final volume of 0.6 ml. The reaction was initiated by the addition of ascorbate oxidase and carried out at 30 °C. AFR reductase was also assayed by a procedure in which AFR was prepared as a mixture of ASC and DHA at equal concentrations of 10 mM. The final concentration of AFR was given by an equilibrium constant, $[\text{AFR}]^2/[\text{ASC}] \times [\text{DHA}]$, of 10^{-8} [14]. The reaction mixture was identical with that stated above, except for the substitution of DHA for ascorbate oxidase. In either case, the rate of NADH oxidation by AFR reductase was linear over the first several min and thus initial velocities were calculated from the decrease of absorbance within 1 min, based on a molar absorption coefficient of $6.22 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ for NADH [15].

Purification of the enzyme

Mycelia of *P. ostreatus* were grown under conditions described previously [11]. The purification was performed at 4 °C, unless otherwise stated. The grown mycelia were collected by filtration on Whatman No. 1 filter paper and washed with 10 mM Tris/HCl buffer, pH 7.4, containing 2 mM dithiothreitol. The washed mycelia were homogenized in the buffer with aluminium oxide. After the removal of cell debris and aluminium oxide by centrifugation, the supernatant was fractionated with ammonium sulphate (55–75%). The precipitate was dissolved in a minimal volume of the buffer and applied to Sephadex G-15 (6 cm × 55 cm) equilibrated with the same buffer to remove the salt. The active fractions were loaded on to Reactive Red 120–agarose CL-4B (3.5 cm × 14 cm) and eluted with a linear concentration gradient of 0–0.5 M NaCl in the buffer (800 ml). Peak fractions containing AFR reductase were pooled, concentrated and equilibrated with the buffer. The enzyme was applied to a column of Mono-Q and eluted with a linear concentration gradient of 60–120 mM NaCl in the buffer (25 ml). This column chromatography was performed at room temperature with a FPLC system equipped with UV and a conductivity flow cell (Amersham Pharmacia Biotech).

Determination of protein concentration and molecular mass

The concentration of protein was determined using the Bio-Rad protein-assay kit after the removal of dithiothreitol using an Amicon concentrator with a YM-10 membrane.

The molecular mass of the purified enzyme was determined using a Superdex 200 HR 10/30 column with a FPLC system (Amersham Pharmacia Biotech). The column was calibrated with ferritin (440 kDa), aldolase (158 kDa), ovalbumin (43 kDa) and ribonuclease A (13.7 kDa). For the determination of the molecular mass of the subunit, SDS/PAGE was performed using the Bio-Rad Mini Protean II kit according to the manufacturer's procedures.

Determination of flavin

The purified enzyme (20 µg of protein) was treated with 5% trichloroacetic acid and the precipitated apoenzyme was discarded by centrifugation at 8000 g for 10 min [16]. The supernatant was analysed using an HPLC system equipped with a fluorescence detector (Hewlett Packard 1046A), using FAD and FMN as reference compounds, according to the method proposed previously [17] with some modifications. An ODS Hypersil column (Hewlett Packard, $4.6 \times 100 \text{ mm}$) was used and the flow rate of the mobile phase was set at 0.5 ml/min. The mobile phase was acetonitrile/water/10% trifluoroacetic acid/phosphoric acid (14:84:1.5:0.09, by vol.). The fluorescence detector was set at 430 nm for excitation and 525 nm for emission, with a 470-nm cut-off filter. The apoenzyme was prepared using potassium bromide as described by Tedeschi et al. [18]. Reconstitution was performed in 10 mM Tris/HCl buffer, pH 7.4, containing 2 mM dithiothreitol at 4 °C for 3 h with an excess of FMN or FAD.

Spectroscopic studies

UV/visible spectra of the enzyme were obtained with a Shimadzu UV-1601 spectrophotometer after the removal of dithiothreitol by ultrafiltration in 0.1 M Tris/HCl buffer, pH 7.4. Fluorescence spectra were recorded with SLM Aminco 48000 spectrofluorimeter at room temperature. EPR spectra were recorded on a Bruker EMX system at room temperature with 9.5 GHz of microwave frequency, 1 mW of microwave power and 0.1 mT of modulation amplitude.

Chemical-modification studies

p-Chloromercuribenzoate (CMB) was prepared in 0.1 M Tris/HCl buffer, pH 9.0. The enzyme was incubated with the indicated concentrations of CMB in 0.1 M Tris/HCl buffer, pH 7.4. To investigate the pH dependence of the inactivation reaction, the incubation of the enzyme with CMB was carried out at various pHs in the range 6.0–8.0. Lysine residues were modified with pyridoxal 5'-phosphate or 2,4,6-trinitrobenzenesulphonic acid and arginine residues with diacetyl or phenylglyoxal. For the modification of histidine and tyrosine residues, diethyl pyrocarbonate and trinitromethane were used, respectively.

RESULTS AND DISCUSSION

Purification of the enzyme

AFR reductase was purified 1143-fold from the cytosolic fraction of *P. ostreatus* with an overall yield of 9.9%, as summarized in Table 1. About 300 µg of AFR reductase was recovered from 300 g (wet weight) of the starting mycelium. The enzyme did not bind to Blue-Sepharose or 5'-AMP-Sepharose, unlike AFR reductases from cucumber [7] and soya bean root nodule [8]. For the purification of *P. ostreatus* AFR reductase, Reactive Red-agarose chromatography was rather efficient. The purity of the final preparation was confirmed by an electrophoretic method (Figure 1).

Reduction of AFR by *P. ostreatus* AFR reductase

The reduction of AFR by *P. ostreatus* reductase was demonstrated by means of EPR spectroscopy. ASC (1 mM) in 0.1 M

Table 1 Purification of AFR reductase from *P. ostreatus*

Step	Total protein (mg)	Total activity (nmol/min)	Specific activity (nmol/min per mg)	Yield (%)	Purification (-fold)
Crude extract	4025	39166	9.7	100	1
Ammonium sulphate precipitation	379	25545	67.4	65.2	7
Reactive Red-agarose chromatography	12	6689	574.2	17.1	57
Mono-Q chromatography	0.35	3880	11085.7	9.9	1143

Figure 1 SDS/polyacrylamide gel electrophoretogram of AFR reductase

A 10% polyacrylamide gel was used. Lane A, molecular-mass markers: myosin (200 kDa), β -galactosidase (116.25 kDa), phosphorylase *b* (97.4 kDa), BSA (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa). Lane B, 5 μ g of the purified enzyme.

Tris/HCl buffer, pH 7.4, under aerobic conditions showed the traces of AFR signal, indicating the auto-oxidation of ASC (Figure 2A). When 0.1 unit of ascorbate oxidase was added to this solution, giving rise to a steady-state generation of AFR, the spectrum with $g = 2.0054$ and $a = 1.82$ G increased (Figure 2B). These spectral parameters were similar to those of AFR reported previously [19], in which $g = 2.0052$ and $a = 1.75$ G had been measured. The subsequent addition of NADH did not diminish the signal intensity of AFR (Figure 2C). On the other hand, *P. ostreatus* AFR reductase in the presence of NADH decreased the signal intensity to the level of the auto-oxidation of ASC (Figure 2D). Therefore, these results exhibited the strong evidence that the enzyme purified from *P. ostreatus* was AFR reductase and, in addition, that the non-enzymic reduction of AFR did not proceed under our experimental conditions, unlike the well-known chemical reaction of DHA and reduced glutathione [20].

Molecular properties

The purified enzyme in 10 mM Tris/HCl buffer, pH 7.4, containing 2 mM dithiothreitol and 25% glycerol, can be stored at -20°C for 1 month without significant loss of activity. However, activity was completely lost within a day in the absence of dithiothreitol at 4°C or on freezing without glycerol.

Figure 2 Reduction of AFR by AFR reductase

(A) 1 mM ASC was dissolved in 0.1 M Tris/HCl buffer, pH 7.4. (B) Ascorbate oxidase (0.1 unit) was added to the solution of (A). (C) 0.1 mM NADH was added to the solution of (B). (D) AFR reductase was added to the solution of (C). EPR spectra were taken under the conditions described in the Materials and methods section.

The molecular mass of the native enzyme was determined to be 127 kDa by gel-permeation chromatography. When the enzyme was subjected to SDS/PAGE, a single band of 62 kDa was found (Figure 1), indicating that the enzyme is composed of two subunits. When the enzyme solution that had been boiled in the presence or absence of 5% β -mercaptoethanol was electrophoresed, no changes were observed on polyacrylamide gel, indicating that intersubunit disulphide bonds were absent in the enzyme. The AFR reductases so far reported are all monomeric enzymes, with molecular masses of 42–47 kDa in potato tuber [21] and cucumber [7], and of 66 kDa in *N. crassa* [10]. Although the enzyme from soya bean root nodule was also a single polypeptide, two isoenzymes, with molecular masses of 39 and 40 kDa, were reported [8]. Thus the AFR reductase from *P. ostreatus* is the first dimeric enzyme of the AFR reductases reported.

The pI value of the purified enzyme was 5.1. That value is slightly higher than those of AFR reductases from soya bean root nodule, which had two isoenzymes with pI values of 4.6 and 4.7 [8]. The enzyme exhibited maximal activity at pH 7–9 and was stable in the range of pH 7–11 (results not shown). The haem-containing ascorbate oxidase reported previously in *P. ostreatus* exhibited maximal activity at pH 4.8–5.5 and was

Table 2 Amino acid compositions of AFR reductases purified from *P. ostreatus* and various plants

The data for cucumber were from [7], and those for soya bean were from [8]

Amino acid	<i>P. ostreatus</i> (residues/mol)	Cucumber (residues/mol)	Soya bean (residues/mol)
Cys	7.6	2.8	—
Asp + Asn	55.1	35.6*	56.4
Glu + Gln	54.9	47.0†	35.5
Ser	37.4	42.8	14.1
Gly	62.9	60.0	33.0
His	10.7	7.3	11.4
Arg	23.6	18.5	16.1
Thr	32.7	19.6	14.4
Ala	58.0	41.6	36.4
Pro	35.2	19.5	14.4
Tyr	10.9	9.7	12.4
Val	44.1	32.1	36.0
Met	11.8	2.0	0.0
Ile	27.0	19.9	19.7
Leu	42.8	31.5	28.6
Phe	21.3	21.6	18.4
Trp	6.4	1.8	—
Lys	39.3	28.8	20.5
Total	581.7	442.1	367.3

* The content of aspartic acid was reported only.

† The content of glutamic acid was reported only.

relatively stable in acidic pH [11]. The similar patterns of pH dependence of AFR reductase and ascorbate oxidase has been observed in the plant system [3,7,8,21]. Thus two constituents of the ascorbate redox system, one catalysing the oxidation of ASC and the other responsible for the reduction of AFR, may be functional in separate compartments within the cell.

The amino acid composition of the monomer of the enzyme is presented in Table 2. A comparison of the composition of the *P. ostreatus* enzyme with those of plant enzymes [7,8] shows the similar molar percentages of the amino acid residues, with the exceptions of serine, glycine, methionine and cysteine, in spite of the differences in molecular mass. *P. ostreatus* reductase is relatively rich in cysteine and methionine, whereas cucumber reductase is rich in serine and glycine. Soya bean reductase has substantially less serine, glycine and methionine. Moreover, 7.6 cysteine residues per monomer of the enzyme coincide with 8.1 thiol groups per monomer of the enzyme, which were determined from the 5,5'-dithiobis-(2-nitrobenzoic acid) assay (results not shown). Thus the eight cysteine residues of *P. ostreatus* reductase do not appear to form disulphide bonds. The N-terminal amino acid sequence could not be determined by Edman degradation, indicating that its N-terminal may be blocked, like the cucumber enzyme [7].

Prosthetic group

The recovery of the flavin in the supernatant after boiling or 5% trichloroacetic acid extraction indicates that the flavin is non-covalently bound to the enzyme. The flavin type of the enzyme was identified as FMN (Figure 3). Under isocratic conditions with a reverse-phase column, FAD and FMN were well resolved and eluted with retention times of 3.41 and 5.21 min, respectively (Figure 3A). The supernatant of the enzyme extract exhibited the retention time of 5.20 min, coinciding with that of FMN (Figure 3B). Incubation of the purified enzyme with FMN up to 100 μ M did not affect the enzyme activity. We prepared the apoenzyme

Figure 3 Analysis of the prosthetic group of AFR reductase with an HPLC-fluorescence detector

(A) HPLC profile of flavin standards. The peaks correspond to FAD and FMN, with retention times of 3.41 and 5.21 min, respectively. The standard solution (10 μ l, 100 μ g/ml) prepared in 0.1 M sodium phosphate buffer (pH 6.0) was injected. (B) HPLC profile of the supernatant of 5% trichloroacetic acid extraction of AFR reductase. The arrow indicates the retention time of 5.20 min, indicating that the prosthetic group is FMN. Other experimental conditions are described in the text.

to investigate whether FMN can restore the enzyme activity. The specific activity of the apoenzyme due to the residual FMN was 7% of the native enzyme, and the enzyme reconstituted with FMN recovered 16% of the specific activity with respect to the native holoenzyme after incubation with an excess of FMN. The apoenzyme treated with FAD did not recover the specific activity, as in the case of cucumber enzyme [7]. We also confirmed the type of flavin as FMN, based on the other criteria (results not shown). First, the fluorescence intensity of the supernatant at 530 nm did not increase at pH 2, compared with that at pH 7.0. Secondly, the treatment of the flavin with snake-venom phosphodiesterase (1 unit) for 30 min did not result in any increase in the fluorescence intensity. Under the same conditions, the authentic FAD produces an increase of fluorescence intensity [22,23]. The FMN content of the supernatant was calculated to be 0.9 ± 0.11 mol per monomer (three independent experiments) using a molar absorption coefficient of $12500 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 445 nm [15]. Thus the enzyme seems to contain 1 mol of FMN per monomer subunit. AFR reductases from cucumber fruits [7] and soya bean root nodule [8] are flavoproteins containing FAD

Table 3 Substrate specificity of AFR reductase

The concentration of electron donors used was 0.1 mM. NADH was used as an electron donor and the concentrations of the electron acceptors tested were 0.1 mM, except for AFR, O_2 and $O_2^{\cdot-}$. The concentration of AFR used was 3 μ M. To detect the activity of glutathione reductase, NADPH was used instead of NADH. For oxygen, a solution saturated with air was used. Xanthine (1 mM) and 0.03 units of xanthine oxidase were used for the generation of $O_2^{\cdot-}$.

Substrate (mM)	Relative activity (%)
Electron donors	
NADH	100
NADPH	0.5
Electron acceptors	
AFR	100
Ferricyanide	464
2,6-Dichloroindophenol	120
1,4-Benzoquinone	52
Menadione	0
Cytochrome <i>c</i> from <i>Saccharomyces cerevisiae</i>	0
GSSG	0
GSSG + NADPH	0
H_2O_2	0
O_2	0
$O_2^{\cdot-}$	0

Figure 4 Double-reciprocal plots of initial velocities of AFR reductase versus NADH with various concentrations of AFR

AFR was generated by various amounts of ascorbate oxidase. Both NADH and AFR were kept at non-saturating concentrations.

as prosthetic groups whereas, in case of the enzymes found in potato tuber [21] and *N. crassa* [10], the occurrence of any prosthetic groups was not reported. Therefore, this is the first AFR reductase containing FMN as a prosthetic group.

Substrate diversity

P. ostreatus AFR reductase utilized only NADH as an electron donor (Table 3), like AFR reductase from *N. crassa* [10]. However, the enzymes from plants could utilize both NADH and NADPH as the electron donor, even though the K_m value for NADPH was higher than that of NADH [7,8,21]. Hence, the

Table 4 Effects of the various reagents on the activity of AFR reductase

The concentrations of $CuCl_2$ and $HgCl_2$ were 0.5 mM and the other reagents were all 10 mM.

Reagent	Relative activity (%)
1,10-Phenanthroline	84
EDTA	89
α,α' -Dipyridyl	79
8-Hydroxyquinoline	85
NaN_3	84
$CuCl_2$	0
$HgCl_2$	0

fungal AFR reductases are distinct from the enzymes found in plants in terms of the specificity for pyridine nucleotides as electron donors.

Besides AFR, a physiological substrate, *P. ostreatus* AFR reductase was capable of catalysing the reduction of ferricyanide and 2,6-dichloroindophenol as artificial electron acceptors, effectively using NADH as an electron donor (Table 3). On the other hand, the reductase showed a measurable activity for only 1,4-benzoquinone, and the activities for NADH peroxidase, NADH oxidase and glutathione reductase were not detected (Table 3). This aspect of utilizing electron acceptors in *P. ostreatus* reductase is similar to that of the enzymes from plant and *N. crassa* [7,8,10,21]. High specificity towards AFR as an electron acceptor and much lower activity towards some quinone compounds shown in the enzymes originating from *P. ostreatus* and cucumber [7] can distinguish the AFR reductase from other flavoproteins, such as glutathione reductase [24] or NADH–quinone reductase [25].

Kinetic studies

From the Lineweaver–Burk plot the apparent K_m values for NADH and AFR were estimated to be 35 ± 0.22 and 2.1 ± 0.03 μ M, respectively (results not shown). These values were calculated using the software annexed to the Shimadzu UV-1601 spectrophotometer, and three independent measurements were carried out. To study the kinetic mechanism of *P. ostreatus* reductase, a two-substrate kinetic analysis was carried out under the conditions of non-saturating concentrations of the substrates. When the activity of the enzyme was assayed against the various NADH concentrations in the range 2.5–12.5 μ M at several fixed AFR concentrations, a series of parallel lines was obtained from the double-reciprocal plot, suggesting the Ping Pong mechanism (Figure 4).

Effects of the various inhibitors

The enzyme was incubated at the indicated concentration of each reagent for 10 min at 4 °C. To avoid the effect on the ascorbate oxidase, AFR was generated by mixing 10 mM ASC and 10 mM DHA in the final volume of 600 μ l of 0.1 M Tris/HCl buffer, pH 7.4. Chelating agents such as 1,10-phenanthroline, EDTA, α,α' -dipyridyl and 8-hydroxyquinoline had little effect on the enzyme activity, indicating that the metal ions were not involved in the catalytic activity. Heavy metals such as Cu^{2+} and Hg^{2+} inhibited the activity (Table 4).

Spectral properties

The purified AFR reductase shows the absorption spectrum of a flavoprotein with absorption bands at 370 and 450 nm and a

Figure 5 UV/visible absorption spectra of AFR reductase in 0.1 M Tris/HCl buffer, pH 7.4, under aerobic conditions

Curve A (—), the native enzyme (1.8 μ M). Curve B (---), the reduced enzyme with NADH (2 μ M). Curve C (···), the reoxidized enzyme with AFR generated by ascorbate (1 mM) and ascorbate oxidase (0.1 unit).

shoulder at 470 nm. In contrast with the absorption spectrum of a flavoprotein observed in cucumber AFR reductase [7], the enzyme from *P. ostreatus* had higher intensity at 370 nm than at 450 nm and lower-intensity absorbance extending to 800 nm (Figure 5, curve A). This long-wavelength absorption with low absorbance in the 500–600 nm region can be ascribed to the charge-transfer interaction of a thiolate anion with FMN, by analogy to glutathione reductase and lipoamide dehydrogenase [26,27]. These two enzymes contain both FAD and redox-active disulphide and catalyse a pyridine nucleotide-disulphide oxidation-reduction. Two-electron reduction of both enzymes produces a spectrally characteristic intermediate. Kosower [28] has proposed that this form, with its broad 530-nm absorption band, is a charge-transfer complex between a thiolate anion (formed on the reduction of the active-centre disulphide) and flavin. In the case of enterococcal flavoprotein, NADH peroxidase, the enzyme contains a cysteine-sulphenic acid instead of the disulphide as a non-flavin redox centre, which cycles catalytically between Cys-SOH and Cys-SH [29]. Reduction of the enzyme with dithionite or NADH leads to the appearance of a charge-transfer absorbance band centred at 540 nm and generates a nascent thiol group. There is a spectral resemblance of *P. ostreatus* AFR reductase to two-electron reduced forms of enzymes belonging to a pyridine nucleotide-disulphide oxidoreductase family. This, combined with the ensuing loss of charge-transfer absorbance and the reductase activity after treatment with CMB, demonstrates that the peak centred at 540 nm in the absorption spectrum of AFR reductase from *P. ostreatus* is due to the formation of charge-transfer complex between a thiolate anion as a charge-transfer donor and the oxidized form of the electron-deficient FMN as a charge-transfer acceptor (see Figure 6). The molar absorption coefficients of the native enzyme at 275, 370 and 450 nm were estimated to be 68 300, 12 000 and 10 500 $\text{M}^{-1} \cdot \text{cm}^{-1}$ respectively,

Figure 6 Absorption spectra of CMB-treated AFR reductase

Curve A (—), the native enzyme (10 μ M). Curve B (---), the inactivated enzyme with 3-fold CMB. Curve C (···), 10 μ M NADH was added to inactivated enzyme.

on the basis of the protein concentration. When the enzyme was reduced with NADH, the absorption of the flavin disappeared and broad absorption above 600 nm increased slightly (Figure 5, curve B), suggesting the formation of the charge-transfer complex of the reduced enzyme with NAD^+ [30]. Excess of NADH did not cause any more lowering of absorbance at 540 nm, or in the 300–500 nm region. The reduced enzyme was rapidly reoxidized by AFR and the absorption of the flavin reappeared (Figure 5, curve C).

Effects of the amino acid residue-specific reagents

To delineate the active site of the enzyme, chemical-modification studies on some amino acids were attempted. The enzyme was incubated at the indicated concentrations of the modifying reagents for 10 min at 4 °C in the appropriate buffers. There was no effect on the activity of ascorbate oxidase during the assay. As shown in Table 5, CMB and phenyl mercuric acetate inhibited the enzyme activity more severely than other thiol-alkylating reagents, such as *N*-ethylmaleimide, iodoacetamide and 5,5'-dithiobis-(2-nitrobenzoic acid). The treatment of the enzyme with a 3-fold excess amount of CMB for 30 min caused the loss of charge-transfer absorbance (Figure 6, curve B) and blocked the electron transfer from NADH to FMN of the enzyme (Figure 6, curve C). The blockage of electron transfer in the thiol-group-modified enzyme has been observed also in cucumber reductase preincubated with CMB, although the charge-transfer interaction of the thiol group with the flavin has not been observed in the cucumber enzyme [7]. This demonstrates that the thiol group is involved in the electron transfer from NADH to the flavin and is thus essential for the catalytic activity of AFR reductase. In Figure 6, curve C, NADH could not reduce the FMN of CMB-modified enzyme; nevertheless, the broad ab-

Table 5 Effects of the reagents for the amino acid modification on the activity of AFR reductase

If not specified, 0.1 M Tris/HCl buffer, pH 7.4, was used for the reagents.

Reagent	Concentration (mM)	Relative activity (%)
None (control)	—	100
CMB*	0.01	50
CMB*	0.1	0
Phenyl mercuric acetate	0.1	15
5,5'-Dithiobis-(2-nitrobenzoic acid)*	1	77
<i>N</i> -Ethylmaleimide	1	18
Iodoacetamide	5	68
Pyridoxal 5'-phosphate†	1	54
Pyridoxal 5'-phosphate (pre-incubated with 1 mM NADH)†	1	82
2,4,6-Trinitrobenzenesulphonic acid†	1	46
Diacetyl	10	45
Diacetyl (pre-incubated with 1 mM NADH)	10	73
Phenyglyoxal†	100	54
Diethyl pyrocarbonate‡	0.1	38
Trinitromethane‡	10	89

* Prepared in 0.1 M Tris/HCl buffer, pH 9.0, and the reaction proceeded in 0.1 M Tris/HCl buffer, pH 7.4.

† Prepared in 0.1 M sodium bicarbonate buffer, pH 8.25, and the reaction proceeded in the same buffer.

‡ Stock solution of diethyl pyrocarbonate was prepared in absolute ethanol and the concentrations of the solutions were determined from the absorbance at 230 nm in 0.1 M imidazole buffer, pH 7.5 [31]. The reaction proceeded in 0.1 M sodium phosphate buffer, pH 6.4, and the final concentration of ethanol in the reaction mixture did not exceed 5%.

sorption above 600 nm also appeared, as in the case of CMB-modified cucumber enzyme [7]. The residual activity of the inactivated enzyme was below 5% of that of the active enzyme. The small increase of the broad absorption might have been due to the interaction of the residual active enzyme with NADH. Chemical-modification data indicate that the thiols of the enzyme are relatively unreactive towards thiol-alkylating reagents. It is noteworthy that the charge-transfer thiols of most of the disulphide reductases are also relatively unreactive towards alkyl-

ating reagents, compared with the interchange thiol. Such preferential modification of thiol by mercurials implies that the environment of the essential thiol is probably somewhat hydrophobic, since mercury itself possesses markedly hydrophobic properties [29].

Despite the severe inhibition of the activity by thiol reagents shown in all other AFR reductases reported, kinetic analyses of the modification process of thiol groups with AFR reductases have not yet been performed. Incubation of AFR reductase with the various concentrations of CMB resulted in a time- and concentration-dependent inactivation of the enzyme (Figure 7A). The inactivation process can be represented as:

where n equals the average order of the reaction with respect to CMB, and E-CMB_n is the modified enzyme. The inactivation of the enzyme can be described by the differential equation:

$$v = -d[E]/dt = k[E][\text{CMB}]^n$$

Under the experimental conditions of $[\text{CMB}] \gg [E]$, the reaction follows pseudo-first-order reaction kinetics:

$$v = k_{\text{obs}}[E]$$

with

$$k_{\text{obs}} = k[\text{CMB}]^n$$

Kinetics were measured under pseudo-first-order conditions and the pseudo-first-order rate constant k_{obs} was determined for each CMB concentration from plots of relative activity versus time, according to the integral equation $-\log(A/A_0) = k_{\text{obs}} \times t$ (where A_0 and A correspond to the initial enzymic activity and activity at time t , respectively) [31]. The rate constant k and the reaction order n with respect to CMB concentration were deduced from eqn. (1):

$$\log k_{\text{obs}} = \log k + n \log [\text{CMB}] \quad (1)$$

Figure 7 Modification of cysteine residues of AFR reductase with CMB

(A) Inactivation of AFR reductase. The enzyme was incubated with the various concentrations of CMB in 0.1 M Tris/HCl buffer, pH 7.4. The concentrations of CMB used were 20 (●), 30 (○), 40 (■), 50 (▽) and 60 μM (◆). (B) The order of the inactivation of the enzyme with respect to CMB (PCMB) concentrations. The slope of the line was 0.85, indicating that modification of a single molecule of cysteine is required to cause inactivation of AFR reductase. (C) The pH dependence of the inactivation rates. AFR reductase was incubated with 100 μM CMB at various pH values between 6.0 and 8.0. Pseudo-first-order rate constants were plotted versus pH. The solid line represents the theoretical curve fitted to eqn. (2).

Figure 8 Quenching of the fluorescence of NADH by AFR reductase

(A) Fluorescence emission spectrum of NADH was recorded in 0.1 M Tris/HCl buffer, pH 7.4 (curve a). Incubation of NADH with the native enzyme resulted in the quenching of the fluorescence of NADH (curve b). (B) Fluorescence of NADH was decreased by diacetyl-treated, arginine-modified AFR reductase (curve b). However, pyridoxal 5'-phosphate-treated lysine-modified enzyme could not quench the fluorescence of NADH (curve a). In (B), the fluorescence spectrum of NADH without the enzyme is identical with curve a in (A). The emission spectra were recorded at an excitation wavelength of 340 nm. The concentrations of NADH and the enzyme were 50 nM. The modified enzyme showed weak fluorescence by an excitation at 340 nm, which might have been due to the enzyme and reagents. Thus the fluorescence spectrum of the modified enzyme was recorded separately and subtracted from the spectrum of the mixture of the modified enzyme and NADH.

As shown in Figure 7(B), a double-logarithmic plot of k_{obs} against [CMB] gave a straight line with slope of 0.85, which is the reaction order with respect to CMB. This indicates that the kinetics are second order and that only 1 mol of CMB reacts with 1 mol of enzyme subunit to cause the inactivation of the enzyme. Therefore, one thiol group may be involved in the catalytic function.

The inactivation rates of AFR reductase by CMB were examined over the pH range 6–8 (Figure 7C). If it is assumed that the protonated residues were modified by CMB, then the following equation, from the Henderson–Hasselbach equation,

can be used to describe the dependence of the rate constant on pH:

$$k_{\text{obs}} = (k_{\text{obs}})_{\text{max}} / (1 + K_{\text{a}} / [\text{H}^+]) \quad (2)$$

where K_{a} is the dissociation constant of the reacting group and $(k_{\text{obs}})_{\text{max}}$ is the pseudo-first-order rate constant of the protonated reacting group. The experimental data were fitted theoretically to eqn. (2) and values of $(k_{\text{obs}})_{\text{max}} = 0.92 \text{ min}^{-1}$ and $\text{p}K_{\text{a}} = 6.9$ were estimated from the least-squares analysis. Taking into account this $\text{p}K_{\text{a}}$ value below that of a cysteine residue ($\text{p}K_{\text{a}}$ near 8.3), the thiol group is likely to be present in a thiolate anion at neutral pH, and then a hydrogen-bonding of the type $\text{S}^- \cdots \text{H}^+ \text{B}$ (where S^- is the thiolate anion, B is the proton donor) is required to stabilize the thiolate anion in the active site. Histidine residues have been established in lipoamide dehydrogenase [27], enterococcal NADH peroxidase [32] and papain [33] as logical candidates as proton donors in this hydrogen-bonding. The AFR reductase from *P. ostreatus* was inactivated rapidly upon incubation with diethyl pyrocarbonate (Table 5). Also, an increase of absorbance at 244 nm without change in absorbance at 278 nm was observed (results not shown), which indicates that the inactivation of *P. ostreatus* reductase with diethyl pyrocarbonate is caused by the *N*-carbethoxylation of histidine residues [31].

Besides cysteine and histidine residues, positively charged amino acid residues such as lysine and arginine deserve attention, since AFR and NADH are mainly present in anionic form, with $\text{p}K_{\text{a}}$ values of -0.45 [34] and 3.9 [35] respectively. Three lysine residues and one arginine around the putative NADH-binding domain, which was found in the sequence of the cucumber AFR reductase cDNA clone, may facilitate the interaction between the flavin and negatively charged substrates [36]. As summarized in Table 5, the inactivation of *P. ostreatus* reductase by the modification of lysine and arginine residues supports the above assumption. The pre-incubation of 1 mM NADH before modification of the enzyme resulted in substantial retention of the activity, showing the protective effect of NADH. Modification of tyrosine with trinitromethane had little effect (Table 5). To distinguish the roles of these two positively charged residues, the fluorescence quenching of NADH was examined. Incubation of NADH with active AFR reductase resulted in the decrease of fluorescence of NADH (Figure 8A). Arginine-modified enzyme, created by treating with diacetyl, was also capable of quenching the fluorescence of NADH. However, lysine-modified enzyme, made by treating with pyridoxal 5'-phosphate, was not (Figure 8B). This result shows that lysine residues can be responsible for the binding of NADH.

Conclusions

AFR reductase was suggested to be a key enzyme in maintaining the ascorbate system in the reduced state [2]. However, in spite of its expected physiological importance and ubiquity, its labile nature and low abundance have hindered the investigation of the enzyme, and a scarcity of spectroscopic and biochemical data has restricted the understanding of the active site and catalytic mechanism of AFR reductase. Here we have described the novel molecular properties of FMN-containing and dimeric AFR reductase purified from *P. ostreatus*. The features of prosthetic-group and subunit composition are peculiar to *P. ostreatus* AFR reductase and can be distinguished from the properties of AFR reductase known previously in plants and *N. crassa*. Another distinctive feature of *P. ostreatus* AFR reductase is related to the absorption spectrum. In contrast with the

absorption spectrum of flavoprotein shown in cucumber AFR reductase [7], the only absorption spectrum of AFR reductase to have been reported previously, the spectrum of *P. ostreatus* AFR reductase presents the characteristic absorption of a charge-transfer interaction of thiolate anion with FMN. Thus in view of the prosthetic-group and spectral characteristics, as well as the molecular mass and dimeric structure, *P. ostreatus* reductase can be regarded as a novel type of AFR reductase. According to the experimental results of the chemical modification using thiol-group-modifying reagents, the charge-transfer-interacting thiol of *P. ostreatus* AFR reductase seems to behave with some hydrophobic characteristics and be directly involved in the electron transfer from NADH to FMN. The thiol group is present as a thiolate anion, with a pK_a value of 6.9 at neutral pH, which was determined from the pH-dependence of the inactivation process of the enzyme with CMB. Although the thiol group is crucial to the function of reductases from both cucumber and *P. ostreatus*, differences between the absorption spectra of the two enzymes point to the variations of the microenvironment around the flavin in the active site of two enzymes. Instead, the charge-transfer interaction and low pK_a value of the thiol group in *P. ostreatus* AFR reductase show remarkable similarities to two-electron reduced forms of the pyridine nucleotide-disulphide reductases. Moreover, the homodimeric structure of *P. ostreatus* AFR reductase implies a closer structural relationship with the disulphide reductases rather than with cucumber AFR reductase. However, nothing can yet be concluded about this assumption and a combination of molecular-biological and biochemical approaches are underway to extract information about the environment of the flavin and the active site.

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