Electron transfer reactions in the alkene mono-oxygenase complex from *Nocardia corallina* B-276

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Nocardia corallina B-276 possesses a multi-component enzyme, alkene mono-oxygenase (AMO), that catalyses the stereoselective epoxidation of alkenes. The reductase component of this system has been shown by EPR and fluorescence spectroscopy to contain two prosthetic groups, an FAD centre and a [2Fe–2S] cluster. The role of these centres in the epoxidation reaction was determined by midpoint potential measurements and electron transfer kinetics. The order of potentials of the prosthetic groups of the reductase were $FAD/FAD^{\bullet} = -216$ mV, $[2Fe-2S]/[2Fe-2S]^{\bullet} = -160$ mV and $FAD^{\bullet}/FAD^{\bullet\bullet} = -134$ mV. Combined, these data implied that the reductase component supplied

the energy required for the epoxidation reaction and allowed a prediction of the mechanism of electron transfer within the AMO complex. The FAD moiety was reduced by bound NADH in a two-electron reaction. The electrons were then transported to the [2Fe–2S] centre one at a time, which in turn reduced the di-iron centre of the epoxidase. Reduction of the di-iron centre is required for oxygen binding and substrate oxidation.

Key words: chiral propylene oxide, methane mono-oxygenase, oxobridged protein, propylene, redox reactions.

INTRODUCTION

Alkene mono-oxygenase (AMO) from *Nocardia corallina* B-276 catalyses the stereoselective epoxidation of alkenes to give predominantly the *R* enantiomer [1]. The enzyme was purified previously and found to consist of three components: epoxidase, reductase and coupling protein [2]. The epoxidase component comprised a large and a small subunit (53 and 35 kDa respectively) and contained a bridged di-iron centre similar to that found in the hydroxylase component of methane mono-oxygenase and thought to be the site of epoxidation [1]. The reductase component was a 37 kDa NADH-dependent oxidoreductase that was believed to supply the energy required for the epoxidation reaction. The third component, the coupling protein, was a 14 kDa polypeptide and was essential for the epoxidation reaction.

AMO bears many biochemical similarities to soluble methane mono-oxygenase (sMMO) from methanotrophs; it consists of a terminal hydroxylase, an NADH-dependent oxidoreductase and a regulatory component, protein B. The hydroxylase component has been extensively characterized [3–9] and contains the active site for the hydroxylation of alkanes and the epoxidation of alkenes. The reductase component of sMMO provides reducing power to the hydroxylase component [10]. It also seems to modulate the redox potential of the hydroxylase by making it more positive and therefore stimulating its reduction to the Fe^{II} – Fe^{II} state by NADH [11]. Furthermore, reduction of the hydroxylase binuclear iron centre to the Fe^{II} – Fe^{II} state is required for the binding of molecular oxygen; protein B of sMMO might be responsible for controlling this [12]. One of these oxygen atoms is then inserted into the substrate, resulting in the conversion of alkanes to alcohols and alkenes to epoxides [13]. A similar mechanism is believed to be involved in the hydroxylation and epoxidation reactions catalysed by alkane ω -hydroxylase from *Pseudomonas oleovorans*, for which the reducing equivalents were supplied by rubredoxin reductase [14].

Saeki and Furuhashi [15] observed that the deduced amino acid sequence of the reductase component of AMO was similar

(extent of similarity not reported) to that of the reductase component of sMMO; they concluded that the AMO reductase may have a similar role in the AMO-catalysed epoxidation reaction to that predicted for the reductase component of sMMO. The reductase component of AMO was originally purified by Miura and Dalton [2], who suggested that it contained an FAD prosthetic group and a possible [2Fe–2S] cluster on the basis of the absorption spectrum of the reductase and the direct chemical detection of iron, acid-labile sulphur and free FAD. Here we present evidence for the nature of the prosthetic groups of the reductase and show how they might be involved in electron transfer within the AMO complex.

MATERIALS AND METHODS

Materials

All chemicals used were of analytical grade and were obtained from commercially available sources.

Growth of *N. corallina* B-276 and preparation of soluble extract

Cultures were maintained on a minimal salts medium supplemented with 20% (v/v) propene, as described previously [2].

Preparation of inoculum

Cells were grown in a 2-litre shake-flask containing 750 ml of supplemented minimal salts medium containing 3.6 g/l $Na_2HPO_4 \cdot 12H_2O$, 1.25 g/l $MgSO_4 \cdot 7H_2O$, 50 mg/l $FeSO_4 \cdot 7H_2O$, and 20 g/l yeast extract. A large loopful of cells grown as described above were used to inoculate the culture. Glucose was added to 1% (w/v) and the culture incubated at 30 °C for 16 h.

Batch fermentation in 20-litre batches

Cells were cultivated in a 20-litre jar-fermenter (MBR) containing 17.5 litre of supplemented minimal salts medium, with 1%

Abbreviations used: AMO, alkene mono-oxygenase; SHE, standard hydrogen electrode; sMMO, soluble methane mono-oxygenase.

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glucose (w/v) as carbon source and 5 ml of propylene glycol as anti-foaming agent. The fermenter was stirred at 600 rev./min, and the starting pH value was 7.2. Growth was initiated by seeding with 750 ml of shake-flask culture grown for 16 h as above.

After 40–48 h of cultivation, cells were harvested by continuous centrifugation at 3000 *g*, washed with Mops buffer, pH 7.5, drop-frozen in liquid nitrogen and stored at -70°C . Cells stored under these conditions maintained propene epoxxygenation activity over a minimum period of 18 months.

Preparation of soluble extract

Soluble extract was obtained as follows: thawed whole-cell paste was centrifuged at 10000 *g* for 10 min and then re-suspended in 25 mM Mops buffer, pH 7.5, containing 1 mM benzamidine, 10 mM MgSO_4 , 1 mM D,L-dithiothreitol and 5% (v/v) glycerol, with the addition of a few crystals of deoxyribonuclease I (Sigma). This cell suspension was passed three times through a high-pressure cell disrupter (Constant Systems, Kenilworth, Warwickshire, U.K.) at 172 MPa and the resultant mixture was centrifuged at 48000 *g* for 50 min. The pellet of cell debris was discarded and the supernatant was used as the soluble extract. Soluble extract could be frozen in liquid nitrogen and stored at -70°C for a minimum of 12 months without loss of epoxxygenation activity.

Purification of AMO components

The purification of the AMO components was performed at 4°C as described previously [2].

AMO assay

AMO activity was followed by the production of propene oxide from propene by GLC of gas-phase samples as described previously [2]. For whole-cell and protein preparations, 0.45 ml of sample was placed in a 7 ml Suba-sealed conical flask; 3 ml of propene was injected and the sample was incubated at 30°C for 1 min to equilibrate. The reaction was initiated by the addition of 50 μl of 10% (w/v) glucose (for whole-cell reactions) or 25 μl of 100 mM ethanol-free NADH (for protein preparations). The production of propene oxide was determined after a further 10 min of incubation at the same temperature by removal of 0.5 ml of gas from the head space of the flask and analysing it by GLC at 180°C on a glass column (2 m) packed with Porapak Q (Phase Separations). For the calibration, 0.5 ml of a 2 mM solution of propene oxide was incubated in a 7 ml sealed conical flask at 30°C for 10 min; 0.5 ml of the gas from the head space was then subjected to GLC on the same apparatus. One unit of AMO activity was defined as the amount that formed 1 nmol of propene oxide/min at 30°C .

NADH oxidase activity

NADH oxidase activity was followed by the oxidation of ethanol-free NADH monitored at 340 nm, with all samples dissolved in buffer B. Specific NADH oxidase activity was determined from the molar absorption coefficient of NADH (ϵ_{340} 6.22 $\text{mM}^{-1}\cdot\text{cm}^{-1}$). One unit of NADH oxidase activity is defined as the amount that oxidized 1 nmol of NADH/min at 30°C .

Reduction of the reductase component for EPR measurements

Ethanol-free aqueous NADH (100 mM) or sodium dithionite (1 mM) was added to the reductase (125 μM , total volume

200 μl) under anaerobic conditions. The protein was incubated for 2 min and frozen slowly in liquid nitrogen.

Preparation of the reductase for midpoint potential measurements

The reductase (100 μM) was titrated with sodium dithionite or potassium ferricyanide in the presence of each of the following mediator dyes at 10 μM : potassium ferricyanide, phenazine methosulphate, Methylene Blue, indigodisulphonate, indigo-trisulphate, anthraquinone 1,5-disulphonate, Safranin T, Benzyl Viologen and Methyl Viologen. The proportion of reduced $[2\text{Fe}-2\text{S}]$ species was monitored by EPR at $g = 1.97$.

Redox potentials were monitored by using a combined platinum and calomel reference electrode (Russell), as described previously [16]. The electrode was set up in an oxygen-impermeable vessel that was made anaerobic by being flushed continuously with argon. Samples and titrant solutions were added with a syringe through a small hole, which also acted as the outlet for the argon. At least 3 min was allowed for equilibration after each change in ambient redox potential on the addition of reductant or oxidant, before the EPR spectra were scanned.

X-band EPR spectroscopy

The reductase was analysed on a Bruker ER200D EPR spectrometer with an Oxford Instruments ESR-900 helium-flow cryostat. The reductase for midpoint potential measurements was analysed on a Bruker ESP300 spectrometer with an Oxford Instruments ESR-900 helium-flow cryostat.

UV absorption spectroscopic determination of the midpoint potential of the FAD centre of the AMO reductase

The midpoint potential for the reduction of the FAD centre of the reductase (28.5 μM) was determined by following the absorbance changes on reduction of the FAD centre after the addition of NADH in the presence of the mediator dye phenosafranin (10 μM). The protein was monitored at 365 nm, at which wavelength there is an isosbestic point for reduction; the flavin semiquinone species was monitored at 582 nm and phenosafranin was monitored at 520 nm. Potentials were calculated from the Nernst equation, as described previously [17].

Chemical removal and reconstitution of the prosthetic groups of the reductase component

Reductase (2 μM) in buffer B was depleted of the iron-sulphur centre by titration with sodium mersalyl, as described previously for the sMMO reductase [10]. Sodium mersalyl was added to the reductase and the protein was desalted by ultrafiltration by using a membrane with a molecular mass cut-off of 30 kDa in a 50 ml stirred cell (Amicon); 2-mercaptoethanol (300 nmol) was added and the protein was similarly desalted again. The iron-sulphur centre could be restored by the addition of 2-mercaptoethanol before the initial desalting step.

The reductase (2 μM) in buffer B was depleted of both the iron-sulphur centre and the flavin by incubation with 8 M urea for 20 min followed at room temperature by desalting by ultrafiltration as described above. The flavin could be reconstituted by the addition of FAD (1 mM).

Other methods

Estimation of total protein concentration

Total protein concentration was estimated colorimetrically by the method of Bradford [18], with BSA as the standard.

Determination of iron content of protein samples

The iron content of the epoxigenase and reductase components was determined by the method described previously [2], with slight modifications as follows. Trifluoroacetic acid (50 μ l) was added to the protein (450 μ l), incubated for 10 min at room temperature and the mixture was centrifuged at 14000 *g* for 5 min. A 250 μ l sample of the supernatant was transferred to a 0.4 ml quartz cuvette containing 200 μ l of satd. sodium acetate, 45 μ l of 20% (v/v) acetic acid and 5 μ l of 50 mM bathophenanthroline sulphonate. The iron content of the protein was estimated from the molar absorption coefficient of an iron standard at 535 nm (ϵ_{535} 22 mM⁻¹·cm⁻¹).

Acid-labile sulphide determination

Acid-labile sulphide was determined by using the method described previously [19]. Protein solution (100 μ l) was added to 300 μ l of zinc acetate (1%, w/v) in an Eppendorf tube. NaOH (15 μ l; 12%, w/v) was added; the mixture was inverted several times and then incubated at room temperature for 2 h. *N,N'*-dimethyl-*p*-phenylenediamine monohydrochloride (75 μ l; 0.1%, w/v) dissolved in 5 M HCl was added and the solution was mixed gently. FeCl₃ (10 μ l; 23 mM) was added and the mixture was inverted several times. After centrifugation at 14000 *g* for 15 min and incubation of the supernatant at 30 °C for 15 min the absorbance at 670 nm was measured. Acid-labile sulphide content was determined from the molar absorption coefficient of Na₂S (ϵ_{670} 34.5 mM⁻¹·cm⁻¹).

Estimation of protein purity

The purity of the purified protein samples was determined by SDS/PAGE with the method of Laemmli [20]. The protein-containing samples (20 μ l) were boiled for 5 min in Tris/HCl, pH 6.8 (9.7 g/l), supplemented with SDS (2%, w/v), dithiothreitol (15.44 g/l), glycerol (10%, v/v) and Bromophenol Blue (1 g/l). The treated protein was loaded on a homogeneous 15% (w/v) polyacrylamide gel with a 4% (w/v) stacking gel. A discontinuous buffer system was used containing 0.375 M Tris/HCl (pH 8.8) resolving gel buffer, 0.125 M Tris/HCl (pH 6.8) stacking gel buffer and 25 mM Tris/glycine (pH 8.3) reservoir buffer. All buffers were supplemented with 0.1% SDS. Gels were stained with methanol/acetic acid/water (3:1:6, by vol.) containing 0.1% Coomassie Brilliant Blue, then destained with methanol/acetic acid/water (4:1:5, by vol.) [20].

RESULTS

The UV-visible and EPR spectra of a flavoprotein can be used to distinguish between FAD and FMN and the type of iron-sulphur clusters present. Examination of the UV-visible spectrum of the AMO reductase (Figure 1) showed that in the oxidized form, absorbance maxima occur at 350, 388, 425 and 454 nm, with a shoulder at 335 nm. These peaks are indicative of the presence of similar redox centres to those found in the reductase of sMMO [10]. The addition of NADH (or sodium dithionite) to a preparation of purified reductase resulted in a decrease of the absorption spectrum over the range 350–520 nm. At a point midway through this reduction (i.e. after the addition of 20 μ M NADH to 25 μ M reductase (Figure 1, spectrum 3 with the dotted line) a band at 580 nm was produced, which is indicative of the neutral semiquinone form of an FAD centre [10]. With the addition of further NADH the reduction proceeded to the fully reduced spectrum (Figure 1, spectrum 4 with the dot-dashed line). An FAD prosthetic group can exist in three redox active states: the fully oxidized form, which is diamagnetic and hence

Figure 1 Absorbance spectra showing anaerobic titration of the AMO reductase with NADH

The reductase (25 μ M) in buffer B was placed in a 500 μ l quartz cuvette (10 mm pathlength) and reduced with NADH under anaerobic conditions at room temperature as described in the Materials and methods section. The curves show AMO reductase plus 0 μ M (—), 10 μ M (---), 20 μ M (·····) and 40 μ M (-·-·-) NADH respectively.

EPR silent; the semiquinone (or partly reduced) form, which is paramagnetic and has a characteristic EPR spectrum ($g = 2.0$ in sMMO reductase); and the hydroquinone (or fully reduced) form, which is diamagnetic and EPR silent. A [2Fe-2S] cluster can exist in only two redox active forms: the oxidized form, which is diamagnetic and hence EPR silent, and the reduced form, which has a characteristic EPR spectrum ($g_{av} = 1.94$ in sMMO reductase [10]).

The oxidized form of the AMO reductase showed essentially no EPR signal, except for a weak signal at $g = 2$ corresponding to a free radical (Figure 2a).

Titration of the reductase with NADH resulted in the formation of a characteristic EPR signal corresponding to the semiquinone form of the FAD prosthetic group and the reduced form of the [2Fe-2S] centre (Figure 2b). The signal at $g = 2$ is typical of the semiquinone form of an FAD centre. The signal at $g_{av} = 1.96$ ($g_x = 2.05$, $g_y = 1.95$ and $g_z = 1.86$) was similar to the signal attributed to the reduced form of the [2Fe-2S] cluster of the reductase component of sMMO from *Methylobacterium* CRL26 [21] and from *M. capsulatus* (Bath) [10] and is typical of a [2Fe-2S] cluster.

On full reduction of the reductase with excess sodium dithionite, the signal at $g = 2$ decreased, suggesting that the FAD prosthetic group was now in the hydroquinone (reduced) form. Simultaneously, the signal at $g_{av} = 1.96$, corresponding to the reduced [2Fe-2S] centre, increased approx. 10-fold (Figure 2d).

These results provide further experimental evidence that the AMO reductase does indeed contain a redox-active FAD group and a [2Fe-2S] centre and is therefore similar in its complement of prosthetic groups to the reductase component of sMMO with which it is homologous [10,21].

Oxidation of the reductase by addition of the epoxigenase

The first direct indication that electron transfer occurs between the reductase and the epoxigenase components was observed during EPR spectroscopic measurements of the reductase component. Under anaerobic conditions the signal observed for the partly reduced form of the reductase did not change appreciably over time (approx. 3 h). With the addition of a small amount of the epoxigenase component, however, the EPR signal decreased, indicating that the reductase was oxidized by the addition of the epoxigenase (Figure 2c).

Addition of the epoxigenase to the reductase in sub-stoichiometric amounts resulted in a decrease in the signal cor-

Figure 2 EPR spectra of the reductase

(a) Reductase (125 μM) was placed in a 3.0 mm EPR tube, frozen slowly in liquid nitrogen and used as prepared. (b) NADH (40 μM) was added to the reductase (125 μM) in a 3.0 mm EPR tube, incubated under anaerobic conditions for 2 min and frozen slowly in liquid nitrogen. (c) The sample from (b) was thawed and epoxygenase (25 μM) in buffer B was added under anaerobic conditions and re-frozen immediately before the sample was analysed by EPR. (d) Sodium dithionite (1 mM) was added to the reductase (125 μM) under anaerobic conditions. The spectra were recorded under the following conditions: temperature 19 K; microwave power 1 mW; microwave frequency 9.63 GHz; modulation amplitude 0.94 mT; signal gain 2×10^4 . Conditions for sample preparation and use of instrumentation were as given in the Materials and methods section. The signal corresponding to the semiquinone form of the FAD centre is at $g = 2$ and the remaining signals centred at $g = 1.96$ correspond to the reduced [2Fe-2S] centre.

responding to the semiquinone form of FAD by approx. 25% and a decrease in the signal corresponding to the reduced [2Fe-2S] cluster by approx. 50%. The oxidation of the reductase clearly shows that the epoxygenase must act as the electron acceptor and therefore that electron transfer takes place from the reductase component to the epoxygenase. Because the [2Fe-2S] centre is reduced approx. 2-fold more than the FAD centre, this implies that it might be the [2Fe-2S] centre that interacts directly with the epoxygenase component. The addition of oxygen to the EPR tube as a control also resulted in a decrease in the EPR signal corresponding to the semiquinone form of FAD and the reduced [2Fe-2S] centre (results not shown).

Role of the reductase prosthetic groups

By analogy with the work performed on the role of the prosthetic groups of the reductase component of sMMO from *M. capsulatus* (Bath) and the mechanism of electron transfer to the hydroxylase component [10,19], an investigation into the mechanism of electron transfer between the reductase and epoxygenase components of AMO was performed. The rate of electron transfer could be monitored by following the decrease in A_{340} due to the

oxidation of NADH. Selective removal of the reductase prosthetic groups allowed the determination of the role of each prosthetic group in the electron transfer reaction.

Removal and reconstitution of the prosthetic groups

Treatment of the reductase component of the sMMO with sodium mersalyl resulted in the removal of the [2Fe-2S] cluster by the covalent modification of cysteine residues and sulphide [10]. The removal of the [2Fe-2S] cluster could be monitored conveniently by following the changes in absorbance over the range 300–600 nm.

The reductase component of AMO was titrated with sodium mersalyl and the resultant absorbance changes were monitored as shown in Figure 3.

Titration of the reductase with sodium mersalyl resulted in a decrease in absorbance in the range 320–600 nm, with the accompanying bleaching of the protein from brown to a pale yellow. After the addition of 21 equiv. (600 μM) of sodium mersalyl, no further decrease in absorbance occurred. After the protein was desalted to remove excess reagent, the iron content was found to be 0.2 mol of Fe/mol of reductase, implying that much of the [2Fe-2S] centre had been removed. After the addition of 300 μM sodium mersalyl (10 equiv.) there was a shift of absorbance from 390 nm, which is predominantly due to the FAD, to 360 nm, which suggested that the environment of the FAD centre was perturbed and therefore that full modification of the ligands to the [2Fe-2S] centre had occurred.

From the changes in absorbance after removal of the [2Fe-2S] centre the contributions to absorbance from the FAD prosthetic group at 385 and 465 nm were determined as approx. 60%, similar to the contribution (65%) made by the FAD group of the sMMO reductase at 465 nm [10].

The addition of Fe^{II} and Na_2S to the [2Fe-2S]-depleted protein did not result in the reconstitution of the [2Fe-2S] centre as found for the sMMO reductase [17]. The [2Fe-2S] centre could be more successfully reconstituted from reductase that had been treated with sodium mersalyl (up to 90%) by the addition of 2-mercaptoethanol before the desalting step. 2-Mercaptoethanol reduces mersalyl sulphide and iron released from the protein and hence reconstitutes the [2Fe-2S] centre.

Treatment of the reductase with 8 M urea for 2 h at room temperature followed by desalting three times [addition of fresh buffer B and concentration by ultrafiltration using a membrane with a molecular mass cut-off of 30 kDa in a 50 ml stirred cell (Amicon)] resulted in the removal of both the FAD and [2Fe-2S] centres as judged by absorption measurements and chemical quantification (see the Materials and methods section). When the apoprotein was desalted as above and then FAD (1 mM) was added, 98% reconstitution of the FAD centre was observed, resulting in the formation of reductase lacking only the [2Fe-2S] centre.

With these techniques it was possible to produce reductase that was depleted of either the [2Fe-2S] centre or the FAD group so that the role of the prosthetic groups could be analysed separately.

Electron transfer rates from the reductase to the epoxygenase

Electron transfer from the reductase to the epoxygenase was monitored by following the decrease in absorbance at 340 nm caused by the oxidation of NADH. Removal of the [2Fe-2S] centre by treatment of the reductase with sodium mersalyl followed by desalting and treatment with 2-mercaptoethanol (to reactivate the cysteine residues) resulted in the loss of the epoxygenation activity but some NADH oxidase activity

Figure 3 Titration of the reductase with sodium mersalyl

Reductase (28.5 μM) in buffer B (0.5 ml) was titrated at room temperature with sodium mersalyl: trace 1, 0 μM ; trace 2, 80 μM ; trace 3, 160 μM ; trace 4, 300 μM ; trace 5, 500 μM ; trace 6, 600 μM ; trace 7, 1.4 mM. The spectra were corrected for volume changes and contributions from the sodium mersalyl.

Table 1 Role of the prosthetic groups of the reductase in NADH oxidation and epoxygenation

For both assays the epoxygenase (5 μM) and coupling protein (5 μM) were added to the reductase (2 μM) in buffer B. The mixture was incubated at 30 °C for 1 min to equilibrate and the assay was initiated by the addition of NADH (25 μM). Reduction of NADH and the formation of propene oxide were determined after a further incubation for 5 min at the same temperature, as described in the Materials and methods section. NADH oxidase activity was determined by following A_{340} . Results are the means for three experiments for each reaction condition (S.D. 10%).

Reductase form	NADH oxidase activity (%)	Epoxygenation activity (%)
Native	100	100
[2Fe-2S] centre removed	56.8	0
[2Fe-2S] centre reconstituted	74.5	60.5
FAD and [2Fe-2S] centres removed	0	0
FAD centre reconstituted	65.6	0

remained (Table 1). The remaining NADH oxidase activity in this preparation was not as high as that of the untreated protein because the reductase was very unstable (half-life 4–5 h) and sensitive to salts (for example, incubation with 2 mM CuSO_4 resulted in the loss of all AMO activity [22]). The [2Fe–2S] centre and the epoxygenation activity could be restored to a certain extent if 2-mercaptoethanol was added before the sodium mersalyl-treated reductase was desalted (Table 1). 2-Mercaptoethanol reconstitutes the [2Fe–2S] centre from mersalyl sulphide and iron. If the sodium mersalyl-treated protein is desalted before the addition of 2-mercaptoethanol it cannot restore the [2Fe–2S] centre because mersalyl sulphide and iron have then been removed [17].

Removal of both the FAD and [2Fe–2S] centres by treatment with 8 M urea resulted in the loss of the epoxygenation and NADH oxidase activities. The NADH oxidase activity of this preparation could be restored to some extent (65.6%) by the removal of urea by desalting with the method of ultrafiltration [membrane with molecular mass cut-off of 30 kDa in a 10 ml stirred cell (Amicon)] followed by the addition of 1 mM FAD (Table 1).

Taken together, these results imply that the [2Fe–2S] centre is responsible for interaction with the epoxygenase component in

the epoxygenation reaction. The [2Fe–2S] centre is not, however, required for the oxidation of NADH. The FAD prosthetic group seems to be responsible for the oxidation of NADH and then the supply of electrons required for the reduction of the [2Fe–2S] centre, because in the absence of the FAD centre no epoxygenation activity was observed. On the basis of these initial observations the flow of electrons in the AMO complex was predicted as:

This order is consistent with the observations of the flow of electrons in the sMMO complex [17].

Midpoint potential measurements

Measurement of the midpoint potentials for reduction of the reductase was used to give an indication of the possible sequence of electron transfer between the prosthetic groups within and between the components of the complex.

FAD centre

The midpoint potentials for the two redox couples (FAD/FAD $^{\bullet}$ and FAD $^{\bullet}$ /FAD $^{2-}$) of the FAD group of the AMO reductase were determined by following the absorbance changes on reduction of the reductase with NADH in the presence of the mediator dye phenosafranin. The oxidized form of phenosafranin absorbs at 520 nm and its reduction can be monitored by following the decrease in absorbance at this wavelength (Figure 4).

Determination of the midpoint potential involved the measurement of the change in absorbance of phenosafranin (at 520 nm) at the point of the maximal semiquinone form of FAD determined at 582 nm. The absorbance was also monitored at 365 nm, where in the presence of the dye there is an isosbestic point for reduction.

Measurement of the midpoint potential for the two-electron reduction of FAD was then calculated from the Nernst equation, which gave the value of the two-electron reduction of the FAD centre of the AMO reductase as -175 mV [against standard hydrogen electrode (SHE)] and the midpoint potentials for the individual couples (FAD/FAD $^{\bullet}$ and FAD $^{\bullet}$ /FAD $^{2-}$) as -216 and -134 mV (against SHE) respectively.

Figure 4 Titration of the reductase with NADH in the presence of phenosafranin

The reductase (25 μM) was titrated with NADH in buffer B at room temperature in the presence of phenosafranin (10 μM). Trace a, oxidized form of the reductase; trace b, semi-reduced form of the reductase (after the addition of 12.5 μM NADH); trace c, reduced form of the reductase (after the addition of 100 μM NADH).

Figure 5 Plot of the proportion of reduced [2Fe-2S] against the redox potential

The reductase (100 μM) was titrated with sodium dithionite or potassium ferricyanide under anaerobic conditions in the presence of mediator dyes, as described in the Materials and methods section. Reduction of the [2Fe-2S] centre was monitored by EPR at $g = 1.97$ under the following conditions: temperature 10 K; microwave power 1 mW; microwave frequency 9.42 GHz; modulation 1 mT; signal gain 2×10^4 . Redox potentials were monitored with a combined platinum and calomel reference electrode; results are from two separate experiments (□, ▲). The arrow indicates the calculated midpoint potential for reduction of the iron-sulphur centre from the data.

[2Fe-2S] centre

The midpoint potential for the reduction of the [2Fe-2S] centre was determined by measurement of the EPR signal at $g = 1.97$, corresponding to the reduced form at different potentials, with a calomel electrode as reference in the presence of mediator dyes (see the Materials and methods section). The reductase was titrated with sodium dithionite for reduction and potassium ferricyanide for oxidation. Figure 5 shows a plot of the proportion of reduced [2Fe-2S] centre against redox potential (against SHE).

From Figure 5 the midpoint potential for the [2Fe-2S]/[2Fe-2S] $^{\cdot-}$ couple was calculated from the EPR measurements to be -160 mV (against SHE).

The potentials for the reductase prosthetic groups are thus:

DISCUSSION

On the basis of the midpoint potentials for the various couples measured here we propose the following model (Scheme 1). The addition of one molecule of NADH results in the reduction of

Scheme 1 Proposed mechanism of electron transfer from the reductase to the epoxigenase

Symbols: ●, electron; $2e^-$, NADH. The cycle represents the proposed steps involved in the transfer of electrons from NADH to the di-iron centre of the epoxigenase via the reductase prosthetic groups. The FAD and [2Fe-2S] centres are represented by the left and right halves of an oval as shown. See the text for further explanation. Adapted from [17] and [25].

the FAD prosthetic group in a two-electron step (by hydride transfer) to give the fully reduced form. The reductase then acts as a two-/one-electron transformase by transferring one electron from the FAD centre to the [2Fe-2S] centre, resulting in the formation of the reduced form of the [2Fe-2S] centre and the semiquinone form of the flavin. The reduced [2Fe-2S] centre then transfers an electron to the binuclear iron centre of the epoxigenase, resulting in the formation of the mixed-valence form of the epoxigenase and the one-electron reduced form of the reductase. The remaining electron in the FAD centre is then transferred to the [2Fe-2S] centre again, resulting in the reduction of the [2Fe-2S] centre. From this stage there are two possible routes to the full reduction of the epoxigenase binuclear iron centre.

1. The electron from the [2Fe-2S] centre could be transferred to the epoxigenase giving the fully reduced epoxigenase, which can now bind molecular oxygen (Scheme 1, left). Regeneration of the fully oxidized form of the reductase then occurs and the cycle continues.

2. Alternatively, the one-electron-reduced reductase could be further reduced by another molecule of NADH in a two-electron step to give the three-electron-reduced reductase (Scheme 1, right). The electron in the [2Fe-2S] centre would then be transferred to the epoxigenase, giving the fully reduced form of the binuclear iron centre. Regeneration of the two-electron reduced reductase would also occur and the cycle would then continue. Because the reductase is not fully oxidized in this scheme, the stoichiometry of the electron transfer is the same as that shown at the left of Scheme 1.

Overall, whichever route is used, two electrons from one molecule of NADH result in the full reduction of the epoxigenase binuclear iron centre.

In the sMMO system, the reductase component not only supplies the electrons required for the reduction but, as described earlier, might alter the redox potential of the hydroxylase di-iron centre, enhancing electron transfer [11]. In addition to the role proposed here, it is possible that the AMO reductase functions in a similar manner by interaction with the epoxigenase component.

As in the sMMO system, reduction of the epoxigenase presumably allows the binding of molecular oxygen, one atom of which is then inserted into the substrate and the other is reduced to water [21,23,24]. In sMMO, protein B is thought to alter the redox potential of the binuclear iron centre (to a more negative value) so that interaction with molecular oxygen is more favourable [12].

Characterization of the prosthetic groups within the AMO complex has demonstrated that although AMO and sMMO catalyse similar reactions, the mechanism of epoxigenation and precise roles of the components of the two mono-oxygenase enzymes differ. This difference might reflect the inability of AMO to catalyse hydroxylation reactions. The ease of reduction of the

AMO reductase might, for example, result in more efficient reduction of the epoxigenase compared with the same reaction in sMMO. Because epoxigenation does not require anywhere near as strong an electrophile as hydroxylation (average C-H bond energy 435 kJ/mol; average C-C bond energy of a double bond 305 kJ/mol), more efficient reduction of the epoxigenase could prevent the production of damaging peroxy species while allowing the appropriate reduction of the epoxigenase required for the binding of molecular oxygen.

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