ELSEVIER

Biochimica et Biophysica Acta 1459 (2000) 106–116

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

www.elsevier.com/locate/bba

# One-electron reduction of quinones by the neuronal nitric-oxide synthase reductase domain

Hiroyuki Matsuda, Shigenobu Kimura, Takashi Iyanagi *

*Department of Life Science, Faculty of Science, Himeji, Institute of Technology, Harima Science Garden City, Hyogo 678-1297, Japan*

Received 26 October 1999; received in revised form 16 March 2000; accepted 22 March 2000

**Abstract**

Flavin electron transferases can catalyze one- or two-electron reduction of quinones including bioreductive antitumor quinones. The recombinant neuronal nitric oxide synthase (nNOS) reductase domain, which contains the FAD-FMN prosthetic group pair and calmodulin-binding site, catalyzed aerobic NADPH-oxidation in the presence of the model quinone compound menadione (MD), including antitumor mitomycin C (Mit C) and adriamycin (Adr). Calcium/calmodulin ($Ca^{2+}$/CaM) stimulated the NADPH oxidation of these quinones. The MD-mediated NADPH oxidation was inhibited in the presence of NAD(P)H:quinone oxidoreductase (QR), but Mit C- and Adr-mediated NADPH oxidations were not. In anaerobic conditions, cytochrome b5 as a scavenger for the menasemiquinone radical ($MD^{\bullet -}$) was stoichiometrically reduced by the nNOS reductase domain in the presence of MD, but not of QR. These results indicate that the nNOS reductase domain can catalyze a only one-electron reduction of bivalent quinones. In the presence or absence of $Ca^{2+}$/CaM, the semiquinone radical species were major intermediates observed during the oxidation of the reduced enzyme by MD, but the fully reduced flavin species did not significantly accumulate under these conditions. Air-stable semiquinone did not react rapidly with MD, but the fully reduced species of both flavins, FAD and FMN, could donate one electron to MD. The intramolecular electron transfer between the two flavins is the rate-limiting step in the catalytic cycle [H. Matsuda, T. Iyanagi, Biochim. Biophys. Acta 1473 (1999) 345–355). These data suggest that the enzyme functions between the $1e^- \rightleftarrows 3e^-$ level during one-electron reduction of MD, and that the rates of quinone reductions are stimulated by a rapid electron exchange between the two flavins in the presence of $Ca^{2+}$/CaM. 

*Keywords:* Neuronal nitric oxide synthase reductase domain; P450 reductase; One-electron reduction of quinone; Mitomycin C; Adriamycin; Menadione

## 1. Introduction

Quinones (Qs) including antitumor Qs undergo facile reduction and oxidation. A one-electron reduction of a Q gives the semiquinone radicals while a two-electron reduction gives hydroquinone. In 1969 and 1970, Iyanagi and Yamazaki [1,2] reported that the reduction of Qs and oxygen by flavin enzymes falls into three mechanistic categories: one-electron, two-electron and mixed-type reactions. NADPH-cy-

Abbreviations: NOS, nitric oxide synthase; nNOS, neuronal NOS; P450 reductase, NADPH-cytochrome P450 reductase; QR, NAD(P)H:quinone oxidoreductase; CaM, $Ca^{2+}$-dependent calmodulin; MD, menadione (2-methyl-,1,4-naphthoquinone); $MD^{\bullet -}$, anionic semiquinone of MD; Adr, adriamycin; Mit C, mitomycin C; FAD and FMN are the oxidized forms, FADH• and FMNH• are the one electron(semiquinone)-reduced forms, and $FADH_2$ and $FMNH_2$ are the fully reduced forms of the two flavins; EPR, electron paramagnetic resonance

* Corresponding author. Fax: +81-791-58-0132;
E-mail: iyanagi@sci.himeji-tech.ac.jp

0005-2728/00/$ – see front matter 
PII: S0005-2728(00)00117-1

tochrome P450 reductase (P450 reductase) catalyzes the one-electron reduction of Qs, including antitumor Qs. On the other hand, NAD(P)H: Q oxidoreductase (QR) catalyzes the obligatory two-electron reduction of Q to hydroquinone [2–5].

Neuronal nitric oxide synthase (nNOS) consists of a C-terminal reductase domain and a N-terminal oxygenase domain. The C-terminal reductase domain contains binding sites for $Ca^{2+}$-dependent calmodulin (CaM), FAD, FMN, and NADPH [6,7] and has redox properties similar to those of P450 reductase [8–12]. This domain can mediate transfer of electrons from NADPH to the cytochrome P450-like heme domain [13–15]. This process is facilitated by the binding of calcium/calmodulin ($Ca^{2+}$/CaM) to the CaM-binding site located near the center of the enzyme [6,7]. The electron transfer from the reductase domain to the heme domain is thought to be stimulated by $Ca^{2+}$/CaM [13,16]. The isoenzymes of NOS can catalyze the reduction of several exogenous electron acceptors, such as cytochrome *c*, ferricyanide, and molecular oxygen [17–19]. The endothelial NOS (eNOS) [20] and its reductase domain [21] can catalyze the one-electron reduction of adriamycin (Adr). nNOS can also catalyze the one-electron reduction of adrenochrome [22] and naphthoquinones [21,23]. These reactions are thought to occur at the reductase domain of the enzyme. However, the role of a FAD-FMN pair in the three NOS isoenzymes in the reaction with exogenous electron acceptors including Q and ferricyanide is not fully understood.

P450 reductase, which contains FAD and FMN in equimolar amounts [8,9], can catalyze a typical one-electron reduction of bivalent exogenous Qs [1–5,23,24]. The one-electron reduction potential of Qs is related to their reduction rates by P450 reductase [25–27]. If the one-electron redox potential of a Q ($Q/Q^{\bullet-}$) is below −155 mV, which is the one-electron redox potential for $O_2/O_2^{\bullet-}$ at pH 7.0 on the molar basis, the semiquinone radical can transfer the electron to molecular oxygen ($O_2$) resulting in the formation of superoxide anion radical ($O_2^{\bullet-}$) [1–4,20,24,28]. Each flavin of the reductase has an individual function, that is, FAD accepts two reducing equivalents from NADPH (dehydrogenase flavin) and FMN acts as a one-electron carrier (flavodoxin-type flavin) [8–10]. The fully reduced FMN is the most reactive species in the one-electron reduction of Qs, and a $1e^- \rightleftarrows 3e^-$ catalytic cycle has been proposed [9,29].

In the present study, we examined whether the recombinant nNOS reductase domain, which contains an FAD/FMN prosthetic group pair and a CaM-binding site (amino acid residues 718–1429) could catalyze the one-electron reduction of menadione (2-methyl-,1,4-naphthoquinone) (MD), mitomycin C (Mit C) and adriamycin (Adr) by a mechanism similar to that of the P450 reductase, and if these activities are stimulated by $Ca^{2+}$/CaM. Furthermore, the role of an FAD-FMN pair of the nNOS reductase domain is discussed in the context with that of the P450 reductase.

## 2. Materials and methods

### 2.1. Materials

The synthetic CaM peptide, amino acid residues 726–746, RRAIGFKKLAEAVKFSAKLM, was kindly supplied by Dr. Hisaaki Taniguchi (Fujita Health University, Japan). The rat NADPH-cytochrome P450 reductase cDNA [30] was kindly supplied by Dr. Yoshiyasu Yabusaki (Biotechnology Laboratory, Sumitomo). MD, Mit C, and Adr were purchased from Sigma. Pig brain calmodulin and cytochrome *c* were obtained from Boehringer Mannheim. NADPH and NADH were obtained from Oriental Yeast. Catalase and glucose oxidase were obtained from Sigma. All other materials were of analytical reagent grade.

### 2.2. Enzymes

The rat nNOS reductase domain (amino acid residues 718–1429) was expressed in *Escherichia coli*, strain BL21, and was purified as described previously [31]. QR was prepared from rat livers [32,33]. Trypsin-solubilized cytochrome b5 [34] was prepared from pig liver microsomes. The rat P450 reductase (amino acid residues 57–676) was constructed in the expression vector $pCWori^+$ and expressed in *E. coli*, strain BL21. The resulting recombinant enzyme was purified as described previously [8,29].

### 2.3. Methods

Optical spectra were measured with a Shimazu Model MPS UV-2000 spectrophotometer in a sample compartment at 25°C. Stopped-flow experiments were performed using a Union Giken Model RA401 stopped-flow spectrophotometer, equipped with photodiode array detection. For anaerobic rapid reaction experiments, 0.1 μM catalase, 10 mM glucose, and 10 units $ml^{-1}$ glucose oxidase were added to the reaction mixtures. The enzyme concentration of nNOS reductase domain was measured at 457 nm using an extinction coefficient of 22.9 $mM^{-1}$ $cm^{-1}$ [31]. The enzyme concentrations of NADPH-cytochrome P450 reductase was measured at 455nm ($\varepsilon = 21.4$ $mM^{-1}$ $cm^{-1}$) [8], and of NADPH:Q oxidoreductase at 452nm ($\varepsilon = 11.3$ $mM^{-1}$ $cm^{-1}$) [35]. The rates of NADPH oxidation by MD and Adr were monitored at 340 nm using an extinction coefficient of 6.22 $mM^{-1}$ $cm^{-1}$. The rate of NADPH oxidation by Mit C was monitored at 320 nm using an extinction coefficient of 4.5 $mM^{-1}$ $cm^{-1}$. For the kinetics studies, the concentrations of Qs were varied between 1.0 and 400 μM and 100 μM NADPH. Apparent *K*m and *k*cat values were determined from a Line–Weaver–Burk plot of the data. The rate of cytochrome *c* reduction was measured at 550 nm using an extinction coefficient of 21 $mM^{-1}$ $cm^{-1}$ [36]. The rate of cytochrome b5 reduction was measured at 556 nm using an extinction coefficient of 19.3 $mM^{-1}$ $cm^{-1}$ [37]. The concentrations of Mit C were determined at 367 nm ($\varepsilon = 21.8$ $mM^{-1}$ $cm^{-1}$) [38] and Adr at 480 nm ($\varepsilon = 12.4$ $mM^{-1}$ $cm^{-1}$) [39].

The reactivities of air-stable semiquinone with MD and ferricyanide were measured at 590 nm ($\varepsilon = 2.4$ $mM^{-1}$ $cm^{-1}$) [31] for nNOS reductase domain, and 585 nm ($\varepsilon = 2.3$ $mM^{-1}$ $cm^{-1}$) [29] for P450 reductase. The air-stable semiquinone forms of the nNOS reductase domain and P450 reductase were prepared by the addition of three times excess NADPH in the presence of air and were allowed to stand for about 20 min, respectively. Five μM air-stable semiquinone was mixed with 40 μM MD in the absence or presence of 200 μM $Ca^{2+}$, 20 μM CaM, and 25 mM Tris-buffer, pH 7.6.

## 3. Results

### 3.1. Absorption spectra of the nNOS reductase domain and P450 reductase

In this study, we compared the absorption spectra of the nNOS reductase domain and P450 reductase. The purified recombinant nNOS reductase domain and P450 reductase contained approximately equi-

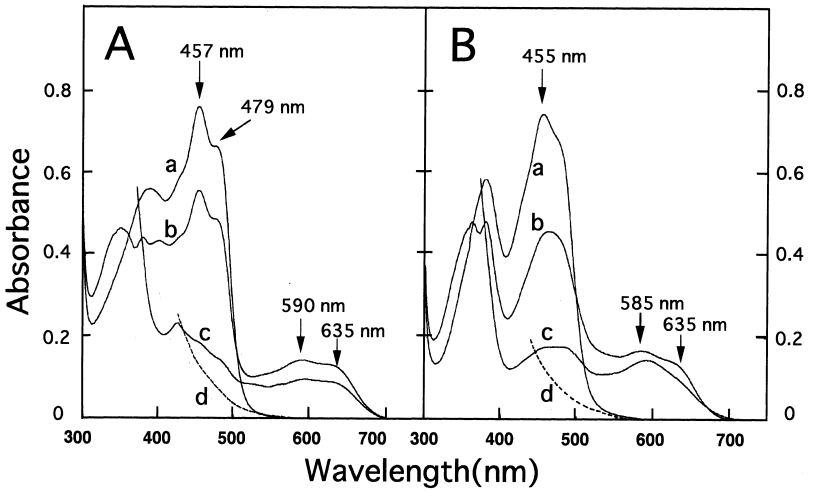



Fig. 1. Absorption spectra of nNOS reductase domain and P450 reductase. The nNOS reductase domain (A) and P450 reductase (B). Air-stable semiquinones of both enzymes were prepared by adding NADPH (105 μM, final concentration) in the presence of air to enzyme (35 μM) in 10 mM potassium phosphate buffer, pH 7.0, containing 5% glycerol. The systems were allowed to stand for about 20 min. (Curve a) oxidized; (curve b) air-stable semiquinone form; (curve c) after addition NADPH (81 μM, final concentration) under anaerobic conditions (NADPH-reduced residual semiquinone); (curve d) hydrosulfite reduced.

molar quantities of FAD and FMN, respectively. The visible absorption spectra of the purified nNOS reductase domain and P450 reductase at several oxidation levels are shown in Fig. 1A,B, respectively. The oxidized enzyme of the nNOS reductase domain had absorption peaks at 387 and 457 nm with a shoulder at 479 nm, but the P450 reductase had no pronounced shoulder in this region. The air-stable semiquinone of both enzymes was obtained by the reduction with NADPH in the presence of air. The spectrum so obtained (Fig. 1A, curve b) was very similar to that of the air-stable semiquinone (FAD-FMNH•) of the P450 reductase (Fig. 1B, curve b). The air-stable semiquinone in both enzymes was not completely reduced by excess NADPH (Fig. 1A,B, curves c). On the basis of NADPH-reduced residual semiquinone of the P450 reductase [8–10], this spectrum (Fig. 1A, curve c) corresponds mainly to a mixture of three-electron and four-electron reduced forms of P450 reductase [31]. In the NADPH-reduced residual semiquinone, a shoulder at 635 nm of the air-stable semiquinone was not seen in the P450 reductase (Fig. 1B, curve c), but it was still retained in the nNOS reductase domain (Fig. 1A, curve c). In both the presence and absence of $Ca^{2+}$/CaM, the spectra of curves b and c did not change (data not shown). These data suggest that the redox potentials of the two flavins are not modulated by the binding of CaM.

### 3.2. The nNOS reductase domain can catalyze one-electron reduction of Qs

NADPH oxidation by the P450 reductase, which is a 'one-electron transfer' enzyme, is greatly stimulated by the addition of MD as reported by Iyanagi and Yamazaki [1,2]. Fig. 2A demonstrates the NADPH oxidation by the nNOS reductase domain in the presence of $Ca^{2+}$/CaM and MD. The level of NADPH-oxidase activity was very low in the absence of MD, even in the presence of $Ca^{2+}$/CaM, but MD-mediated NADPH oxidation was increased approximately 12 times by the addition of $Ca^{2+}$/CaM (Fig. 2B). This activation was inhibited by the addition of excess synthetic CaM peptide, amino acid residues 726–746, RRAIGFKKLAEAVKFSAKLM, (0.5 mM) or EGTA (0.5 mM) (data not shown). When QR, which is a 'two-electron transfer' enzyme was

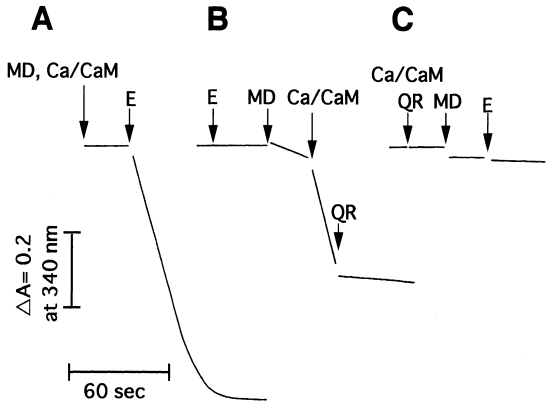



Fig. 2. MD-mediated aerobic oxidation of NADPH in the nNOS reductase domain. Concentrations: 0.25 μM nNOS reductase domain (E), 2.4 nM QR, 100 μM NADPH, 10 μM MD, 200 μM $Ca^{2+}$, 3 μM CaM, and 10 mM Tris buffer, pH 7.6. In this system, QR completely reduced MD to the hydroquinone form within the mixing time (approximately 2 s). The rates of NADPH-oxidation were monitored in the presence or absence of QR (A, B and C), respectively. Additions are indicated by arrows.

added during the reaction, the rates for NADPH oxidation in both the absence and presence of $Ca^{2+}$/CaM were significantly decreased (Fig. 2B,C).

P450 reductase catalyzes a one-electron reduction of both Mit C and Adr, which are antitumor drugs containing the Q structure, whereas QR catalyzes a two-electron reduction of these Qs [4,5]. In this work, we tested whether the nNOS reductase domain catalyzes a one- or two-electron reduction of these antitumor Qs. As shown in Fig. 3A, aerobic NADPH-oxidation was observed with the addition of Adr and was stimulated in the presence of $Ca^{2+}$/CaM. A change in absorbance at 480 nm, which indicates the absorption peak of oxidized Adr, was not observed during the reaction (Fig. 3A,B), suggesting that Adr is in the oxidized form without accumulation of the fully reduced form. If the rate of autoxidation of the fully reduced form, which is formed by a two-electron reduction, is rate-limiting in the overall NADPH-oxidation, Adr would accumulate in the fully reduced form in the presence of high concentrations of the nNOS reductase domain. However, such a product was not observed even in the presence of a 10-times excess of the enzyme concentration, as indicated in Fig. 3A (data not shown). These observations indicate that Adr was reduced by a one-electron transfer. Similar results were obtained in the

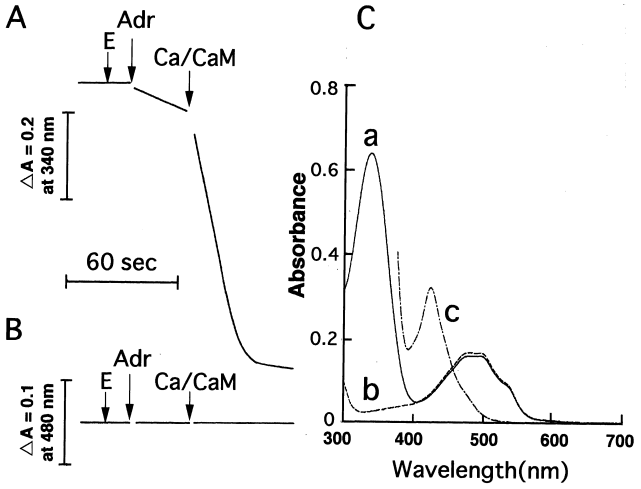



Fig. 3. Adr-mediated aerobic NADPH-oxidation (A), change of absorption with time at 480 nm (B) and its absorption spectra (C) observed during the nNOS reductase reaction. Concentrations: (A) 0.25 μM nNOS reductase domain, 100 μM NADPH, 200 μM $Ca^{2+}$, 3 μM CaM, 13 μM Adr, 10 mM Tris buffer, pH 7.6. (B) The oxidation-reduction state of Adr was monitored at 480 nm in the same conditions as A. (C) (curve a) the spectra of the mixture containing Adr, NADPH and $Ca^{2+}$/CaM; (curve b) 20 min after the start of the reaction by addition of nNOS reductase domain; (curve c) curve b was reduced by the addition of hydrosulfite. Additions are indicated by arrows.

reaction of Mit C (data not shown). The present data also indicate that the nNOS reductase domain catalyzes a one-electron reduction of antitumor Qs, Adr and Mit C, as well as simple Q MD. The kinetic parameters are summarized in Table 1.

We next used cytochrome b5 as a scavenger for the menasemiquinone radical: anionic semiquinone of MD ($MD^{\bullet -}$) [1,2,40]. Cytochrome b5 was not reduced directly by the nNOS reductase domain, but it was effectively reduced in the presence of MD (Fig. 4A). The stoichiometry of NADPH-oxidation by MD versus cytochrome b5 reduction (as determined from −d[NADPH]/dt versus d[cytochrome $b5^{2+}$]/dt) corresponded to 0.5 ($0.5 \pm 0.1$, $n = 5$), as shown in Fig. 4A. This ratio indicates that 1 mol of NADPH can reduce 2 mol of cytochrome b5, and that MD mediates transfer of electrons from NADPH to cytochrome b5 as a one-electron carrier [1,2,40]. On the other hand, QR did not effectively stimulate the reduction of cytochrome b5 in the presence of MD, although the rate of MD reduction was the same between the nNOS reductase domain and QR, as shown in Fig. 4A,B. These results also confirm that the nNOS reductase domain can catalyze only a one-electron reduction of MD, and the resulting semiquinone radical which has the rate constant of $3 \times 10^8$ $M^{-1}$ $s^{-1}$ for cytochrome b5 reduction [40], can rapidly react with cytochrome b5 [1].

### 3.3. Reactivity of the air-stable semiquinone with MD and ferricyanide.

The air-stable semiquinone of the nNOS reductase domain is more reduced by one equivalent than is the fully oxidized enzyme, and its reactivity with cytochrome *c* is very slow [31]. In this study, we measured the reactivities of the air-stable semiquinone with MD and ferricyanide. The air-stable semiquinone of the nNOS reductase domain did not react significantly with MD. The second order rate constant (as determined from −d[air-stable semiquinone form]/dt in the absence of $Ca^{2+}$/CAM) was $3.3 \times 10$ $M^{-1}$ $s^{-1}$, but its rate constant was $2.8 \times 10^2$ $M^{-1}$ $s^{-1}$ in the presence of $Ca^{2+}$/CaM.

The data indicate that the activity of the air-stable semiquinone with MD was increased nine times by the addition of $Ca^{2+}$/CaM, but these values can not explain the overall turnover shown in Table 1. A similar rate constant, $10.5 \times 10^2$ $M^{-1}$ $s^{-1}$, was obtained in the reaction of MD with the air-stable semiquinone of P450 reductase.

The second order rate constant of the air-stable semiquinone with ferricyanide was $1.1 \times 10^2$ $M^{-1}$ $s^{-1}$ in the absence of $Ca^{2+}$/CaM, but its rate constant was $1.3 \times 10^3$ $M^{-1}$ $s^{-1}$ in the presence of $Ca^{2+}$/CaM. The data also indicate that the activity of the air-stable semiquinone with ferricyanide was increased 12 times by the addition of $Ca^{2+}$/CaM.

Table 1
Kinetic parameters[a] for quinone-induced NADPH oxidation by the nNOS reductase domain

| | Ca/CaM | MQ | Adr | Mit C |
|---|---|---|---|---|
| *k*cat ($min^{-1}$) | − | 126 ± 9 | 136 ± 26 | 55 ± 11 |
| | + | 2368 ± 144 | 1664 ± 260 | 1016 ± 120 |
| *K*m (μM) | − | 11.2 ± 1.5 | 13.6 ± 4.4 | 113.8 ± 37.0 |
| | + | 24.6 ± 2.3 | 12.6 ± 3.4 | 206.8 ± 30.4 |

[a]Kinetic parameters were determined by monitoring NADPH oxidation in three independent experiments, as described in the text

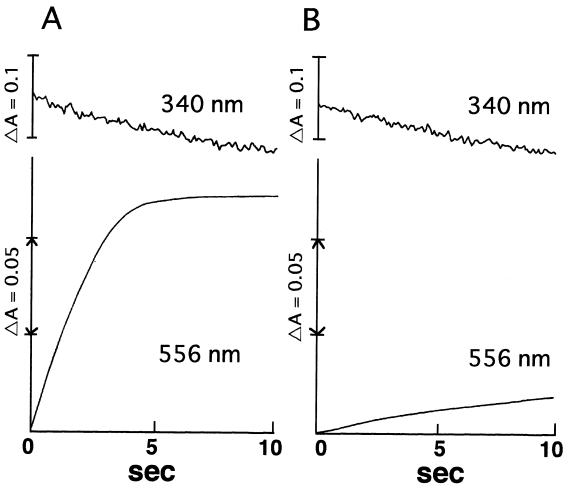



Fig. 4. MD-mediated reduction of cytochrome b5 in the nNOS reductase domain (A) and QR (B) systems. (A) The solution of the nNOS reductase domain containing Ca/CaM was mixed with the solution of cytochrome b5, NADPH, and MQ, anaerobically. (B) The solution of QR was mixed with solution of cytochrome b5, NADPH, and MQ, anaerobically. Final concentrations after mixing: 0.25 μM nNOS reductase domain, 100 μM NADPH, 7 μM cytochrome b5, 20 μM MQ, 200 μM $Ca^{2+}$, 3 μM CaM, 0.75 nM QR, 10 mM potassium phosphate buffer, pH 7.0. NADPH-oxidation was measured in the absence of cytochrome b5 in both systems (A and B).

### 3.4. Flavin intermediates observed during oxidation of the reduced enzyme by MD

We studied the role of an FAD-FMN pair in one-electron reduction of MD. On the basis of similar redox properties with P450 reductase (Fig. 1A,B, and [15,31]), the absorbance changes at 457 nm (the sum of the formation of semiquinone and reduced forms), 504 nm (the formation of the fully reduced species), and 590 nm (the formation of both fully reduced and semiquinone flavin species) were monitored (Fig. 5). The oxidized P450 reductase (FAD-FMN) was mixed with NADPH plus MD solution. The results are shown in Fig. 5A. The decrease in the absorbance at 455 nm parallels the increase at 585 nm, but the absorbance change at 503 nm, which would decrease on the formation of a fully reduced flavin species, is relatively constant. When the air-stable semiquinone (FAD-FMNH•) was mixed with NADPH plus MD, a significant change at all three wavelengths was not observed (Fig. 5A). These data confirm the previous evidence that the air-stable semiquinone is a predominant intermediate observed during the oxidation of the reduced enzyme by MD [29]. In the presence of $Ca^{2+}$/CaM, the oxidized nNOS reductase domain (FAD-FMN) was mixed with NADPH plus MD solution. The results are shown in Fig. 5B. The decrease in the absorbance at 457 nm parallels the increase at 590 nm, but the absorbance change at 504 nm was relatively constant. These results indicate that the fully

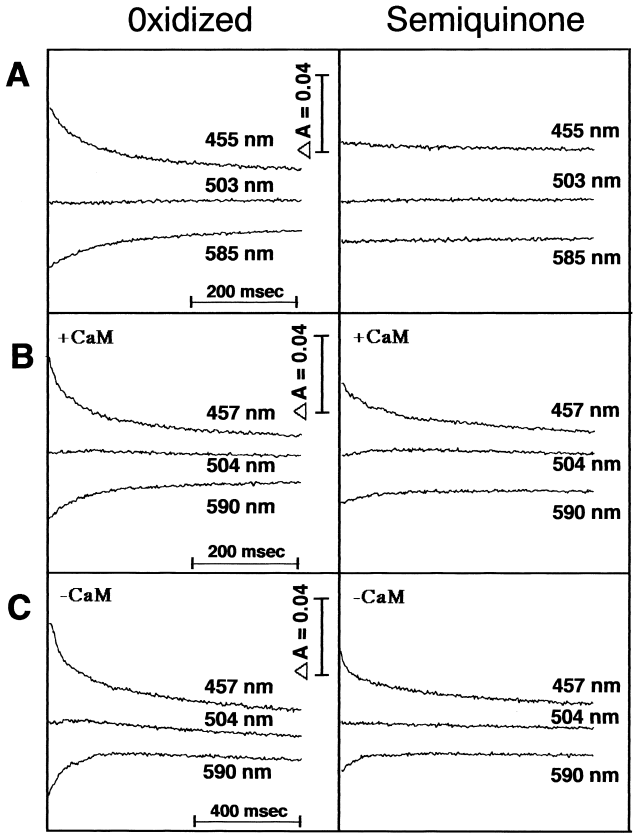



Fig. 5. Changes of absorption with time at three wavelengths during reaction of MD and the nNOS reductase domain with NADPH followed in a stopped-flow apparatus. Oxidized (left panel) or air-stable semiquinone (right panel) of P450 reductase and the nNOS reductase domain were mixed anaerobically with NADPH plus MD and the absorbance changes were recorded at three wavelengths. (A) The oxidized or air-stable semiquinone of P450 reductase. (B) The oxidized or air-stable semiquinone of nNOS reductase domain in the presence of $Ca^{2+}$/CaM. (C) The oxidized or air-stable semiquinone of nNOS reductase domain in the absence of $Ca^{2+}$/CaM. Final concentrations after mixing: 5 μM oxidized enzyme or air-stable semiquinone, 20 μM NADPH, 40 μM MD, and 25 mM HEPES buffer, pH 7.0. Before mixing, 10 μM nNOS reductase domain solutions were incubated for 20 min with 400 μM $Ca^{2+}$, and 20 μM CaM. In the P450 reductase system, CaM was removed from the enzyme solutions.

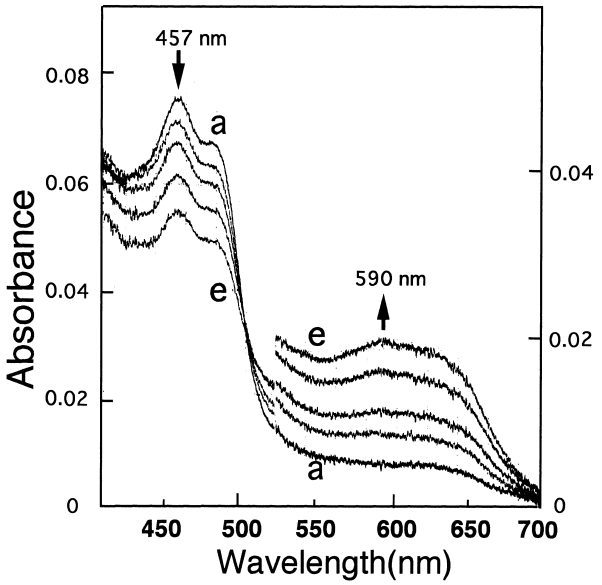



Fig. 6. Spectral changes of the oxidized enzyme followed by a rapid scan spectrophotometer after mixing with NADPH plus MD. The oxidized enzyme was mixed anaerobically with NADPH plus MD and each spectrum was recorded at 15, 37, 57, 117, and 237 ms (a–e, respectively). Final concentrations after mixing: 8 μM nNOS reductase domain, 500 μM $Ca^{2+}$, 20 μM NADPH, 40 μM MD and 25 mM HEPES buffer, pH 7.0. The spectra are recorded from 410 to 520 and 520 to 700 nm, respectively.

reduced species of the two flavins do not accumulate measurably during the oxidation of the reduced enzyme by MD. When the air-stable semiquinone form was mixed with NADPH plus MD (Fig. 5B), changes in the absorbance at 457 and 590 nm were observed. The increase of absorbance at 590 nm strongly suggests the formation of another semiquinone species, probably a semiquinone radical derived from the FAD moiety (Fig. 5B), but such an absorbance change was not observed in the P450 reductase (Fig. 5A). Its concentration was approximately 30% of the air-stable semiquinone. When the oxidized nNOS reductase domain (FAD-FMN) was mixed with NADPH plus MD in the absence of $Ca^{2+}$/CaM, absorbances at 457 nm and 590 nm were increased more than those in the presence of $Ca^{+2}$/CaM (Fig. 5C). The increase of absorbance at 590 nm occurred without a lag phase, and its rate was more rapid than that in the presence of $Ca^{2+}$/CaM. Furthermore, when the air-stable semiquinone was mixed with NADPH plus MD, similar results to that of Fig. 5B were observed (Fig. 5C). In both the presence and absence of $Ca^{2+}$/CaM, the semiquinone species accumulated after the reaction, as shown in Fig. 5B,C. The spectra were monitored by rapid-scan spectrophotometry (Fig. 6). These data strongly suggest that the air-stable semiquinone form is the predominant intermediate observed during the oxidation of the enzyme reduced by MD, but another semiquinone species is also observed during the reaction. These semiquinone species do not react significantly with MD.

### 3.5. *Flavin intermediates observed during oxidation of the reduced enzyme by ferricyanide*

The reduction rate of ferricyanide was approximately doubled in the presence of $Ca^{2+}$/CaM [31]. In this study, we directly observed the reactivity of the reduced enzyme with ferricyanide. The oxidized nNOS reductase domain (FAD-FMN) was mixed with NADPH in the presence of excess ferricyanide. The results are shown in Fig. 7A,B. In the presence

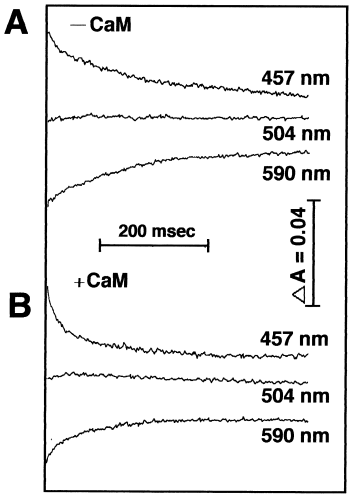
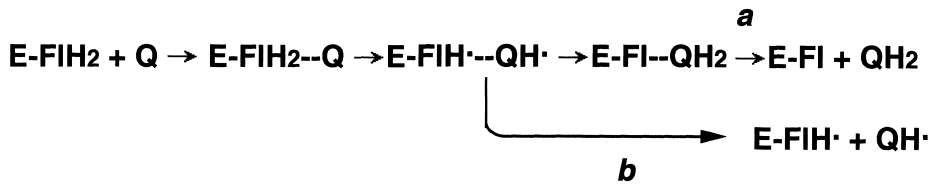



Fig. 7. Changes of absorption with time at three wavelengths during the reaction of ferricyanide and the nNOS reductase domain with NADPH followed in a stopped-flow apparatus. Oxidized enzyme of the nNOS reductase domain was anaerobically mixed with NADPH plus ferricyanide and the absorbance changes were recorded at three wavelengths. The oxidized enzyme of nNOS reductase domain absent (A) or present (B). Final concentrations after mixing: 8.7 μM oxidized enzyme 21.6 μM NADPH, 500 μM ferricyanide, and 25 mM HEPES buffer, pH 7.0. Before mixing, the nNOS reductase domain solutions, 17.7 μM were incubated for 20 min with 400 μM $Ca^{2+}$, and 21 μM CaM.




Scheme 1. The mechanism of electron transfer in the overall bivalent oxidation-reduction reaction. E-$FlH_2$ indicates enzyme-bound reduced flavin, and E-$FlH^{\bullet}$–$QH^{\bullet}$ corresponds to a primarily formed one-electron transfer intermediate [3].

of $Ca^{2+}$/CaM, the decrease in the absorbance at 457 nm parallels the increase at 590 nm, and its rate at 590 nm was approximately twice as high as that in the absence of $Ca^{2+}$/CaM (Fig. 7A,B).

## 4. Discussion

Reduced flavin can be oxidized sequentially via two one-electron processes involving the semiquinone radical [41]. In 1971, Yamazaki suggested a tentative definition of one-electron and two-electron transfer mechanisms [42]. For the electron transfer between reduced flavin enzyme (E-$FlH_2$) and Q the following Scheme 1 can be drawn. In some cases the reverse reaction also takes place. When the reaction proceeds along route ***a***, it is termed a two-electron mechanism regardless of the lifetime of the one-electron transfer intermediate (E-$FlH^{\bullet}$–$QH^{\bullet}$). The hydride transfer also belongs to this category. A one-electron transfer mechanism then implies that the intermediate (E-$FlH^{\bullet}$–$QH^{\bullet}$) releases only semiquinone radicals ($QH^{\bullet}$) via a reaction path indicated by route ***b***. The semiquinone radical ($QH^{\bullet}$) thus formed will transfer one electron between the same semiquinone radical species, and the final product is $QH_2$. The semiquinone radical intermediate formed in route ***b*** is detected with the aid of the electron paramagnetic resonance (EPR) technique [1,2]. When the steady state concentration of a semiquinone radical is below the level of EPR sensitivity, semiquinone radical scavengers, including molecular oxygen and cytochromes, can be used for detection of the semiquinone radical [1,2]. On the basis of this background, we studied the mechanism of Q reduction by the nNOS reductase domain (FAD-FMN). As shown in Figs. 2–4, the present data clearly demonstrate that the nNOS reductase domain catalyzes a typical one-electron reduction of Qs, such as MD (−210 mV) [28], Mit C (−271 mV) [39] and Adr (−292 mV) [39] (Fig. 8). Therefore, the reduction of these Qs proceeds along route ***b***. The one-electron reduction potential of various Qs is related to their reduction rates by the nNOS reductase domain[1]. On the other hand, QR can catalyze the two electron reduction of MD (Figs. 2 and 4, and [2]), but QR did not significantly reduce Adr and Mit C (data not shown).

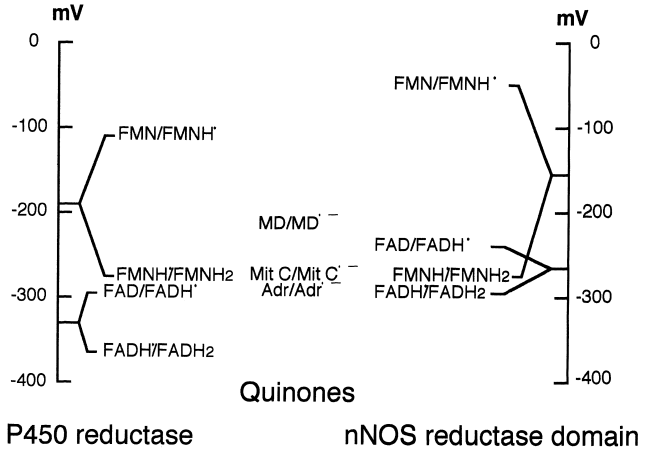



Fig. 8. One-electron oxidation-reduction potentials of microsomal P450 reductase, the nNOS reductase domain, and various Qs. The redox potentials of the flavins of P450 reductase (FAD-FMN) and the nNOS reductase domain (FAD-FMN) are given as reported in [9] and [45], respectively. MD (−210 mV) [28], Mit C (−271 mV) [39], and Adr (−292) [39]. It is possible that $NADP^+$ or NADPH binding to the nNOS reductase domain stabilized the FAD semiquinone by shifting the reduction potential to a more negative value [45].

The nNOS reductase domain has redox properties similar to those of P450 reductase (FAD-FMN) [31]. Iyanagi, Makino and Mason measured one-electron redox potentials of FAD and FMN of the P450 re-

[1] Kitamura, M and Iyanagi, T, unpublished data.

ductase [9], and assigned values to the redox couples at −110, −270, −290 and −365 mV, which represent the couples FMN/FMNH$^{\bullet}$, FMNH$^{\bullet}$/FMNH$_2$, FAD/FADH$^{\bullet}$, and FADH$^{\bullet}$/FADH2, respectively [43,44] (Fig. 8). Recently, Nobel et al. reported one-electron redox potentials of FAD and FMN of the nNOS reductase domain (amino acid residues 695–1429) and determined values for the redox couples at −49, −274, −232, and −280 mV, which represent the couples FMN/FMNH$^{\bullet}$, FMNH$^{\bullet}$/FMNH$_2$, FAD/FADH$^{\bullet}$, and FADH$^{\bullet}$/FADH$_2$, respectively [45] (Fig. 8). The redox couple FMNH$^{\bullet}$/FMNH$_2$ = −274 mV of nNOS reductase is very similar with that of P450 reductase (Fig. 8), and its value is unaffected by the binding of CaM [45]. The FMN-depleted enzyme of the P450 reductase (FAD) is unable to catalyze either reduction of MD (−210 mV) and cytochrome *c* (+250 mV), although these reactions are thermodynamically favored. This enzyme can catalyze the reduction of ferricyanide, but its reactivity with the FAD semiquinone is slower than that of the fully reduced FAD [29]. The air-stable semiquinone FAD-FMNH$^{\bullet}$ does not react significantly with MD (see Section 3). These data indicate that the redox couple FMNH$^{\bullet}$/FMNH$_2$ = −270 mV of P450 reductase can reduce directly various Qs with low one-electron redox potentials [3–5,28] (Fig. 8). The air-stable semiquinone of the nNOS reductase domain has similar oxidation levels to that of P450 reductase [15,31,45,46], and it also does not react significantly with MD (see Section 3). This state is rapidly reduced by NADPH in the three-electron reduced species (Fig. 1, and [31]). These data suggest that the redox couple FMNH$^{\bullet}$/FMNH$_2$ = −274 mV of the nNOS reductase domain also can donate an electron to various Qs (Fig. 8). In the presence of $Ca^{2+}$/CaM, the air-stable semiquinone, FAD-FMNH$^{\bullet}$ is the predominant intermediate observed during oxidation of the reduced nNOS reductase domain by MD (Fig. 5B). In addition to this intermediate, other semiquinone species, probably FADH$^{\bullet}$-FMNH$^{\bullet}$ and FADH$^{\bullet}$-FMN, could be formed during the oxidation of the reduced enzyme by MD, as shown in Fig. 5B,C. Such an absorbance change was not observed in P450 reductase (Fig. 5A). This difference suggests that the fully reduced FAD of the nNOS reductase domain can react directly with MD, and the resulting semiquinone species of FAD has low reactivity toward MD. In fact, the formation of semiquinone, inferred from the increase of absorbance at 590 nm, occurred with a lag phase in the absence of $Ca^{2+}$/CaM [31], but its formation occurred without a lag phase when MD is added to the reaction mixture (Fig. 5C). This observation was directly confirmed by rapid-scan spectrophotometry (Fig. 6). These data strongly support the idea that the semiquinones (FADH$^{\bullet}$-FMN or FADH$^{\bullet}$-FMNH$^{\bullet}$) derived from FAD could be formed during the oxidation of the reduced enzyme by MD. When ferricyanide, which can react directly with FAD [31], was used as a one-electron acceptor, similar results to those of Fig. 5B,C were observed (Fig. 7A,B). The fully reduced flavin species, as judged by the absorbance change at 504 nm, did not significantly accumulate during the oxidation of the reduced enzyme by MD under these conditions (Fig. 5B,C). These accumulated data indicate that both the fully reduced flavins, FADH$_2$ and FMNH$_2$, can donate electrons to MD molecules, but their semiquinone states do not react rapidly with MD. The two-electron reduced enzyme (FADH$^{\bullet}$-FMNH$^{\bullet}$ ⇄ FAD-FMNH$_2$) can donate electrons to MD. Therefore, the FAD-FMN pair shuttles between the one-electron (FADH$^{\bullet}$-FMN ⇄ FAD-FMNH$^{\bullet}$) and three-electron (FADH$_2$-FMNH$^{\bullet}$ ⇄ FADH$^{\bullet}$-FMNH$_2$) reduced states, and the redox couple, FMNH$^{\bullet}$/FMNH$_2$ is the most reactive flavin species with MD at the catalytic cycle. In the presence of $Ca^{2+}$/CaM, the rate of Q reduction is stimulated by a rapid electron exchange between the two flavins during catalytic turnover. Further analysis of the kinetic modeling to justify the mechanisms of these complex reactions remains to be done.

In conclusion, we demonstrated that the nNOS reductase domain does catalyze the one-electron reduction of Qs with fairly low one-electron redox potentials (Fig. 8). The role of each flavin of the nNOS reductase domain has been proposed based on similarities with that of the P450 reductase [9,21,29,31,44–47] and the X-ray crystal structure of P450 reductase [48,49]: FAD accepts the two-electron transfer from NADPH as a hydride ion, while the FMNH$_2$ is the most reactive species with Qs. The enzyme catalyzes only a one-electron reduction of Qs during the $1e^- \rightleftarrows 3e^-$ catalytic cycle, and the rate of

Q reduction is stimulated by a rapid electron exchange between the two flavins in the presence of $Ca^{2+}$/CaM.

## Acknowledgements

This study was supported, in part, by Grant-in-Aid for Scientific Research (B)(09558089) from the Ministry of Education, Science, Sports and Culture of Japan.

## References

[1] T. Iyanagi, I. Yamazaki, Biochim. Biophys. Acta 172 (1969) 370–381.
[2] T. Iyanagi, I. Yamazaki, Biochim. Biophys. Acta 216 (1970) 282–294.
[3] T. Iyanagi, Chem. Scr. 27A (1987) 31–36.
[4] G. Powis, Free Radic. Biol. Med. 6 (1989) 63–101.
[5] E. Cadenas, Biochem. Pharmacol. 49 (1995) 127–140.
[6] D.S. Bredt, P.M. Hwang, C.E. Glatt, C. Lowenstein, R.R. Reed, S.H. Snyder, Nature 351 (1991) 714–718.
[7] E.A. Sheta, K. McMillan, B.S.S. Masters, J. Biol. Chem. 269 (1994) 15147–15153.
[8] T. Iyanagi, H.S. Mason, Biochemistry 12 (1973) 2297–2308.
[9] T. Iyanagi, N. Makino, H.S. Mason, Biochemistry 13 (1974) 1701–1710.
[10] T. Iyanagi, F.K. Anan, Y. Imai, H.S. Mason, Biochemistry 17 (1978) 2224–2230.
[11] K. McMillan, D.S. Bredt, D.J. Hirsch, S.H. Snyder, J.E. Clark, B.S.S. Masters, Proc. Natl. Acad. Sci. USA 89 (1992) 11141–11145.
[12] P. Klatt, K. Schmidt, G. Uray, B. Mayer, J. Biol. Chem. 268 (1993) 14781–14787.
[13] H.M. Abu-Soud, L.L. Yoho, D.J. Stuehr, J. Biol. Chem. 269 (1994) 32047–32050.
[14] K. McMillan, B.S.S. Masters, Biochemistry 34 (1995) 3686–3693.
[15] R. Gachhui, A. Presta, D.F. Bentley, H.M. Abu-Soud, R. McArthur, G. Brudvig, D.K. Ghosh, D.J. Stuehr, J. Biol. Chem. 271 (1996) 20594–20602.
[16] H.M. Abu-Soud, D.J. Stuehr, Proc. Natl. Acad. Sci. USA 90 (1993) 10769–10772.
[17] S. Pou, W.S. Pou, D.S. Bredt, S.H. Snyder, G.M. Rosen, J. Biol. Chem. 267 (1992) 24173–24176.
[18] P. Klatt, B. Heinzel, M. John, M. Kastner, B. Bohme, B. Mayer, J. Biol. Chem. 267 (1992) 11374–11378.
[19] K.J. Baek, B.A. Thiel, S. Lucas, D.J. Stuehr, J. Biol. Chem. 268 (1993) 21120–21129.
[20] J. Vasquez-Vivar, P. Martasek, N. Hogg, B.S.S. Masters, K.A. Pritchart Jr., B. Kalyanaraman, Biochemistry 36 (1997) 11293–11297.
[21] A.P. Garner, M.J.I. Paine, I. Rodriguez-Crespo, E.C. Chinje, P. Ortiz de Montellano, I.J. Stratford, D.G. Tew, C.R. Wolf, Cancer Res. 59 (1999) 1929–1934.
[22] R.T. Miller, P. Martasek, L.J. Roman, J.S. Nishimura, B.S.S. Masters, Biochemistry 36 (1997) 15277–15284.
[23] Y. Kumagai, H. Nakajima, K. Midorikawa, S. Homma-Takeda, N. Shimojo, Chem. Res. Toxicol. 11 (1998) 608–613.
[24] G. Powis, P.L. Appel, Biochem. Pharmacol. 29 (1980) 2567–2572.
[25] J. Butler, B.M. Hoey, Biochim. Biophys. Acta 1161 (1993) 73–78.
[26] Z. Anusevicius, M. Martinez-Julvez, C.G. Genzor, H. Nivinskas, C. Gomez-Moreno, N. Cenas, Biochim. Biophys. Acta 1320 (1997) 247–255.
[27] N.K. Cenas, D. Ascott, C.H. Williams Jr., J.S. Blanchart, Biochemistry 33 (1994) 2509–2515.
[28] T. Iyanagi, Free. Radic. Res. Commun. 8 (1990) 259–268.
[29] T. Iyanagi, R. Makino, F.K. Anan, Biochemistry 20 (1981) 1722–1730.
[30] H. Murakami, Y. Yabusaki, H. Ohkawa, DNA 5 (1986) 1–10.
[31] H. Matsuda, T. Iyanagi, Biochim. Biophys. Acta 1473 (1999) 345–355.
[32] B. Hojeberg, K. Blomberg, S. Stenberg, C. Lind, Arch. Biochem. Biophys. 207 (1981) 205–216.
[33] M. Haniu, H. Yuan, S. Chen, T. Iyanagi, T.D. Lee, J.E. Shively, Biochemistry 27 (1988) 6877–6883.
[34] T. Omura, S. Takesue, J. Biochem. (Tokyo) 67 (1970) 249–257.
[35] M.T. Huang, G.T. Miwa, A.Y.H. Lu, J. Biol. Chem. 254 (1979) 3930–3934.
[36] V. Massey, Biochim. Biophys. Acta 34 (1959) 255–256.
[37] T. Yubisui, K. Murakami, M. Takeshita, T. Takano, Biochim. Biophys. Acta 936 (1988) 447–451.
[38] G.S. Kumar, R. Lipman, J. Gummings, M. Tomasz, Biochemistry 36 (1997) 14128–14136.
[39] B.A. Svingen, G. Powis, Arch. Biochem. Biophys. 209 (1981) 119–126.
[40] T. Ohnishi, H. Yamazaki, T. Iyanagi, T. Nakamura, I. Yamazaki, Biochim. Biophys. Acta 172 (1969) 357–369.
[41] L. Michaelis, M.P. Schubert, Chem. Rev. 22 (1938) 437–470.
[42] I. Yamazaki, Adv. Biophys. 2 (1971) 33–76.
[43] J.L. Vermilion, M.J. Coon, J. Biol. Chem. 253 (1978) 8812–8819.
[44] J.L. Vermilion, D.P. Ballou, V. Massey, M.J. Coon, J. Biol. Chem. 256 (1981) 266–277.
[45] M.A. Nobel, A.W. Munro, S.L. Rivers, L. Robledo, S.N. Daff, L.J. Yellowees, T. Shimizu, I. Sagami, G. Guillemette, S.K. Chapman, Biochemistry 38 (1999) 16414–16418.
[46] R.T. Miller, P. Martasek, T. Omura, B.S.S. Masters, Biochem. Biophys. Res. Commun. 265 (1999) 184–188.

[47] B.S.S. Masters, H. Kamin, Q.H. Gibson, C.H. Williams Jr., J. Biol. Chem. 240 (1965) 921–931.
[48] M. Wang, D.L. Roberts, R. Paschke, T.M. Shea, B.S.S. Masters, J.-J. Kim, Proc. Natl. Acad. Sci. USA 94 (1997) 8411–8416.
[49] Q. Zhao, S. Modi, G. Smith, M. Paine, P.D. McDonagh, C.R. Wolf, D. Tew, L.-Y. Lian, G.C.K. Roberts, H.P.C. Driessen, Protein Sci. 8 (1999) 298–306.