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LOCATION OF FUNCTIONAL -SH GROUPS IN NADPH-CYTOCHROME *P*-450 REDUCTASE FROM RABBIT LIVER MICROSOMES

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The total -SH content of purified NADPH-cytochrome *P*-450 reductase (NADPH: ferricytochrome oxidoreductase, EC 1.6.2.4) from rabbit liver microsomes accessible to an excess equivalent of PCMB was 7.0 ± 0.3 mol thiol groups/mol protein. The modification of four -SH groups at low concentrations of PCMB stimulated the activity of the enzyme. On the other hand, further blocking of -SH groups (6–7 mol -SH groups/mol protein) with an excess amount of PCMB completely inhibited cytochrome *c* (or DCPI) reductase activity. The fluorescence quenching of the flavin was rapidly removed by binding of PCMB to a fifth and sixth -SH group during a gradual titration. Kinetic and fluorimetric analyses confirmed the suggestion that these two -SH groups essential for catalytic function were partly protected by $NADP^+$ or 2′-AMP against the reaction with PCMB. Excess PCMB begins to compete with the ligand preincubated with the enzyme. The spectral perturbation on the addition of approx. 6–7 equiv. PCMB/mol enzyme is accompanied by a slight blue shift of the absorbance maximum at 380 nm, with the appearance of a pronounced shoulder at 475 nm. In contrast to the native enzyme, 3-electron-reduced semiquinone form of PCMB-treated enzyme showed the same absorption spectrum as 1-electron-reduced semiquinone which has an absorption maximum at 585 nm with a broad shoulder around 635 nm. An inhibitory effect may be attributable to the fact that NADPH is less accessible to the FAD binding site as well as the pyridine nucleotide binding site, since the rate of FAD reduction becomes extremely slow after complete modification.

Introduction

The detergent solubilized-hepatic NADPH-cytochrome *P*-450 reductase (NADPH: ferricytochrome oxidoreductase, EC 1.6.2.4), the flavoprotein component of a liver microsomal mixed-function oxidase contains 1 mol each of FAD and FMN/mol enzyme. The two flavins per polypeptide chain of molecular weight 78 000 have different oxidation-reduction characteristics [1–3]. The FMN-depleted enzyme lost the ability to catalyze electron transfer to the phenobarbital-inducable form of liver microsomal cytochrome *P*-450 in the reconstituted hydroxylation system, as well as to cytochrome *c* and some other artificial acceptors, but retained activity toward ferricyanide [4].

Previous reports have provided evidence for the presence of thiol groups at or near the catalytic site [5]. In this publication, it is noted that at pH 7.7 addition of 1–2 mol PCMB/mol flavin resulted in activation of cytochrome *c* reductase activity; at higher concentrations inhibition appeared, and became virtually complete between 7 and 10 mol PCMB/mol flavin [5]. However, the correlation between -SH groups and the enzymatic properties has not yet been clarified. Neither the flavin-binding domain nor the NADPH-binding site has been characterized. In the recent publication, only three -SH

Abbreviations: PCMB, p-hydroxychloromercuribenzoate; DCPI, 2,6-dichlorophenol-indophenol.

groups of the protease-solubilized NADPH-cytochrome *P*-450 reductase from porcine liver microsomes could be modified by 5,5′-dithio-bis(2-nitrobenzoate), and more than 95% of the original enzymatic activity was lost during this treatment [6]. Experiments with PCMB showed that the enzymatic activity of NADPH-cytochrome *P*-450 reductase depends strongly on its sulfhydryl residues. Using the holoenzyme and FMN-depleted enzyme, the gradual modification of the accessible thiol groups gave detailed information of the functional and structual roles of thiol groups in the reductase.

Materials and Methods

Materials. NADPH, FAD, FMN, cytochrome *c*, Triton N-101 and 2′-AMP were purchased from Sigma, DEAE-Sepharose CL-6B, 2′,5′-ADP-Sepharose 4B and Sephadex G-50 from Pharmacia. All other reagents were of the highest grade commercially available.

Solubilization and purification of NADPH-cytochrome P-450 reductase. Detergent-solubilized NADPH-cytochrome *P*-450 reductase was prepared by the method of Iyanagi et al. [7] with some modifications. A suspension of microsomes, about 8 g protein in 1 h 0.1 M Tris-acetate buffer, pH 7.6, was stirred for 30 min at 0°C with 200 ml glycerol, 450 mg EDTA, 5 g deoxycholate, 25 ml Triton N-101 and 6.5 g KCl; the precipitate obtained by centrifugation at 78 000 × *g* for 120 min was discarded. The clear supernatant fluid was applied to a DEAE-Sepharose CL-6B column (4.5 × 20 cm) previously equilibrated with 0.1 M Tris-acetate buffer, pH 7.6/20% glycerol/0.1% Triton N-101/1 mM EDTA (buffer A) containing 0.1 M KCl. The column was washed with 500 ml equilibration buffer to remove cytochrome *P*-450 and cytochrome b_5. The reductase was then eluted with buffer A containing 0.5 M KCl. The active fractions containing more than 3 units reductase activity/mg were combined and dialyzed for 48 h against 5 l 25 mM potassium phosphate buffer, pH 7.0/20% glycerol/0.1% Triton N-101 (25 mM buffer B) according to the method of Yasukochi and Masters [8], then applied to a 2′,5′-ADP-Sepharose 4 B column (1.8 × 10 cm) equilibrated with 25 mM buffer B. The column was washed with 500 ml 25 mM B/0.2% deoxycholate and the reductase was then eluted with 25 mM buffer B containing 2 mM 2′-AMP. The bright yellow active fraction was applied to a hydroxyapatite column (1.5 × 15 cm) equilibrated with 25 mM buffer B; the column was washed with 200 ml of the same buffer and the reductase was then eluted with 100 mM potassium phosphate buffer/20% glycerol. The enzyme contained 1.91–1.95 mol flavin/mol protein; the rate of FMN to FAD was 0.93.

Preparation of FMN-depleted enzyme. FMN-depleted enzyme was prepared by the method described by Vermillion and Coon [4] except as noted below. The holoenzyme was diluted to a final concentration of approx. 0.5–1.0 μM with 2 M KBr in 0.1 M Tris-acetate buffer, pH 7.7/10% glycerol/0.1 mM EDTA/0.1 mM dithiotheitol. The enzyme solution was dialyzed at least 1 week against 2 l of the same buffer, pH 7.7, with seven changes of the buffer solution. KBr-treated reductase preparation was dialyzed 48 h against 4 l 25 mM potassium phosphate buffer, pH 7.7/10% glycerol and KBr was removed. The enzyme solution was applied to a column (1.0 × 8.0 cm) of hydroxyapatite previously equilibrated with the same buffer, pH 7.7. The column was washed with 100 ml equilibration buffer and the reductase was eluted with 100 mM potassium phosphate buffer, pH 7.7/20% glycerol. The preparation of FMN-depleted reductase obtained by this procedure contained 95% or more of the FAD present in the native enzyme but only 2–5% of the original amount of FMN.

Assay for cytochrome c reductase activity. Rates of cytochrome *c* reduction were determined at 25°C in a potassium phosphate buffer, pH 7.7. The 1.0 ml assay mixture contained the following components: 200 μmol phosphate buffer/40 nmol cytochrome *c*/0.1 μmol NADPH. The reaction was initiated by the addition of the reductase, and the rate of cytochrome *c* reduction was determined at 550 nm using an extinction coefficient of 21 $mM^{-1} \cdot cm^{-1}$ [9] for the conversion of the oxidized cytochrome to the reduced form. Specific activities are expressed as μmol cytochrome *c* reduced min per mg protein.

Activity of NADPH-cytochrome P-450 reductase with other electron acceptors. Reaction rates were determined at 25°C under aerobic conditions in 0.1 M phosphate buffer, pH 7.7, at the following concentration of electron acceptor: DCPI, 50 μM; ferricyanide, 0.2 mM. For assay of DCPI and ferricyanide reduc-

tion the concentration of NADPH was 0.1 mM. The following extinction coefficients, given as $mM^{-1} \cdot cm^{-1}$, were used to calculate the rates of reduction of the substrates: DCPI, 21 at 600 nm [10]; 1.02 ($\Delta\epsilon$ between ferri- and ferrocyanide) at 420 nm [11].

Determination of protein and flavin content. Protein was estimated according to Lowry et al. [12] with bovine serum albumin as reference protein. The individual determination of FMN and FAD concentrations in mixtures of the two compounds was carried out at both pH 7.7 and 2.6 as described by a fluorimetric method of Faeder and Siegel [13].

Estimation of -SH content in the reductase. The number of reactive -SH groups/mol NADPH-cytochrome *P*-450 reductase was estimated with PCMB using an absorption difference at 250 nm between PCMB reacted with thiol groups and free PCMB [14] and a molecular weight of 78 000 for the detergent-solubilized NADPH-cytochrome *P*-450 reductase [2]. The modified degree of -SH group was calculated from the amount of PCMB consumed assuming 1 : 1 stoichiometry between PCMB and the mol thiol group reacted. Estimation of reactive -SH group in FMN-depleted reductase was also performed according to the same method as holoenzyme. The -SH content accessible to *N*-ethylmaleimide was calculated using a molar absorption coefficient of 620 $M^{-1} \cdot cm^{-1}$ at 300 nm.

Absorption spectra of holo- and FMN-depleted NADPH-cytochrome P-450 reductase. Optical spectra were measured with a Cary Model 17 spectrophotometer, in a sample compartment thermostatted at 5 ± 1°C. The total flavin concentration was determined spectrophotometrically using the molar absorption coefficient, 11.5 $mM^{-1} \cdot cm^{-1}$ at 455 nm by the method of Mayhew and Massey [15].

Emission measurement. Fluorescence intensity changes of the flavin in NADPH-cytochrome *P*-450 reductase on reaction of PCMB were measured using a Shimazu RF-501 spectrofluoriphotometer. A fluorescence square cell ($1 \times 1 \times 3$ cm) was thermostated at 5°C with dry N_2 circulated through the sample chamber. Excitation and emission wavelengths for flavin were 455 and 525 nm, respectively. Dependence of the fluorescence intensity of flavin on PCMB concentration was expressed as the logarithm of the ratio of fluorescence intensity after PCMB addition to that before addition.

Results

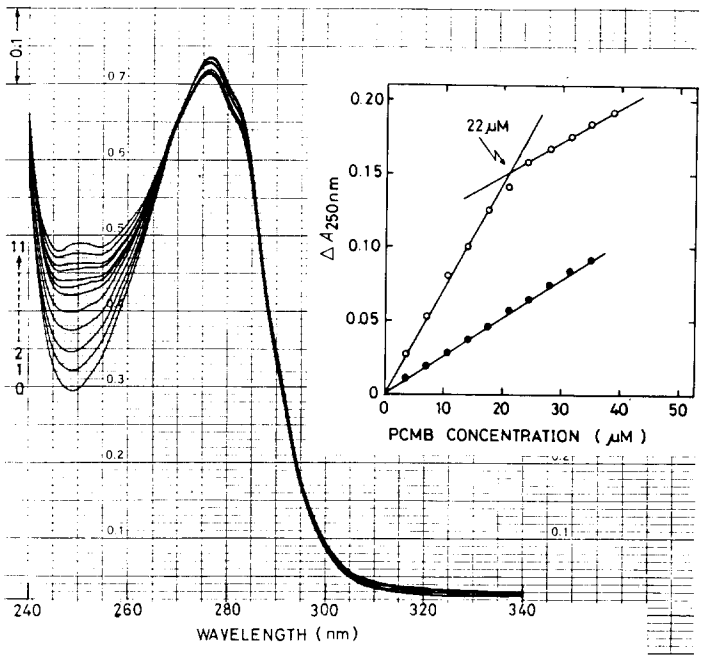
Reaction of thiol reagents with NADPH-cytochrome P-450 reductase

Titration of the holoenzyme with PCMB at 25°C revealed that about seven sulfhydryl groups/mol enzyme protein were accessible to the reagent, however, *N*-ethylmaleimide does not appear to react with sulfhydryl groups (Figs. 1 and 2). No inhibition was observed with even high concentrations of *N*-ethylmaleimide.

It is noted that addition of 1–4 mol PCMB/mol protein resulted in 50–70% activation of cytochrome *c* reductase activity; at higher PCMB concentration (5 mol PCMB/mol protein) inhibition appeared, and became complete between 6 and 7 mol PCMB/mol protein. Furthermore, it was found that this reagent irreversibly inactivated the reductase in a time-dependent fashion. As show in Fig. 3, PCMB causes time-dependent activation and inactivation of NADPH-cytochrome *c* reductase at pH 7.0 and 25°C. However, extended incubation for more than 60 min did not change the enzyme activity at each concentration of PCMB.

Effect of ligands on the inactivation of the enzyme by PCMB

The competitive inhibitors $NADP^+$ and 2′-AMP protect the enzyme against inactivation by PCMB (Fig. 4), indicating that the fifth and sixth -SH groups modified by the reagent are likely to be present at the vicinity of the active site (probably the NADPH binding site) of NADPH-cytochrome *P*-450 reductase. When 1 mM $NADP^+$ was included in the incubation of PCMB and the reductase, practically no loss of catalytic activity was evident even after 60 min. From this result, we could suggest the importance of sulfhydryls to catalysis even though *N*-ethylmaleimide was not inhibitory. Essentially similar results were obtained with DCPI and ferricyanide as electron acceptor. As shown in Fig. 5, the rate of activation of the reductase by this reagent does not increase linearly with increasing reagent concentration and exhibits a different degree of stimulative effect among different electron acceptors. The activation of the enzyme was found to be accompanied by the reaction of four sulfhydryl residues with PCMB whether 2′-AMP is present or not in the incubation. However,



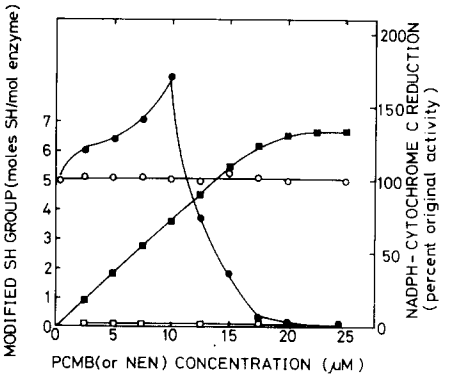


Fig. 1. Determination of sulfhydryl groups in the holoenzyme. Determination of sulfhydryl groups modified was carried out by A_{250nm} change. Holoenzyme (3.24 μM protein; 1.92 mol flavin/mol enzyme) was titrated with PCMB at 25°C. Each 20 μl 5 · 10^{-4} M PCMB was added after 30 min intervals and monitored by following the absorbance at 250 nm (Curve 0,1,2, ---- 11). Modification was completed in this time as judged by the absorption change. The inset shows the absorption changes occurring at 250 nm in the reductase (○) with the absorbance of the native enzyme without PCMB taken as zero and only the buffer (●).

Fig. 2. Effect of PCMB or *N*-ethylmaleimide on NADPH-cytochrome *c* reductase activity. The incubation mixture contained the holoenzyme (3.38 μM protein/6.5 μM flavin) and either PCMB or *N*-ethylmaleimide (NEM) in 0.1 M potassium phosphate buffer, pH 7.0/10% glycerol. The changes of the activity treated with PCMB (●) or *N*-ethylmaleimide (○) and the number of modified -SH groups reacted with PCMB (■) or *N*-ethylmaleimide (□).

the reactivity of these four sulfhydryls is dependent on the existence of the ligand in the incubation mixture. The NADPH-DCPI reductive activity indicated about 2-fold enhancement in the presence of the ligand within the indicated extent of -SH groups modified. This conspicuous activation was not observed when ferricyanide was used as electron acceptor instead of DCPI. On the other hand, the inactivation rate increases linearly with higher concentration of PCMB in the same way as shown in NADPH-cytochrome *c* reductase activity. In the presence of 1 mM 2′-AMP the lower accessibility of the essential two thiol groups toward PCMB was distinctively observed, and it is likely that at higher concentrations of PCMB begins to compete with AMP for the active site (probably the NADPH-binding site).

The fluorescence enhancement of the flavin bound with the protein was observed in both titrations with and without 2′-AMP, however, the flavin fluorescence rapidly increased with an accompanying loss of the enzyme activity upon binding of PCMB to five or more sulfhydryl groups. While approximately the same fluorescence quenching as for native enzyme could be observed when four -SH groups were titrated with PCMB.

Anaerobic reduction of PCMB-treated NADPH-cytochrome P-450 reductase

As shown in Fig. 6, considerable molar excess of

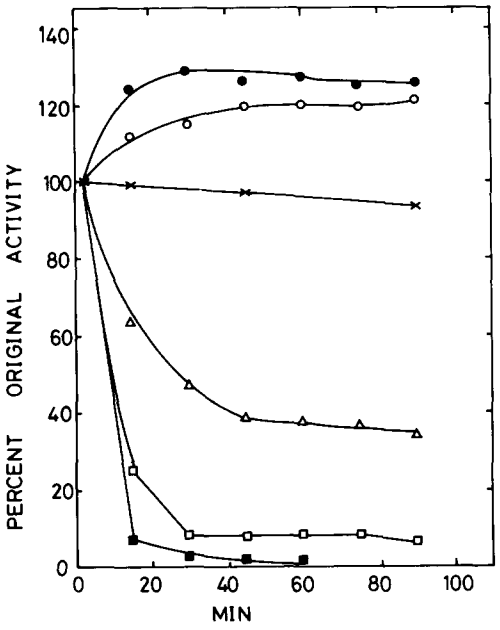


Fig. 3. Incubation of the holoenzyme with PCMB. Holoenzyme (3.38 μM protein/6.5 μM flavin) was incubated with PCMB at the indicated concentrations in 0.1 M potassium phosphate buffer, pH 7.0/10% glycerol (total volume, 0.5 ml). After preincubation for 60 min, 10 μl were removed and used directly in the standard activity assay (cytochrome *c* reductase activity). A control was prepared without PCMB (X). The concentrations of PCMB used were 5 μM (○), 10 μM (●), 15 μM (△), 20 μM (□) and 30 μM (■), respectively.

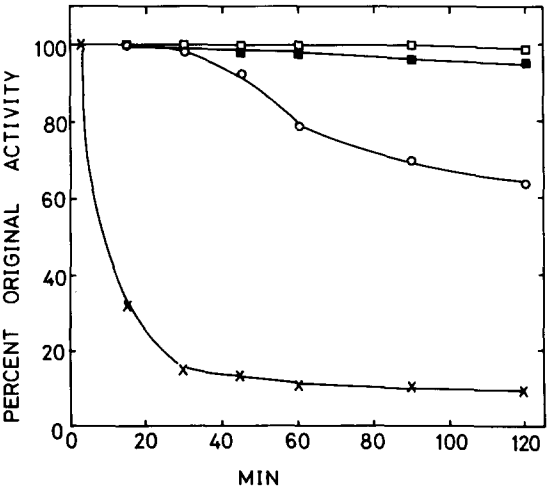


Fig. 4. Effect of ligands on the inactivation of NADPH-cytochrome *P*-450 reductase by PCMB. The incubation mixture contained the holoenzyme (3.52 μM protein/1.92 mol flavin/mol protein), 20 μM PCMB and the indicated concentration of the ligand. □, 1 mM $NADP^+$; ■, 0.1 mM $NADP^+$; ○, 1 mM 2′-AMP and X, no ligand. The reaction was carried out at 25°C.

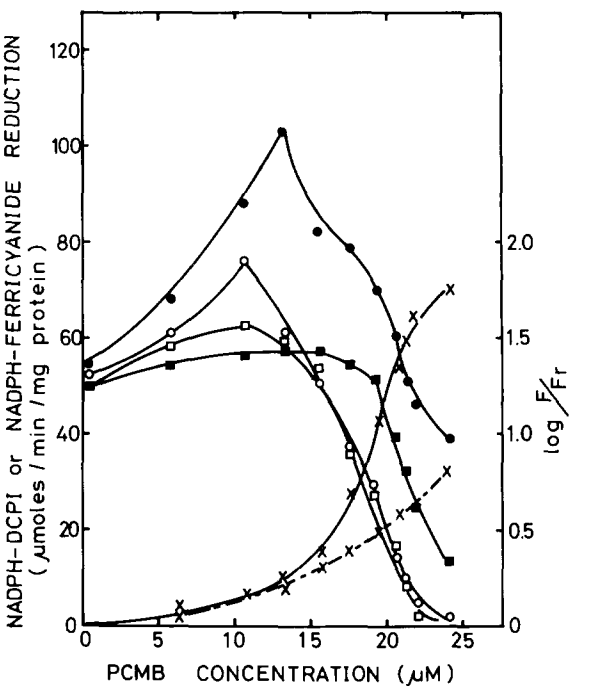


Fig. 5. Activation and inactivation of the holoenzyme with PCMB. NADPH-cytochrome *P*-450 reductase (3.38 μM protein/1.92 mol flavin/mol protein) in 0.1 M potassium phosphate buffer, pH 7.0/20% glycerol were treated with PCMB in the presence or absence of 1.0 mM 2′-AMP at 25°C for 60 min. Total volume was 2.0 ml. 10-μl aliquots were used for assay of DCPI reductase activity (○, without 2′-AMP; ●, with 2′-AMP) and ferricyanide reductase activity (□, without 2′-AMP; ■, with 2′-AMP). Flavin fluorescence at 525 nm when excited at 455 nm was measured under the same condition (X———X, without 2′-AMP; X- - - - -X, with 2′-AMP). The logarithm of the ratio of fluorescence intensity after PCMB addition (F) to that before addition (F_r) is plotted.

PCMB (6–7 mol PCMB/mol protein) produces the appearance of a pronounced shoulder centered around 475 nm and a weak blue shift of the absorbance maximum at 380 nm (spectral curve 4 and 5). After addition of more equivalents of PCMB, no further blue shift and distinctive shoulder occurs. In connection with this spectral change, it should be noted that the flavin fluorescence abruptly increases and an inhibitory effect by PCMB begins to appear.

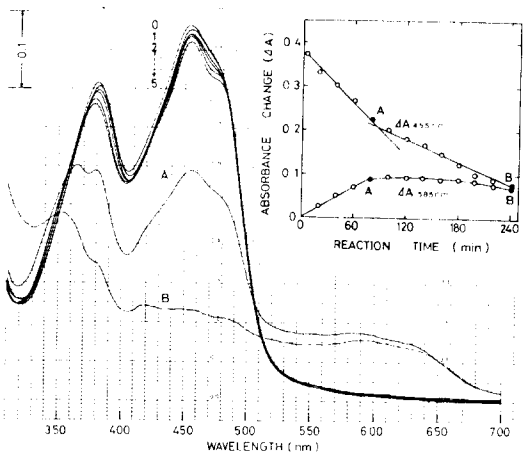


Fig. 6. Titration of the holoenzyme with PCMB and subsequent reduction by NADPH under anaerobic condition. The enzyme (41.7 μM flavin, 1.92 mol flavin/mol protein) in 2 ml of 0.1 M potassium phosphate buffer, pH 7.0/10% glycerol was titrated aerobically with PCMB at 25°C. Curve 0, 0 M; curve 1, 30 μM; curve 2, 60 μM; curve 3, 90 μM; curve 4, 120 μM; curve 5, 150 μM PCMB. After PCMB titration, spectral changes during the anaerobic reduction with 0.2 mM NADPH were measured at 25°C in the presence of 1 μM methylviologen. Curve A and B, the spectra at the stage representing approx. 25 and 75% reduction of flavin, respectively. The same concentration of NADPH and the buffer were added to the reference cuvette. The inset shows the absorption changes occurring at 455 and 585 nm during the reduction at each interval with the absorbance of fully-reduced enzyme taken as zero.

After a completion of the titration of NADPH-cytochrome *P*-450 reductase with 7 equiv. of PCMB/mol protein, the anaerobic reduction of the reductase with NADPH was also carried out in the presence of a small amount of methylviologen. During the stepwise addition of reducing equivalent (0.25–1.0 mol NADPH/mol flavin), spectroscopic changes did not take place at 455 and 585 nm similar to those observed with the native enzyme, but further additions of NADPH up to about 5.0 mol/mol flavin caused very slow reduction of the flavin. The process is brought to completion by addition of an excess amount of NADPH and by spending a long time at 25°C (about 4 h). From these results, it was suggested that the lack of cytochrome *c*, DCPI or ferricyanide reductase activity by the enzyme treated with PCMB to excess may be ascribed to the extremely slow reduction of flavin. The flavin semiquinone formed during the anaerobic reduction of the reductase with NADPH was also followed by simultaneous observation of absorption at 455 and 585 nm as depicted in the inset. During the first phase of the reduction, the decrease in absorbance at 455 nm paralleled the increase in absorbance at 585 nm. During the second phase, the absorbance at 455 nm continued to decrease, and there was little change in flavin semiquinone concentration. Since the reduction of the modified flavoprotein can be conveniently slow to be followed by a recording spectrophotometer over 4 h, we found that these partially reduced oxidation-reduction intermediates shown in curve A and curve B were identified as 1-electron reduced semiquinone (FAD-FMNH ·) and 3-electron reduced species ($FADH_2$-FMNH ·) of the enzyme [1,4,16]. In addition, curve B is easily converted to curve A upon exposure to air and curve A is stable for a few hours in air. As reported already [1,4], the spectrum of the air-stable semiquinone form of the native enzyme is characterized by an absorption shoulder at 635 nm other than 585 nm, but O_2-sensitive state does not show the shoulder at 635 nm while the semiquinone absorption at 585 nm remains. The has been interpreted as the following: that the semiquinone species (FAD-FMNH·, O_2-stable and a shoulder at 635 nm) is converted to the semiquinone of the other flavin (FADH·-$FMNH_2$, O_2-sensitive and absorption at 585 nm) because the shoulder at 635 nm disappears [1]. However, we observed the absorption spectrum of 3-electron reduced semiquinone with a prominent shoulder at 635 nm similar to that observed in anaerobic titration of the enzyme with dithionite (Fig. 7 and inset). This may be a basic difference in the absorption spectrum of 3-electron reduced form between the native enzyme and the inactivated enzyme caused by the modification of catalytic -SH groups. After similar spectral perturbations were observed with the complete modification of -SH groups, it was possible to titrate NADPH-cytochrome *P*-450 reductase stoichiometrically (three steps) with sodium dithionite in the presence of a small amount of methylviologen. However, the rate of reduction of the PCMB-treated flavoprotein was much slower than that of the native enzyme even if a mediator was present. In the first 1.0-electron equivalent was consumed per two flavins (curve C). A further addition of dithionite

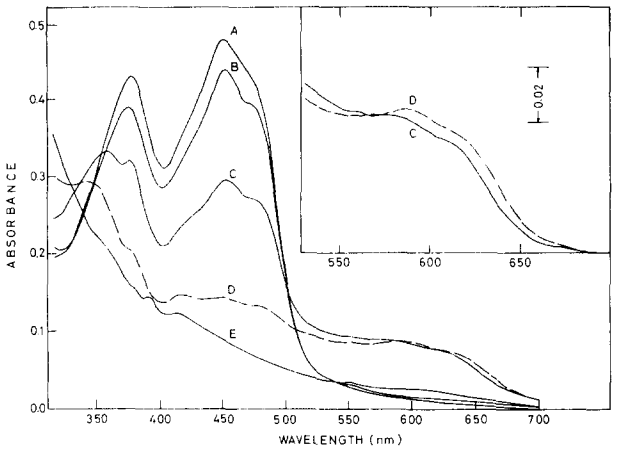


Fig. 7. Spectra of oxidized, air-stable and air-sensitive semiquinone forms of the reductase inactivated with PCMB. 2 ml of the native oxidized enzyme (curve A, 41.7 μM flavin; FMN/FAD, 0.93) in 0.1 M potassium phosphate buffer, pH 7.0/20% glycerol was incubated with 150 μM PCMB at 25°C for 60 min. After observations of spectral changes and inactivation (curve B), 12 μM (curve C), 35 μM (curve D) and 50 μM dithionite (curve E) were anaerobically added to the enzyme in the presence of 2 μM methylviologen, and then each spectrum was recorded when the system had reached each reduction level of flavin. The inset shows the magnified absorption spectra (curve C and D) at near infrared region.

(2-electron equivalent per two flavins) caused a decrease of absorbance at 455 nm and showed the same type of semiquinone with a shoulder around 635 nm (curve D). An additional 1.0-electron equivalent per two flavins produced an extremely slow change of absorbance at 585 nm to the fully reduced state (curve E). It is therefore important to determine whether this 3-electron reduced form of semiquinone is FMN semiquinone ($FADH_2$-FMNH ·) or FAD semiquinone FADH ·-$FMNH_2$).

Modification of FMN-depleted enzyme by PCMB

The preparation of FMN-depleted reductase used contained 95% or more of FAD present in the native enzyme but only a few percent of the original amount of FMN. The activity of the FMN-depleted reductase was very low with DCPI and cytochrome *c*. These relative activities correspond closely to the amount of FMN remaining in the preparation.

In order to determine whether 3-electron-reduced semiquinone of PCMB-treated holoenzyme is attributable to FAD or FMN, the spectral properties of the FMN-depleted and holoenzyme were compared. As indicated in Fig. 8, the exidized form (curve 2) after inactivation is spectrally similar to the holoenzyme. However, NADPH is scarcely possible to reduce the flavin in FMN-depleted enzyme after complete PCMB titration. When dithionite was used in place of NADPH, the inactivated FMN-depleted reductase was extremely slowly reduced in the presence of a small amount of methylviologen. We could indicate that the flavin semiquinone state derived from enzyme treated to excess with PCMB is air unstable and spectrally corresponds to FAD semiquinone without a shoulder at 635 nm of native FMN-depleted enzyme (curve 3, 0.25 mol dithionite/mol flavin; curve 4, 0.5 mol dithionite/mol flavin). The absorbance change at 585 nm observed during the anaerobic titration paralleles that at 455 nm. These results suggest that 3-electron-reduced flavin semiquinone with a shoulder band at 635 nm observed in the inactivated holoenzyme may not be designated as FAD semiquinone (FADH ·-$FMNH_2$) but FMN semiquinone ($FADH_2$-FMNH ·).

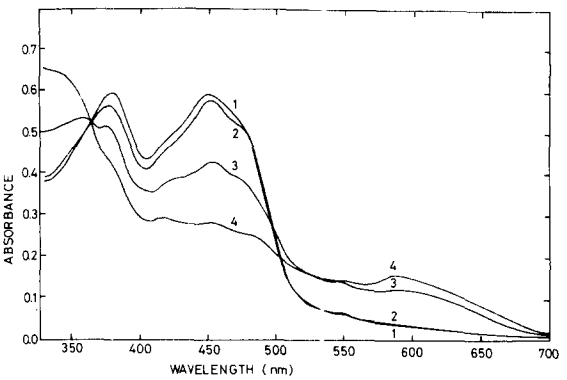


Fig. 8. Anaerobic titration of FMN-depleted enzyme with dithionite. For the oxidized spectrum (curve 1), the cuvette contained 50.5 μM flavin (FMN/FAD, 0.02) in a total volume of 2 ml 0.1 M potassium phosphate buffer, pH 7.0/20% glycerol. Curve 2 was obtained upon addition of 0.5 mM PCMB in the oxidized from. After complete modification of -SH groups by PCMB, the FMN-depleted enzyme was titrated at 25°C in the presence of methylviologen (2 μM) with 5 mM sodium dithionite dissolved in 10 mM potassium phosphate buffer, pH 8.3; curve 3, 15 μM dithionite; and curve 4, 30 μM dithionite.

Discussion

NADPH-cytochrome *P*-450 reductase has 6–7 reactive sulfhydryl groups with PCMB, which may be differentiated as follows: four are relatively exposed and, therefore, easily modified by PCMB but are not essentially involved in the catalytic reaction; two or three are relatively buried and become reactive to the modifying reagent only upon some configurational change of the enzyme after four easily accessible -SH groups are blocked. These critical sulfhydryl groups for catalytic function appear to exist in a part of the NADPH-binding site, which is located very near to the isoalloxazine ring of FAD. Because active-site sulfhydryl groups in both holoenzyme and FMN-depleted enzyme could be protected in the same way against inactivation by $NADP^+$, which is a competitive inhibitor of the reductase with respect to the cosubstrate NADPH. In addition, the rapid release of the fluorescence quenching of FAD at 525 nm when two essential -SH groups were attacked by PCMB is probably due to the loss of tight binding of the flavin. It has been shown that an excessibly PCMB-treated holoenzyme was progressively reduced to the semiquinone and the hydroquinone as the concentration of either NADPH or dithionite was increased, although the rate was very slow. Similar effects were observed in FMN-depleted enzyme, however, NADPH was no longer the effective reductant to produce the FAD semiquinone and hydroquinone form after titration with 6–7 equiv. PCMB/mol protein. In contrast to the native enzyme, 3-electron-reduced semiquinone derived from the holoenzyme inactivated by PCMB has an absorption shoulder at 635 nm (Figs. 6 and 7), whereas, the FAD semiquinone of FMN-depleted enzyme subjected to blocking of the essential -SH groups is O_2-sensitive and without a shoulder at 635 nm (Fig. 8). In addition, an appreciable spectral perturbation of oxidized flavin chromophore with the appearance of a pronounced shoulder at 475 nm and a slight blue shift at 380 nm begins to occur after approx. 5–7 equiv. PCMB/mol protein are added to FMN-depleted enzyme as well as holoenzyme. From these results, it is likely that some small difference in the redox properties of the FAD moiety cannot be excluded in addition to a steric hinderance of NADPH-binding site caused by the block of catalytically essential -SH groups. In marked contrast, the four easily modified -SH groups at the lower concentration pf PCMB could particularly influence the FMN-binding site which plays an essential role in the reduction of cytochrome *P*-450, cytochrome *c* or DCPI, as evidenced by comparing the stimulative effect on the holoenzyme with that observed for the FMN-depleted enzyme [4].

We postulate that the process of -SH modification in NADPH-cytochrome *P*-450 reductase could probably occur by two steps of steric changes. The limited titration of four sulfhydryl groups with a low concentration of PCMB shows a global conformational change of the reductase which may increase the reactivity of cytochrome *c* and DCPI to FMN moiety. On the other hand, it seems likely that the block of two essential -SH groups by additional PCMB titration completed the loss of activity dependent probably on the local conformation change of the active center involving the NADPH-binding site being located in close proximity to the FAD moiety.

In connection with this complicated behavior, it should be noted that the conceivable perturbation of the flavin chromophore may diminish flavin (FAD) - flavin (FMN) interaction and consequently, electron transfer between the two flavins is interrupted. Because in enzyme modified to excess, we could not observe the same 3-electron-reduced semiquinone ($FADH\cdot$-$FMNH_2$) without a shoulder at 635 nm as confirmed in native enzyme, which is supposed to be the active form for dominating the catalytic reduction of cytochrome *P*-450 [1,4,17], suggesting that for FAD-FMN interaction, the 3-electron-reduced state ($FADH_2$-$FMNH\cdot$) does not undergo an internal electron transfer to give $FADH\cdot$-$FMNH_2$, followed by a 1-electron reoxidation. Clearly, much work is required to elucidate the functional mechanism of these -SH groups in NADPH-cytochrome *P*-450 reductase.

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