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Properties of the Stable Aerobic and Anaerobic Half-Reduced States of NADPH–Cytochrome *c* Reductase†

Bettie Sue Siler Masters,* Russell A. Prough, and Henry Kamin

ABSTRACT: The microsomal flavoprotein, NADPH-cytochrome *c* reductase, has been reexamined to determine: (1) the nature of the flavine bound to the enzyme and (2) the oxidation-reduction state of the "half-reduced" form of the flavoprotein. Iyanagi and Mason [Iyanagi, T., and Mason, H.S. (1973), *Biochemistry 12,* 2297] have recently proposed that NADPH-cytochrome *c* reductase contains both FAD and FMN as prosthetic groups in lieu of FAD as the sole constituent, as suggested by all previous studies of this enzyme. The data presented herein, utilizing the recently published fluorometric procedure of Faeder and Siegel [Faeder, E. J., and Siegel, L. M. (1973), *Anal. Biochem. 53,* 332] for the determination of FAD and FMN in mixtures, confirm the conclusions of Iyanagi and Mason for both rat and pig liver reductase preparations. Data for other flavoproteins are also presented. Iyanagi and Mason have also concluded that the air-stable "semiquinone" is a form of NADPH-cytochrome *c* reductase reduced by one electron per two flavines (F-FH). The present studies, however, do not agree with this conclusion, but instead support our previous results which indicate that *both* the aerobic and anaerobic half-reduced states of this flavoprotein exist in the two-electron reduced form (FH-FH). Removal of $NADP^+$ does not affect the spectrum of the air-stable half-reduced form of the flavoprotein, nor does it affect the back titration of this intermediate by potassium ferricyanide. The possible implications of these observations on the catalytic cycle of the flavines of NADPH-cytochrome *c* reductase are discussed.

The isolation of liver NADPH-cytochrome *c* reductase (EC 1.6.2.4) was first achieved by Horecker (1950) from pig liver acetone powder. The enzyme was subsequently identified as a microsomal constituent by Williams and Kamin (1962) and Phillips and Langdon (1962). Both groups purified the enzyme and studied its kinetic and physical properties. The studies of Horecker (1950), Williams and Kamin (1962), Phillips and Langdon (1962), Nishibayashi *et al.* (1963), and Omura and Takesue (1970) all indicated that FAD was the prosthetic group of NADPH-cytochrome *c* reductase. Masters *et al.* (1965b) suggested that the flavoprotein contained 2 mol of flavine with a minimal molecular weight of 35,000–40,000 g mol^{-1} while Masters and Ziegler (1971) reported that the homogeneous enzyme had two flavines per mol, and a molecular weight of 68,000 determined by sedimentation equilibrium. In the latter studies, however, only spectrophotometric determinations of flavine content were performed, since it was assumed on the basis of all of the previous data that FAD was the sole constituent of the flavoprotein.

Recently, Iyanagi and Mason (1973) reported that their preparations of NADPH-cytochrome *c* reductase from rat and pig liver microsomes contained both FAD and FMN in equimolar quantities, rather than FAD alone. We, therefore, reexamined our own available preparations of this enzyme, utilizing the recently described spectrofluorometric procedure of Faeder and Siegel (1973). The data to be presented show that our preparations do indeed contain both FAD and FMN in approximately equimolar quantities.

The studies of Masters *et al.* (1965a,b) and of Kamin *et al.* (1966) led to the proposal that the mechanism of catalysis by NADPH-cytochrome *c* reductase involved a redox cycle in which the two flavines acted cooperatively, cycling

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between a fully reduced and a "half-reduced" form. The reduction of both one- and two-electron acceptors appeared to involve a similar mechanism. A number of observations led to the proposal of this mechanism (Masters *et al.*, 1965a,b; Kamin *et al.*, 1966). These observations included as an important parameter the interpretation of the reduction state of a spectral species of enzyme (Masters *et al.*, 1965b) obtained either (1) by anaerobically adding 0.5 mol of NADPH to fully oxidized enzyme or (2) by adding excess NADPH aerobically, and permitting air oxidation to proceed until all NADPH was oxidized and a stable flavine spectrum was achieved. This "air-stable" flavine spectrum was indistinguishable from that of the anaerobically produced spectrum resulting from the addition of 0.5 mol of NADPH per mol of total flavine. This air-stable species was considered to represent "half-reduced flavin" (FH–FH), containing one reducing equivalent per flavine moiety. The authors (Masters, *et al.*, 1965b; *vide infra*, footnote 3) carefully refrained from using the term semiquinone, since there was not sufficient evidence for describing the nature of the partition of the two electrons between two, presumably cooperating, flavines. However, it was certainly apparent that this "half-reduced" form was refractory to oxidation by O_2 or by acceptors such as ferricytochrome *c* or 2,6-dichlorophenolindophenol. Thus, this state of the enzyme represented the functionally "oxidized" form of the flavine during its redox cycle.

The recent studies of Iyanagi and Mason (1973) and of Iyanagi *et al.* (1974) have also challenged the designation of this form as a two-electron intermediate, and suggest that it contains one electron per two flavines, *i.e.*, F–FH. The present paper addresses itself not only to the description of the nature of the flavine prosthetic groups but to experimental support of the designation of the oxidation state of the "air-stable intermediate" as FH–FH.

Experimental Procedure

Materials. FAD, FMN, and cytochrome *c* (horse heart, type VI) were obtained from Sigma Chemical Co. Pancreatic steapsin was obtained from Nutritional Biochemical Co. and purified according to the procedure described by Masters *et al.* (1967). Potassium ferricyanide was Baker Analyzed reagent and the concentrations of the stock solutions were determined spectrophotometrically using the $\epsilon_M^{420nm} = 1.03 \times 10^3\ M^{-1}\ cm^{-1}$. NADPH was obtained from P-L Biochemicals and the concentrations of stock solutions were determined from molar absorptivities at 260 ($\epsilon_M = 14.9 \times 10^3\ M^{-1}\ cm^{-1}$) and at 340 nm ($\epsilon_M = 6.2 \times 10^3\ M^{-1}\ cm^{-1}$).

Adrenodoxin reductase from beef adrenal cortex, purified by Mr. David Lambeth, and salicylate hydroxylase from a soil bacterium, purified by Mr. Robert Presswood by the method of White-Stevens and Kamin (1972), were obtained from Dr. Kamin's laboratory. Purified NADPH-dependent mixed-function amine oxidase (N-oxidase) was provided by Dr. Daniel M. Ziegler, The University of Texas, Austin, Tex. (Ziegler and Mitchell, 1972).

Preparation of Microsomes. Microsomes were prepared as described previously (Masters *et al.*, 1967) and frozen until solubilized. The solubilization was performed according to the procedure of Masters *et al.* (1967) with a 15-min incubation at 37° with the partially purified pancreatic steapsin, and the supernatant was further purified as described by Prough and Masters (1973). The purified reductase preparations had turnover numbers of 1250–1350 mmol of cytochrome *c* /min per mmol of total flavine. Similar preparations tested in the reconstituted drug metabolism assay system of Coon *et al.* (1973) were capable of reducing cytochrome P-450 only very slowly and could not support hydroxylation of benzphetamine when added to cytochrome P-450 and lipid fractions.

Methods. Spectra were recorded with a Cary 14R recording spectrophotometer thermostatically controlled at 25°. Kinetic determinations were performed in a Hitachi 124 recording spectrophotometer at 25°. Spectrofluorometric analyses were performed in a Farrand or an Aminco-Bowman spectrofluorometer outfitted with a Sargent SRG recorder. The fluorescence excitation maximum was 450 nm; the emission maximum was recorded at 535 nm. The fluorescence emission spectra were recorded for each sample for comparison with standard FAD and FMN samples. Commercial samples of FAD and FMN were chromatographed on DEAE-cellulose using 0.1 M potassium phosphate (pH 6.8) as the eluting buffer. The FAD and FMN solutions gave absorbance ratios (375 nm:450 nm) of 0.81 and 0.83, respectively, in agreement with Beinert (1960). All operations including chromatographic, spectrophotometric, and spectrophotofluorometric procedures were performed under low illumination.

The flavine concentrations were determined by measuring the absorbance of flavine at 453 and 500 nm and utilizing the absolute molar absorptivities of 11.7×10^3 (453 nm) and $4.85 \times 10^3\ M^{-1}\ cm^{-1}$ (500 nm). As reported earlier (Masters *et al.*, 1965b) 10–20% of the total flavine is not reduced by NADPH and probably does not represent catalytically active flavine (see Discussion).

Results

Spectrophotofluorometric Determination of the Flavine Content of NADPH–Cytochrome c Reductase. It was recently reported by Iyanagi and Mason (1973) that NADPH–cytochrome *c* reductase, purified from both rabbit and pig liver microsomes by either trypsin or detergent solubilization techniques, contains equimolar quantities of FAD and FMN. In view of these data, a reexamination of the flavine content of NADPH–cytochrome *c* reductase, prepared by lipase solubilization, was undertaken. This lipase-solubilized preparation was reported by Masters and Ziegler (1971) to have a molecular weight of 68,000 g mol^{-1} as determined by sedimentation equilibrium ultracentrifugation analysis. The flavine contents obtained from a number of preparations of NADPH–cytochrome *c* reductase by the spectrophotofluorometric procedure of Faeder and Siegel (1973) are listed in Table I. In addition, Table I shows data obtained with three other flavoproteins containing FAD as the only prosthetic group, including pork liver microsomal mixed-function amine oxidase (N-oxidase). These results show unequivocally that NADPH–cytochrome *c* reductase contains equimolar quantities of FAD and FMN. Earlier studies by Masters *et al.* (1965b), Baggott and Langdon (1970), and Masters and Ziegler (1971) established a minimal molecular weight of 1 mol of flavine per 35,000 g of enzyme protein and the report of Iyanagi and Mason (1973) confirms these calculations.

Aerobic Titration of Pig Liver NADPH–Cytochrome c Reductase with NADPH and Ferricyanide. It is notable that the aerobic addition of less than 0.5 mol of NADPH/mol of flavine, *i.e.*, 1 electron equivalent of NADPH per 2 electron equivalents of flavine, results in a decrease in absorbance at 453 nm and a concomitant increase at 585 nm

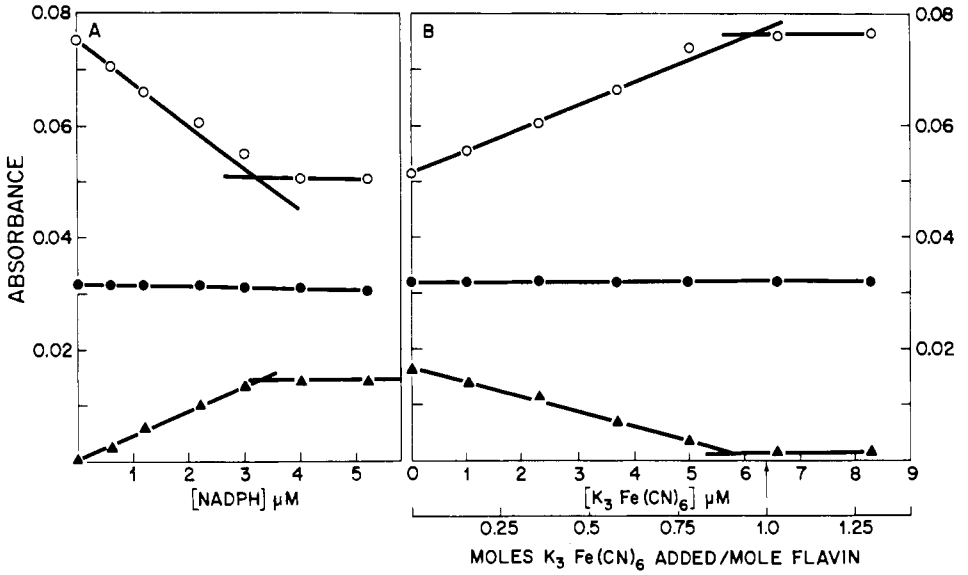

FIGURE 1: Aerobic titration of porcine liver microsomal NADPH-cytochrome *c* reductase with NADPH and $K_3Fe(CN)_6$. Absorbance changes at 453 (○), 500 (●), and 585 (▲) nm are plotted against either NADPH (A) or $K_3Fe(CN)_6$ (B) added. The abscissa in B is also labeled as moles of $K_3Fe(CN)_6$ added per mole of flavine as the data of Figure 7 of Iyanagi and Mason (1973). Pig liver microsomal NADPH-cytochrome *c* reductase was diluted in 0.1 M potassium phosphate (pH 7.7) to a final concentration of 6.4 μM in a total volume of 3 ml and titrated with NADPH and then $K_3Fe(CN)_6$ sequentially under aerobic conditions at 25°. The total volume change was less than 3.5% and was corrected for upon each addition.

Table I: FAD and FMN Contents of Various Flavoproteins Including NADPH-Cytochrome *c* Reductase.[a]

Flavoprotein	Rel Fluorescence Intensity (pH 2.6–7.7)	% Flavine
Pig liver NADPH–cytochrome *c* reductase	1.14 ± 0.04 mean ± S.D. (n = 5)	FAD 54.6 ± 2.2 FMN 45.4 ± 2.3
Rat liver NADPH–cytochrome *c* reductase	1.2	FAD 58 FMN 42
Salicylate hydroxylase	3.9	FAD 98 FMN 2
Adrenodoxin reductase	3.9	FAD 98 FMN 2
Pig liver mixed function amine oxidase (N-oxidase)	3.8	FAD 98 FMN 2

[a] Performed according to the method of Faeder and Siegel (1973).

in the flavoprotein spectrum. Such a titration would not be possible with a flavoprotein which reacted at an appreciable rate with oxygen. Figure 1A shows the results of a titration of NADPH-cytochrome *c* reductase under aerobic conditions. This titration was performed in 0.1 M potassium phosphate buffer (pH 7.7) containing 1 mM EDTA in accordance with the experiment of Iyanagi and Mason (1973). The 453-nm point monitors the reduction of oxidized flavine to its various reduced states; the 500-nm point represents an isosbestic point between oxidized and half-reduced forms of the flavoprotein (see Figure 3); the 585-nm point monitors the longer wavelength absorbing species representing the half-reduced state (FH–FH) of the flavoprotein. As shown in Figure 1A, the addition of NADPH to 6.4 μM NADPH-cytochrome *c* reductase under aerobic conditions results in equivalence points in both the 453- and 585-nm titration curves at 3.2μM NADPH. As can be seen, the isosbestic point at 500 nm remains constant, since the fully reduced flavoprotein is rapidly reoxidized to its stable half-reduced form at concentrations greater than 0.5 mol of NADPH/mol of flavine. Figure 1B shows a back-titration of the same enzyme preparation with $K_3Fe(CN)_6$. It can be seen that the flavoprotein returns to its oxidized form after the addition of approximately 6 μM $K_3Fe(CN)_6$, as seen from the equivalence points at 453 and 585 nm. Again, the 500-nm isosbestic point remains constant throughout the titration. The production of the air-stable intermediate results in a redox state which upon reoxidation by $K_3Fe(CN)_6$ requires the removal of 2 electron equivalents. The data of Figure 1B are also plotted on the abscissa in a manner identical with that of Iyanagi and Mason (1973; Figure 7). It can be seen clearly that the break point in the titration curve occurs at approximately 0.9 mol of $K_3Fe(CN)_6$/mol of flavine, also indicating that 2 electron equivalents remain in the air-stable half-reduced form of the flavoprotein.

Aerobic and Anaerobic Titration of Rat Liver NADPH-Cytochrome c Reductase with NADPH. In order to discount the possibility that the source of the enzyme preparation might affect the titration data, a similar experiment was performed on rat liver NADPH-cytochrome *c* reductase under the conditions originally utilized by Masters *et al.* (1965a,b), *i.e.*, 0.05 M potassium phosphate (pH 7.7), containing 0.1 mM EDTA, with the addition of 0.02% sodium azide. As can be seen by comparison of the aerobic data of Figure 2 with Figure 1A, identical results are obtained, although the ionic strength and enzyme sources have been changed. It is of particular interest to note that under anaerobic conditions, the initial slopes of the titration curves at 453 and 585 nm are similar to those obtained under aerobic conditions. Note that the curves diverge only after the equivalence point of 0.5 mol of NADPH/mol of total flavine is reached. This is in accord with our previous observations (Masters *et al.*, 1965b) that although reduced flavine is formed rapidly under both aerobic and anaerobic conditions, the comproportionation between fully oxidized and fully reduced flavines to yield the half-reduced species is

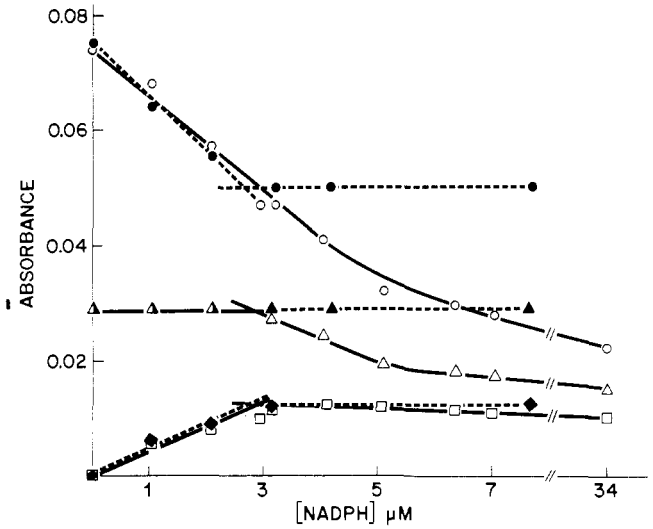

FIGURE 2: Anaerobic and aerobic titrations of rat liver microsomal NADPH–cytochrome *c* reductase. Absorbance changes under aerobic conditions at 453 (●), 500 (▲), and 585 (♦) nm and under anaerobic conditions at 453 (○), 500 (△), and 585 (□) nm are plotted against NADPH added. Rat liver microsomal NADPH–cytochrome *c* reductase was dissolved in 0.05 M potassium phosphate (pH 7.7), containing 0.1 mM EDTA and 0.02% NaN_3, to a final concentration of 6.4 μM in a total volume of 3 ml and titrated with NADPH either anaerobically or aerobically at 25°. The total volume change was less than 3.5% and was corrected for upon each addition.

substantially more rapid than the reoxidation of fully reduced flavoprotein by O_2. Beyond the equivalence point of 0.5 mol of NADPH/mol of flavine (Figure 2; open circles and open squares), no attempt has been made to draw a straight line. This is not only because of the nature of the actual data, but because of previously reported observations (Masters *et al.*, 1965a,b) that a thermodynamic barrier prevents full reduction of the enzyme by NADPH. The anaerobic curve at 453 nm extrapolates to an equivalence point at 6.3 μM NADPH and maximum formation of the longer wavelength species occurs at 2.9 μM. The 500-nm isosbestic point remains until the addition of 2.9 μM NADPH (0.5 mol of NADPH/mol of flavine) and a second break point occurs at 6.1 μM NADPH when formation of fully reduced flavine is maximal. In other experiments, varying the concentration of potassium phosphate from 0.05 to 0.1 M, the concentration of EDTA from 0.1 to 1 mM, and the pH from 7.2 to 8.2, had no effect on these experimental results. Assuming the change in molar absorptivity for fully oxidized to half-reduced states to be 4.3×10^3 M^{-1} cm^{-1} at 453 nm as reported by Masters *et al.* (1965b), the amount of flavine reduced is calculated to be 5.8 μM which corresponds closely to the utilization of 3.1 μM NADPH in the experiment of Figure 1A.

A similar experiment was performed aerobically by titrating trypsin-solubilized (Omura and Takesue, 1970) rat liver microsomal NADPH–cytochrome *c* reductase with NADPH indicating that the equivalence point occurred at 0.5 mol of NADPH/mol of flavine with this enzyme preparation as well.

It is interesting to note that higher ratios of NADPH to flavine than 1:1 were required to reduce the flavoprotein completely under anaerobic conditions. The addition of NADase allowed the anaerobic titration of the flavoprotein with stoichiometric quantities of NADPH to proceed to fully reduced flavoprotein. This effect of NADase suggests that an equilibrium exists between the fully reduced and half-reduced flavine. Since small amounts of $NADP^+$ appear to prevent the complete reduction under anaerobic conditions, the equilibrium constant may be near unity.

Spectra of Aerobic and Anaerobic Half-Reduced NADPH–Cytochrome c Reductase. Figure 3 shows the absorption spectra of rat liver NADPH–cytochrome *c* reductase in the oxidized and half-reduced states and the spectrum obtained upon the addition of excess NADPH (5.4 mol of NADPH/mol of flavine) aerobically. For purposes of comparison, spectra of the half-reduced enzyme produced under aerobic and anaerobic conditions are presented. It is evident that these spectra are identical except for a slight over-reduction in the anaerobic spectrum noted in the 360- and 500-nm (isosbestic point for oxidized and half-reduced) regions. These spectra are presented to demonstrate that there are no anomalies appearing in the aerobically stable half-reduced spectrum as opposed to the anaerobically produced species.

Treatment of Aerobically Produced Half-Reduced

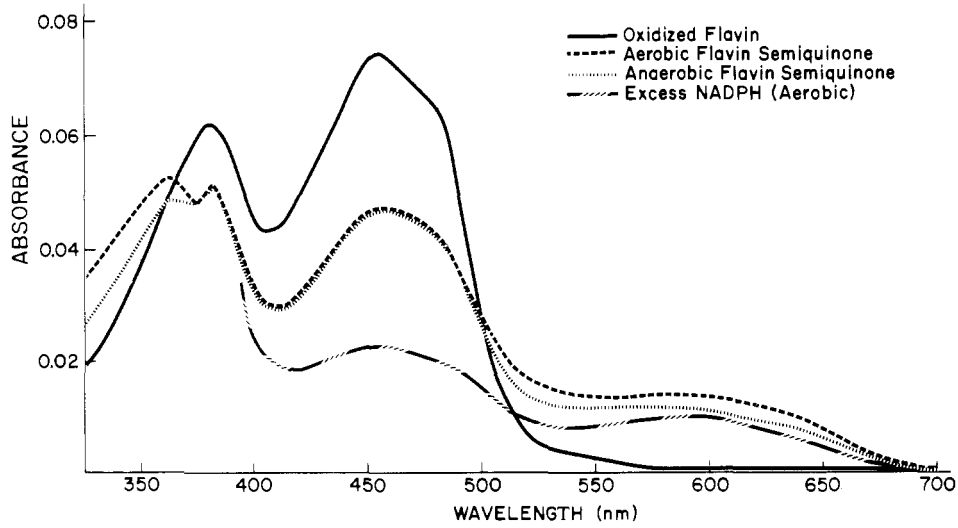

FIGURE 3: Aerobic and anaerobic spectra of rat liver microsomal NADPH–cytochrome *c* reductase. NADPH was added aerobically (- - -), and anaerobically (· · ·) to 6.3 μM oxidized flavoprotein (—) dissolved in 0.05 M potassium phosphate (pH 7.7), containing 0.1 mM EDTA and 0.02% NaN_3. Excess NADPH (34 μM final concentration) was added aerobically (– · · ·).

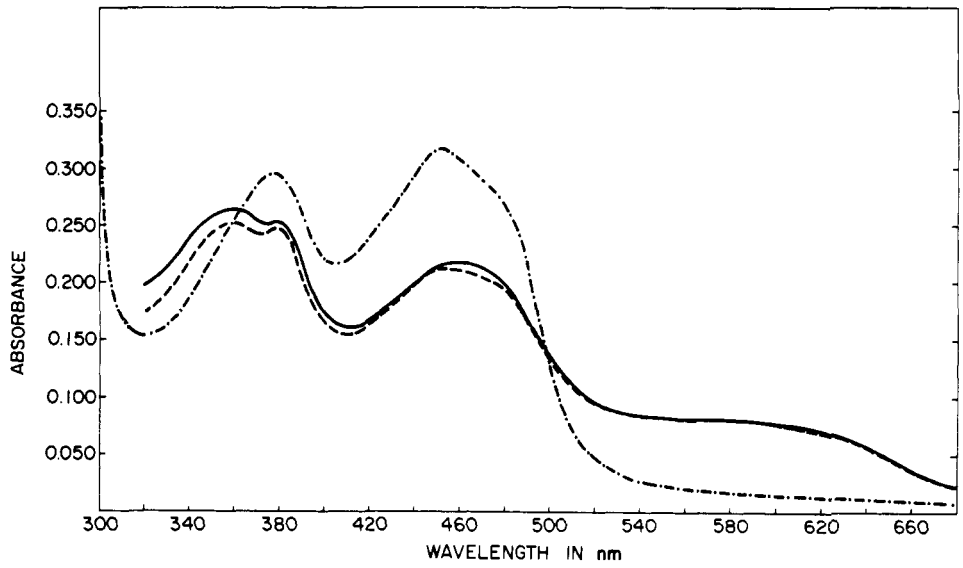

FIGURE 4: Treatment of oxygen-stable half-reduced porcine liver microsomal NADPH-cytochrome *c* reductase with charcoal. The spectra are shown for oxidized (- ·) and half-reduced (—) flavoprotein, and half-reduced flavoprotein passed through charcoal (- - -). To obtain the oxidized spectrum, NADPH-cytochrome *c* reductase was dissolved to a final concentration of 28.1 μM in 3 ml of 0.05 M potassium phosphate (pH 7.7), containing 0.1 mM EDTA. For the air-stable half-reduced spectrum, 2 mol of NADPH/mol of flavine was added aerobically and the flavoprotein was allowed to reoxidize to the half-reduced state. The resulting enzyme solution was then filtered through an acid-washed charcoal-Celite (1:2) column and the spectrum was recorded on a Cary Model 14 recording spectrophotometer. All spectra were corrected for dilution during the various procedures. Such a procedure results in the removal of 99.5% of the $NADP^+$ formed[1].

NADPH-Cytochrome c Reductase with Charcoal. The possibility that $NADP^+$, the oxidized product of the half-reaction with NADPH, could remain attached to the flavoprotein, contribute to the absorption spectrum, and/or act as an electron sink for additional electron equivalents was examined. The experiment shown in Figure 4 was designed to establish the identity of the air-stable half-reduced flavoprotein with the same enzyme preparation after passage through acid-washed charcoal to remove bound and free $NADP^+$.[1] As can be seen, the absorption spectra obtained before and after charcoal treatment are identical.

A preparation similar to the charcoal-treated half-reduced NADPH-cytochrome *c* reductase shown in Figure 4 was back-titrated with $K_3Fe(CN)_6$ to the fully oxidized state. This reoxidation, shown in Figure 5, required 1 mol of $K_3Fe(CN)_6$/mol of reductase flavine, *i.e.*, 2 electron equivalents of $K_3Fe(CN)_6$ per 2 electron equivalents of enzyme-bound half-reduced flavine, thus establishing that $NADP^+$ plays no role as an electron sink in the oxidation-reduction states of NADPH-cytochrome *c* reductase.

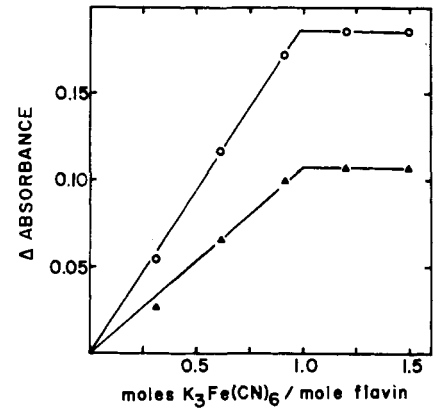

FIGURE 5: Titration of charcoal-treated half-reduced NADPH-cytochrome *c* reductase with $K_3Fe(CN)_6$. The reductase was reduced with 1 mol of NADPH/mol of flavine and allowed to reoxidize in air until no further change in absorbance at 453 nm was observed. One milliliter of this stable half-reduced intermediate was then filtered through 20 mg of acid-washed charcoal-Celite (1:2); 0.95 ml of eluate was collected by vacuum. The enzyme solution containing 54 μM flavine was then back-titrated with increments of $K_3Fe(CN)_6$ until the absorbance at 453 nm remained constant. Plots of the absorbance changes at 453 (○) and 585 nm (▲) are shown in the figure.

Discussion

The reexamination of NADPH-cytochrome *c* reductase became of interest after the recent report of Iyanagi and Mason (1973). These investigators reported that NADPH-cytochrome *c* reductase, isolated from rabbit and pig liver microsomes by either trypsin or detergent solubilization procedures, contained equimolar concentrations of FAD and FMN. Depending upon the method employed, determinations of molecular weight varied from 68,000 to 79,000 g mol^{-1}, resulting in a minimal molecular weight of 1 mol of flavine (FAD or FMN) per 34,000–39,500 g of reductase protein. This paper presents data on spectrophotofluorometric determinations of flavine content in a number of preparations of NADPH-cytochrome *c* reductase, among other flavoproteins. The present study supports the data of Iyanagi and Mason (1973) in that equimolar amounts of FAD and FMN were found in both pig and rat liver microsomal reductase preparations. The minimal molecular weight of the preparations used in this paper was slightly lower (1 mol of flavine/34,000–35,000 g of reductase protein), possibly due to the use of pancreatic lipase (Masters *et al.*, 1967) as solubilizing agent. The use of this lipase preparation was continued in order to maintain continuity with the earlier

[1] The removal of $NADP^+$ from the enzyme preparation was effected through an acid-washed charcoal-Celite (1:2) column. This resulted in the removal of at least 99.5% of added NADPH-*4-t* from the enzyme, as assessed by recovery of pyridine nucleotide from the charcoal-Celite after an ammonia-water-ethanol (1:49:50) wash. These experiments also revealed that NADPH-cytochrome *c* reductase removed hydrogen stereospecifically from the A side of the pyridine nucleotide ring, as reported by Drysdale (1966).

studies of Masters *et al.* (1965a,b) and Masters and Ziegler (1971). In our hands, the trypsin solubilization procedure of Omura and Takesue (1970) yields a preparation quite similar in catalytic and immunochemical properties to the lipase-solubilized preparation originally described by Williams and Kamin (1962). We have observed extra minor bands on disc gel electrophoresis of the trypsin-solubilized preparation which give positive activity stains, indicating that several proteolytic fragments have resulted from the trypsin solubilization procedure. These minor bands are not observed with the lipase-solubilized preparations.

The present study, however, cannot support the conclusions of Iyanagi and Mason (1973) on the reduction state of the aerobically stable partially reduced flavoprotein intermediate. We conclude that this form contains, as previously reported (Masters *et al.*, 1965b), 1 electron equivalent per flavine, *i.e.*, FH-FH. As shown in the experiments of Figures 1 and 2 the oxygen-stable intermediate of reduced NADPH-cytochrome *c* reductase, formed under aerobic conditions, contains 2 electrons. The molar absorptivity used to calculate the concentration of oxidized flavoprotein was assumed to be 11.7×10^3 M^{-1} cm^{-1} at 453 nm, which represents an equimolar mixture of FAD ($\epsilon_M^{450nm} = 11.3 \times 10^3$ M^{-1} cm^{-1}; Beinert, 1960) and FMN ($\epsilon_M^{450nm} = 12.2 \times 10^3$ M^{-1} cm^{-1}; Whitby, 1953). This assumption was found to be valid when comparisons of flavine concentrations, determined by the spectrofluorometric method of Faeder and Siegel (1973) utilizing rechromatographed FAD and FMN standards for the estimation of flavine content, were made with spectrophotometric determinations of flavine (see Table I). Using the assumed molar absorptivity of the enzyme-bound flavine and the known molar absorptivities of pure FAD and FMN, the amount of free flavine released upon boiling was calculated to be 91–96% of the original flavoprotein concentration.

In agreement with Masters *et al.* (1965a,b), between 80 and 90% of the flavine of lipase- or trypsin-solubilized pig or rat NADPH-cytochrome *c* reductase is reducible by *excess* NADPH, as measured by a decrease in absorbance of 450 nm. Since all mechanism studies on this flavoprotein have been performed with the physiological electron donor, NADPH, it is pertinent to consider only NADPH-reducible flavine to calculate the flavine concentration. However, when either an absolute molar absorptivity of 10.7×10^3 M^{-1} cm^{-1} (Iyanagi and Mason, 1973) or the absolute molar absorptivity of 11.7×10^3 M^{-1} cm^{-1} (present paper) is utilized, the discrepancies between the data of Figure 1 and that of Iyanagi and Mason (1973, Figure 7) still cannot be explained. In our hands, the titration of the air-stable half-reduced form of NADPH-cytochrome *c* reductase with $K_3Fe(CN)_6$ demonstrated that 2 electrons remain in this oxidation-reduction state of the enzyme. These results were obtained regardless of the method of formation of the stable intermediate, *i.e.*, reduction of the enzyme aerobically with *excess* NADPH and reoxidation in air to the intermediate *or* titration to the aerobically stable intermediate with graded amounts of NADPH. Virtually identical data were also obtained at several phosphate and EDTA concentrations and at several pH values with both pig and rat liver enzyme preparations.

Furthermore, the conclusion of Iyanagi and Mason (1973) that the air-stable intermediate is F_{ox}-FH remains incompatible with several other experiments of Masters *et al.* (1965a,b). The simultaneous reaction of oxidized enzyme with an approximately equal number of equivalents of NADPH and cytochrome *c* permitted reduction of approximately 1 mol of cytochrome *c*/mol of added NADPH, strongly implying that the other NADPH reducing equivalent remained with the enzyme (Masters *et al.*, 1965b). These experiments, because of the high absorbance of ferrocytochrome *c* at 550 nm, are inherently reliable. In similar experiments, Siegel *et al.* (1972, 1974) using the flavoprotein moiety of *Salmonella* NADPH-sulfite reductase (a 4FAD-4FMN enzyme) obtained results (*vide infra*) which could be clearly differentiated from those obtained with NADPH-cytochrome *c* reductase (Masters *et al.*, 1965b). In addition, Masters *et al.* (1965b) performed experiments with 2,6-dichlorophenolindophenol analogous to those just described with cytochrome *c*. Again, approximately half of the NADPH reducing equivalents entered the dye when enzyme-flavine was present in equimolar quantities with NADPH, implying that one electron per flavine was retained with the enzyme. In the case of 2,6-dichlorophenolindophenol (which has little absorbance at 450 nm), it was possible to demonstrate that the enzyme had the same absorbance at 450 nm at the end of the experiment as that of the "air-stable, half-reduced" form.

Thus, we maintain our previously stated viewpoint that in the stable "half-reduced" enzyme, the flavines exist in the FH-FH oxidation state. Nevertheless, it is most interesting that another FAD-FMN enzyme, NADPH-sulfite reductase of enterobacteria, does, indeed, have the form F_{ox}-FH as a prominent catalytic intermediate (Siegel *et al.*, 1972). In this case, the two types of flavines have been assigned specific roles: the FAD serves as the entry port for NADPH electrons and the FMN is required for transmission of electrons along the complex redox chain of the enzyme (Siegel *et al.*, 1972, 1974). In the case of sulfite reductase, the actual experimental data differ from that with NADPH-cytochrome *c* reductase in two important aspects: (1) in cytochrome *c* reduction experiments (Siegel *et al.*, 1972) analogous to those of Masters *et al.* (1965b), 25% of the NADPH electrons (rather than approximately 50%) remain with the enzyme after one turnover, implying that the residual enzyme form in sulfite reductase is the F_{ox}-FH species; (2) when oxidized sulfite reductase is allowed to react in the stopped-flow spectrophotometer with NADPH, a slight but distinct lag phase is observed in the formation of the FH, but not FH_2, species, whereas with NADPH-cytochrome *c* reductase no such lag is observed. The latter observation with sulfite reductase is compatible with a flavine catalytic cycle (Siegel *et al.*, 1972) in which the flavines have nonequivalent roles. The finding that NADPH-cytochrome *c* reductase has equimolar FAD and FMN makes the model for flavine interaction proposed in Figure 4 by Kamin *et al.* (1966) considerably less attractive (though by no means impossible). This model suggested a functional equivalence for the two flavines which is more credible with an FAD-FAD pair than with an FAD-FMN pair. Further studies will be required to clarify this facet of the mechanism.

It is also pertinent to point out that the data of this paper are consistent with the earlier studies of Masters *et al.* (1965a,b) in which a similar redox intermediate of the NADPH-cytochrome *c* reductase flavoprotein was rapidly formed upon reaction of the reduced enzyme with other one- *or* two-electron acceptors, in addition to oxygen. Other studies, based on stopped-flow kinetic measurements (Masters *et al.*, 1965b; Figure 8) showed that the aerobically stable half-reduced enzyme can be reduced further at rates

more than sufficient to account for catalysis, thus making possible the involvement of this intermediate as the more oxidized form in the catalytic turnover of the flavoprotein.

The electron paramagnetic resonance (epr) studies of NADPH-cytochrome *c* reductase have led to a number of inconsistencies in the establishment of which redox form of the flavoprotein constitutes the true free radical ("semiquinone") form. Exploratory epr experiments were performed[2] in which a free radical was obtained, but the signal yield was low, thus casting some doubt upon the conclusions which could be drawn from our data. We intend to extend these studies further in an attempt to correlate the appearance of free-radical signal in the epr spectrometer with an absorption spectral species of the enzyme. Thus, we have made no attempt to assign the term "semiquinone" or "free radical" to the air-stable, half-reduced form of NADPH-cytochrome *c* reductase.

It should be noted that the studies of Iyanagi and Mason (1973) and Iyanagi *et al.* (1974) did not utilize the solubilization and preparation procedures of Masters *et al.* (1965a,b; 1967), and kinetic data to permit evaluation of the activity of their enzyme were not furnished. Iyanagi and Mason (1973) utilized a modification of the trypsin solubilization procedure of Omura and Takesue (1970) and a deoxycholate-Triton N-101 detergent solubilization procedure for their preparations of NADPH-cytochrome *c* reductase. Our experiments have been performed with both lipase- and trypsin-solubilized preparations under the conditions of Masters *et al.* and of Omura and Takesue (1970) and, in either case, the air-stable, half-reduced intermediate appears to exist in a two-electron reduced state.

As previously pointed out, we have become aware over the years that various preparations of NADPH-cytochrome *c* reductase are reducible by NADPH to varying extents. The reducibility by NADPH is a function of both preparation procedure and age of sample. We have carefully restricted our studies to preparations which are *at least* 80-90% reducible by NADPH. The discrepancies between the results of the two laboratories may well lie in the reducibility of the enzyme preparations, *i.e.*, only flavine bound to active enzyme is reducible by NADPH.

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[2] H. Beinert, G. Palmer, and W. H. Orme-Johnson, unpublished observations.

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