Regulation of Acetyl-CoA Carboxylase by ADP-Ribosylation†

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ABSTRACT: Because of certain similarities between acetyl-CoA carboxylase (ACC) and tubulin, and the recent demonstration of the ADP-ribosylation of tubulin by cholera toxin, we have investigated a potential role for ADP-ribosylation in the regulation of ACC activity. Incubation of purified rat liver ACC with cholera toxin in the presence of millimolar concentrations of [*adenylate*-^{32}P]NAD results in a time-dependent incorporation of ADP-ribose into ACC of greater than 2 mol/mol of enzyme subunit, accompanied by a marked inactivation of enzyme activity. This effect is not mimicked by pertussis toxin, ADP-ribose, or ribose 5-phosphate. Incubation of labeled ACC with snake venom phosphodiesterase and alkaline hydrolysis release ^{32}P-products tentatively identified by high-performance liquid chromatography as 5′-[^{32}P]AMP and [^{32}P]ADP-ribose, respectively. These data are consistent with a mono-ADP-ribosylation of ACC catalyzed by cholera toxin. Phosphodiesterase treatment of inactivated ACC partially restores enzyme activity. The effects of ADP-ribosylation of ACC are expressed both as a decrease in the enzyme V_{max} and as an increase in the apparent K_a for citrate. These results suggest that ACC might be a substrate for endogenous ADP-ribosyltransferases and that this covalent modification could be an important regulatory mechanism for the modulation of fatty acid synthesis in vivo.

Acetyl-CoA carboxylase (ACC), the rate-limiting enzyme of fatty acid biosynthesis, is regulated both by allosteric mechanisms and by covalent phosphorylation (Witters, 1985). In recent studies, we have been exploring other potential modes of regulation of its catalytic activity. We were struck by several similarities between ACC and tubulin. Both proteins are interconverted between protomeric and polymeric forms, and this interconversion is temperature sensitive, the polymer being formed at elevated temperatures and dissociating at cold temperatures (Timasheff & Grisham, 1980; Buechler & Gibson, 1984). Guanine nucleotides may participate in the polymerization reaction for each protein (Timasheff & Grisham, 1980; Buechler & Gibson, 1984; Witters et al., 1981). The polymeric state of both microtubules and ACC can be dissociated by colchicine (Timasheff & Grisham, 1980; Buechler & Gibson, 1984).

Tubulin has been shown to be a substrate in vitro for ADP-ribosylation catalyzed by cholera toxin (Amir-Zaltsman et al., 1982; Hawkins & Browning, 1982). While the potential functional significance in the intact cell for this covalent modification of tubulin is as yet unclear, we were attracted to this possible mechanism for ACC regulation and have thus investigated it in the present study.

Materials and Methods

Materials

Sprague-Dawley rats were purchased from Charles River Breeding Laboratories. Cholera toxin, NAD, ADP-ribose, ribose 5-phosphate, 5′-AMP, guanine nucleotides, thymidine, spermine, and dithiothreitol were obtained from Sigma. Pertussis toxin was obtained from List Biological Laboratories. [*adenylate*-α-^{32}P] NAD (178 Ci/mmol) was obtained from ICN Radiochemicals. Snake venom phosphodiesterase was purchased from Cooper. All other reagents used for ACC isolation and assay were as previously published (Tipper & Witters, 1982).

Methods

Acetyl-CoA Carboxylase Isolation and Assay. Acetyl-CoA carboxylase was isolated from livers of male Sprague-Dawley rats (130–160 g) which had been fasted for 48 h and then refed for 48 h with a low-fat, high-carbohydrate chow (Bioserv). Isolation by the technique of monomeric avidin–Sepharose chromatography was performed by a previously reported method (Tipper & Witters, 1982). Enzyme activity was assayed with the $^{14}CO_2$ fixation assay (Witters et al., 1979) modified by the substitution of acetate salts of the tris(hydroxymethyl)aminomethane (Tris) buffer and magnesium for the chloride ones. Standard assays were carried out in the presence of a saturating concentration of citrate (2 mM); in kinetic studies, the concentrations of citrate, bicarbonate, acetyl-CoA, and ATP/Mg^{2+} were varied while the concentrations of other reactants were held constant. Activity is expressed in units where 1 unit equals 1 μmol of CO_2 fixed/min at 37 °C.

Incubation of ACC with Cholera and Pertussis Toxins. In the standard incubations, ACC (1.25 mg/mL; 5 μM) was incubated in potassium phosphate (50 mM; pH 7.4), dithiothreitol (DTT) (4 mM), thymidine (10 mM), NAD (10 μM–8 mM), and activated toxin at 30 °C. Cholera or pertussin toxin was preactivated by incubation in buffer with 20 mM DTT at 30 °C for 10 min prior to addition. In select experiments, the final concentration of toxin was varied between 5 and 125 μg/mL; standard incubations in most experiments were carried out with 100 μg/mL cholera toxin. For radiolabeling of ACC, incubations were carried out in the presence of a range of concentrations of NAD containing 80 μCi/mL [*adenylate*-^{32}P]NAD.

For the assessment of ACC activity after toxin incubation, an aliquot of the reaction mixture was withdrawn at selected time intervals and immediately added to the ACC reaction

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mixture. Radiolabeling of ACC was assessed in two ways. In the first, the reaction was terminated by the addition of a quench containing sodium dodecyl sulfate (SDS) (2% w/v), DTT (50 mM), sucrose (4% w/v), and pyronin Y, followed by heating in a boiling water bath for 5 min. These samples were then subjected to SDS–polyacrylamide gel electrophoresis by the method of Laemmli (1970), followed by radioautography. In the second, the reactions were terminated by the addition of 0.5 mg of bovine serum albumin (BSA) followed by trichloroacetic acid (TCA; 25% w/v final concentration). The tubes were kept on ice for 10 min followed by centrifugation at 10000*g* for 10 min. The pellet was then washed 5 times with 25% TCA prior to quantification of radiolabel by Cerenkov counting.

Phosphodiesterase Incubation and Alkaline Hydrolysis. Following reaction of ACC with activated cholera toxin in the presence of cold or ^{32}P-labeled NAD, the reaction mixture was immediately cooled on ice and then dialyzed for 2 h against 1000 volumes of Tris-HCl (160 mM; pH 8.0), α-glycerophosphate (40 mM), and DTT (2 mM) at 4 °C with five buffer changes. This mixture was then reacted with snake venom phosphodiesterase (25 units/mL) in the presence of $MgCl_2$ (10 mM) at 30 °C. In some incubations, authentic 5′-AMP was added during the reaction to serve as an internal standard for subsequent high-performance liquid chromatography (HPLC). For assay of ACC activity, an aliquot of this reaction mixture was directly added to the ACC assay. For identification of the nature of the liberated ^{32}P-product, the reaction was terminated by the addition of 10% TCA, cooling on ice, and centrifugation (10000*g* for 10 min). The resulting supernatant was neutralized with KOH prior to HPLC and diluted with the starting HPLC buffer.

Alkaline hydrolysis of TCA-precipitated radiolabeled ACC was performed by incubation of labeled pellets in 0.1 N NaOH for 3 h at 37 °C. In some incubations, authentic ADP-ribose was added to serve as an internal standard for subsequent HPLC. Following incubation, the mixture was centrifuged at 10000*g* for 10 min, the supernatant neutralized with HCl, and the sample diluted in HPLC buffer (see below).

HPLC Separation of 5′-AMP and ADP-Ribose. Anion-exchange HPLC was employed to separate the reaction products from phosphodiesterase incubation and alkaline hydrolysis. Separations were performed on a Whatman SAX-10 column with isocratic elution in potassium phosphate (7 mM; pH 4.0) using a Beckman Model 334 instrument. Chromatography was performed at room temperature at a flow rate of 1.5 mL/min. Eluted compounds were identified by UV absorption at 254 nm and by collection of fractions for Cerenkov counting of radiolabel. Comigration of eluted peaks with authentic standards was determined at the time of each separation, as there was slight variability from day to day runs. The column was washed with potassium phosphate (0.25 M) and KCl (0.25 M) after each day's use followed by extensive washing with water. It was then reequilibrated with fresh starting buffer prior to daily use.

Results

Incubation of purified rat liver ACC with activated cholera toxin and [*adenylate*-^{32}P]NAD (10 μM) results in the incorporation of ^{32}P into the M_r 240000 subunit of ACC, as judged by gel electrophoresis and radioautography (Figure 1). With several different preparations of ACC and cholera toxin, however, only approximately 0.03 mol of phosphate per mole of subunit was incorporated in incubations up to 3 h, as determined by TCA precipitation of labeled enzyme. These incubations resulted in no change in ACC activity measured

FIGURE 1: SDS gel electrophoresis of ACC after incubation with cholera toxin. ACC was labeled by incubation with cholera toxin in the presence of [*adenylate*-^{32}P]NAD (10 μM) for 60 min and then prepared for gel electrophoresis, as detailed under Methods. Shown is a 9% polyacrylamide gel; lane 1 represents the Coomassie Blue stain ACC preparation with the major protein of M_r 240000 and a minor contaminant at M_r 130000. Lane 2 is the corresponding radioautograph.

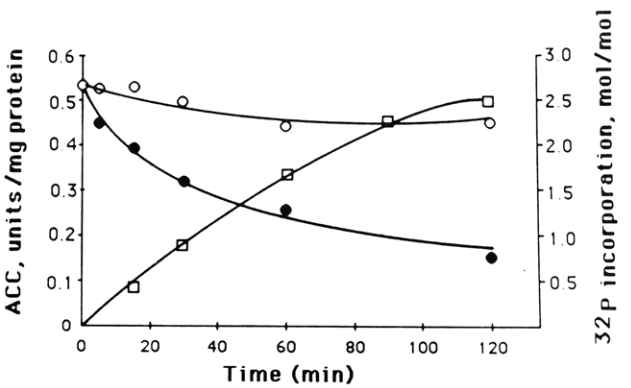

FIGURE 2: ^{32}P labeling and activity of ACC on incubation with cholera toxin. ACC was incubated with (●, □) or without (○) cholera toxin (100 μg/mL) in the presence of either unlabeled NAD (●, ○) or [*adenylate*-^{32}P]NAD (□) at 6 mM. ACC activity was measured at 0.5 mM citrate at selected time intervals (●, ○) and ^{32}P incorporation measured by TCA precipitation (□), as detailed under Methods. No radiolabel incorporation was observed in the absence of cholera toxin or in the absence of substrate. ^{32}P incorporation is expressed as moles of ^{32}P incorporated per mole of 240000-dalton subunit.

at V_{max} and no alteration in the K_a for citrate (not shown). No radiolabel incorporation into ACC was detected in the absence of added cholera toxin or in the presence of activated pertussis toxin at this concentration of NAD (see below).

The K_m for NAD of other cholera toxin mediated ADP-ribosylation reactions is 3–4 mM (Watkins et al., 1980). Therefore, we reexamined the labeling of ACC and ACC activity after incubation with activated cholera toxin at 6 mM NAD (Figure 2). At this concentration of NAD, up to 2.5 mol of ^{32}P can be incorporated into ACC after 2 h of incubation. This incorporation is accompanied by a marked time-dependent inactivation of ACC, measured at a subsaturating citrate concentration (0.5 mM). Little change in ACC activity is noted, if cholera toxin or NAD is omitted from the incubations. The apparent K_m for NAD for the cholera toxin mediated inactivation of ACC, determined from a Lineweaver–Burk plot, is 4 mM, consistent with the reported NAD K_m for other cholera toxin catalyzed reactions (not shown).

Given this dependency of high NAD concentrations for the inactivation of ACC in the presence of cholera toxin, we then reexamined the effects of activated pertussis toxin at variable NAD concentrations. Unlike cholera toxin, this ADP-

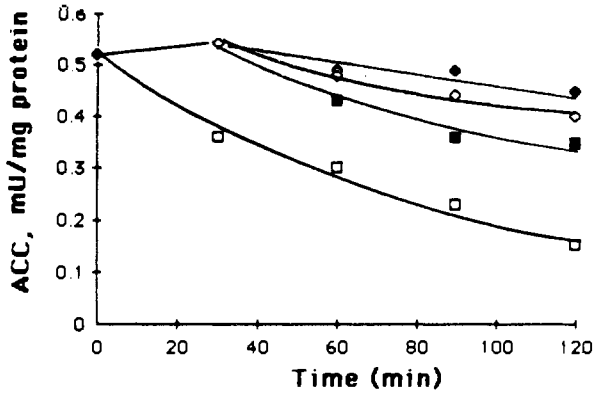

FIGURE 3: Effects of ADP-ribose and ribose 5-phosphate on ACC activity. ACC was incubated with activated cholera toxin and NAD (6 mM) (□), ADP-ribose alone (6 mM) (■), ribose 5-phosphate alone (6 mM) (●), or no additions (○) at 30 °C. Additions were made at time zero, and aliquots were removed at selected times for assay of ACC activity at 0.5 mM citrate.

ribosyltransferase has no effect on ACC activity and does not catalyze ^{32}P incorporation at NAD concentrations up to 8 mM (not shown). As determined by ACC inactivation, the concentration of activated cholera toxin necessary to achieve half-maximal inhibition is 40 μg/mL with maximal inhibition at 100 μg/mL (not shown). To date, we have observed no effects of guanine nucleotides [GTP, GDP, GMP, or the nonhydrolyzable GTP analogues Gpp(NH)p and guanosine 5′-*O*-(thiotriphosphate)] on either cholera toxin mediated ^{32}P incorporation or ACC inactivation.

Cholera toxin has an endogenous NAD glycohydrolase activity which may lead to the generation of free ADP-ribose on incubation with NAD (Ueda & Hayaishi, 1985). ADP-ribose may then form adducts with a number of proteins through a Schiff base linkage. In order to establish that the ^{32}P incorporation and activity change seen with ACC was a true ribosyltransferase reaction, we examined the effects of unlabeled ADP-ribose and ribose 5-phosphate on ACC activity. In contrast to the cholera toxin mediated inactivation, these compounds, added in a concentration equal to that of the NAD, have little effect on ACC activity (Figure 3). In other experiments (not shown), a 10-fold molar excess of unlabeled ADP-ribose has no effects on the labeling of ACC from [*adenylate*-^{32}P]NAD catalyzed by cholera toxin. Thus, significant adduct formation from free ADP-ribose seems unlikely.

To establish that the cholera toxin was mediating the expected mono-ADP-ribosylation of ACC rather than poly-ADP-ribosylation, several different approaches were taken. Inhibitors of polyribosylation (spermine, thymidine, and nicotinamide) (Ueda & Hayaishi, 1985) have no effect of cholera toxin mediated ACC activity changes or ^{32}P incorporation (not shown). ACC was also labeled by incubation with [*adenylate*-^{32}P]NAD and activated toxin, dialyzed, and then digested with snake venom phosphodiesterase. The acid-soluble reaction products were then separated by anion-exchange HPLC. This incubation released 65% of the incorporated ^{32}P to the acid-soluble fraction. All recovered counts eluted in a peak comigrating with authentic 5′-AMP (Figure 4); recovery of injected counts in this peak was 72%. Alkaline hydrolysis of labeled ACC liberated 79% of incorporated counts; on HPLC, all recovered counts (60% total recovery) coeluted with authentic ADP-ribose that had undergone parallel alkaline hydrolysis (Figure 5). These data taken together are consistent with catalysis of a mono-ADP-ribosylation of ACC by cholera toxin.

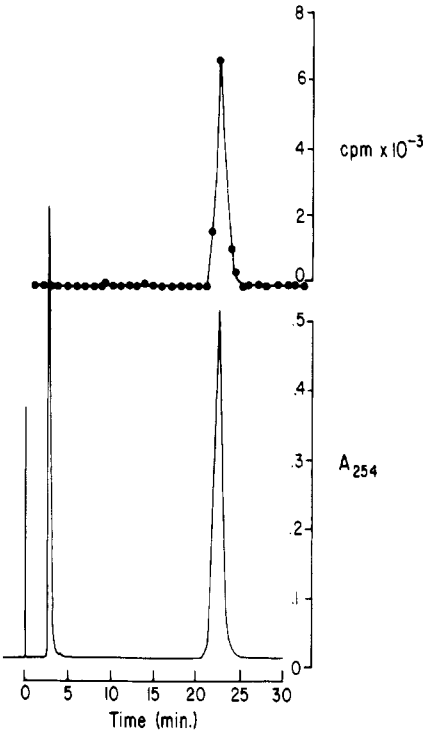

FIGURE 4: HPLC separation of ^{32}P-labeled reaction products of phosphodiesterase incubation. ACC was labeled by incubation with activated cholera toxin and [*adenylate*-^{32}P]NAD (6 mM) for 120 min, dialyzed, and digested with snake venom phosphodiesterase, as described under Methods. Unlabeled 5′-AMP (100 nmol) was added as an internal standard to the phosphodiesterase incubation. This incubation was terminated with TCA and the sample prepared for HPLC, as detailed. Shown is the A_{254} absorbance (lower panel) with the corresponding cpm (upper panel) for the HPLC elution, performed as described under Methods.

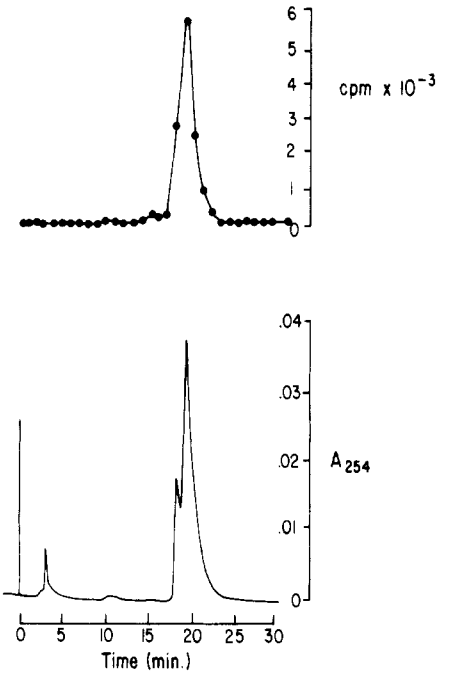

FIGURE 5: HPLC separation of ^{32}P-products of alkaline hydrolysis. Labeled ACC was prepared as in Figure 4, precipitated with TCA in the presence of carrier BSA, washed, and subjected to alkaline hydrolysis, as described under Methods. Unlabeled ADP-ribose (10 nmol) was added as an internal standard to the hydrolysis incubation. The supernatant from this reaction was neutralized for HPLC injection. Shown is the A_{254} absorbance (lower panel) with the corresponding cpm (upper panel) for the HPLC elution, performed as described under Methods.

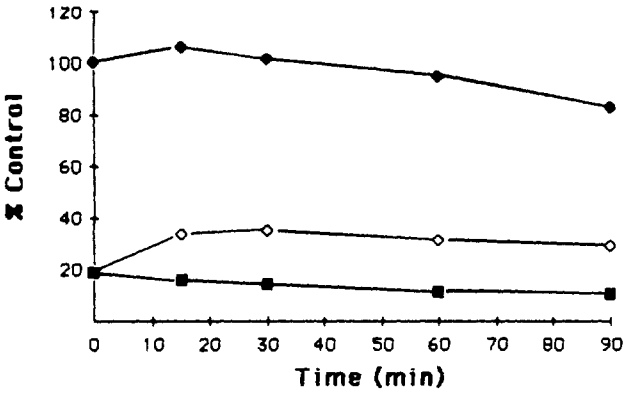

FIGURE 6: ACC activity after phosphodiesterase incubation. ACC was incubated with (○, ■) or without (●) activated cholera toxin in the presence of NAD (6 mM) for 2 h and then dialyzed, as described under Methods. This preparation was then incubated with (○, ●) snake venom phosphodiesterase or with phosphodiesterase that had been previously boiled (■) added at time zero. At intervals, aliquots were removed for assay of ACC activity at 0.5 mM citrate, as detailed. Results are expressed as the percent zero time value for ACC previously incubated without added toxin.

Surprisingly, the release of 5′-AMP from ACC by snake venom phosphodiesterase is accompanied by partial reversal of the inactivation catalyzed by cholera toxin. ACC was incubated with NAD with or without cholera toxin, dialyzed, and then digested with snake venom PDE. A control experiment included PDE that had been boiled prior to use. In this experiment (Figure 6), PDE digestion led to a doubling of ACC activity, although the activity reached only 40% of the noninactivated control.

The cholera toxin mediated ADP-ribosylation of ACC leads to both a decrease in V_{max} (50%) and an increase in the apparent K_a for citrate (0.3 to 0.9 mM) (not shown). No effects of ADP-ribosylation are observed on the K_m values for acetyl-CoA, ATP/Mg^{2+}, or bicarbonate. The K_m for ACC is greater than 5 μM, the greatest concentration tested (not shown).

Discussion

The results of these experiments establish that acetyl-CoA carboxylase is a substrate for cholera toxin mediated ADP-ribosylation in vitro. The ADP-ribosylation of ACC is accompanied by inhibition of enzyme activity characterized both by a reduction in V_{max} and an increase in the apparent K_a for the allosteric activator citrate. On the basis of inhibitor studies and the tentative identification of reaction products, we conclude that the inactivating reaction is consistent with the expected mono-ADP-ribosylation of ACC by cholera toxin. Furthermore, we have been unable to demonstrate that these phenomena are due to Schiff base formation from free ADP-ribose that might be liberated by the inherent NAD glycohydrolase activity of cholera toxin. Thus, the labeling and inactivation of ACC are due to a ribosyltransferase reaction. Some specificity of the reaction is demonstrated by the inability of activated pertussis toxin to catalyze either labeling or inactivation of ACC. The inactivation of ACC appears to be partially reversed by removal of 5′-AMP from the covalently bound ADP-ribose by snake venom phosphodiesterase.

These observations extend the list of potentially important acceptor proteins for ADP-ribosylation by exogenous and endogenous ribosyltransferases. These include the GTP binding proteins (N_s, N_i, and transducin), elongation factor 2, glutamine synthetase, and phosphorylase kinase (Ueda & Hayaishi, 1985; Watkins et al., 1985; Tsuchiya et al., 1985). They also point to an additional similarity between tubulin and ACC beyond the recognized characteristics of the protomer–polymer interconversion, sensitivity to guanine nucleotides, and disruption of polymer by agents such as colchicine.

ADP-ribosylation of phosphorylase kinase by a hen liver nuclear ADP-ribosyltransferase suppresses both cAMP-dependent phosphorylation and autophosphorylation and results in a suppression of phosphorylation-dependent activation of the enzyme (Tsuchiya et al., 1985). Interactions between ADP-ribosylation and phosphorylation by protein kinases have also been demonstrated for nuclear proteins and whole histones (Tanigawa et al., 1983). It will be of interest to determine the effects of ADP-ribosylation on the phosphorylation of ACC by cAMP-dependent and cAMP-independent protein kinases.

The nature of the covalently bound alkali-labile phosphate on ACC is not resolved. ACC, as isolated by a variety of procedures by several groups, may contain 3–6 mol of alkali-labile phosphate per mole of ACC subunit (Witters, 1985). While much of this phosphate is removable on incubation with various protein phosphatase preparations associated either with activation of ACC or with no change in catalytic activity (Ingebritsen et al., 1983; Krakower & Kim, 1981; Thampy & Wakil, 1985), in our hands, we have been unable to remove all of the measured alkali-labile phosphate with a number of protein phosphatases. It is possible that some of this measured phosphate might represent prior incorporation of ADP-ribose in vivo.

In ongoing experiments, we are attempting to establish that ACC is ADP-ribosylated in intact cells and that it is a substrate for endogenous ribosyltransferases present in a variety of tissues. These experiments may identify an important role for this covalent modification in the regulation of an important metabolic enzyme. Such a modification could be a mechanism by which certain hormones and other agonists modify the rate of fatty acid biosynthesis in vivo.

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Registry No. ACC, 9023-93-2; NAD, 53-84-9; NAD–protein ADP-ribosyltransferase, 58319-92-9; citrate, 77-92-9.

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Localization of the Free Radical on the Flavin Mononucleotide of the Air-Stable Semiquinone State of NADPH–Cytochrome P-450 Reductase Using ^{31}P NMR Spectroscopy†

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ABSTRACT: Microsomal NADPH–cytochrome P-450 reductase is the only mammalian flavoprotein known to contain both FAD and FMN as prosthetic groups. The discovery of the air-stable semiquinone [Masters, B. S. S., Kamin, H., Gibson, Q. H., & Williams, C. H., Jr. (1965) *J. Biol. Chem. 240*, 921–931] and its identification as a one-electron-reduced state [Iyanagi, T., & Mason, H. S. (1973) *Biochemistry 12*, 2297–2308] have engendered a number of studies to elucidate its unique catalytic mechanism. In this paper, ^{31}P NMR spectroscopy is utilized to probe the localization of the free radical in this air-stable semiquinone form and to ascertain the environments of the FAD and FMN prosthetic groups as affected by the paramagnetic ion Mn(II). Consistent with conclusions drawn from studies utilizing FMN-free reductase [Vermilion, J. L., & Coon, M. J. (1978) *J. Biol. Chem. 253*, 8812–8819], the free radical was shown to reside on the FMN moiety by the broadening of its characteristic resonance in the ^{31}P NMR spectrum. In addition, the effect of the paramagnetic ion Mn(II) was determined on the four resonances attributable to FAD and FMN and the additional ones contributed by $NADP^+$ resulting from the oxidation of the physiological reductant NADPH. The addition of Mn(II) had little effect on the line widths of the FMN and FAD signals but resulted in an increase in their intensities due to a decrease in T_1 relaxation times. On the other hand, the pyrophosphate resonances of bound $NADP^+$ were only minimally affected by the paramagnetic ion, indicating that the pyrophosphate moiety of $NADP^+$ is more sequestered from the solvent than the pyrophosphate of FAD. These studies demonstrate the utility of ^{31}P NMR as a direct probe of the environments of the phosphorus-containing cofactors of NADPH–cytochrome P-450 reductase under various conditions, including changes in redox state.

NADPH–cytochrome P-450 reductase (EC 1.6.2.4) was first purified and shown to contain 2 mol of flavin/mol of enzyme protein by Masters et al. (1965a). These workers also demonstrated the existence of an air-stable semiquinone form of the flavoprotein. The identification of the prosthetic groups of the reductase as FAD *and* FMN and the determination of the air-stable semiquinone as a one-electron-reduced state were made by Iyanagi and Mason (1973). Subsequently, a number of studies have appeared in the literature addressing the nature of its mechanism of catalysis (Iyanagi et al., 1974, 1981; Vermilion & Coon, 1978a,b; Yasukochi et al., 1979; Vermilion et al., 1981; Oprian & Coon, 1982). Various biophysical methods have been applied to the study of NADPH–cytochrome P-450 reductase in order to understand the spatial and electronic relationships governing the interactions of the two flavin prosthetic groups, including EPR spectroscopy (Iyanagi & Mason, 1973), fluorescence quantum yield and emission anisotropy measurements (Blumberg et al., 1982), X-ray diffraction of enzyme crystals (Sugiyama et al., 1983), and resonance Raman spectroscopy (Sugiyama et al., 1985). These experimental approaches have not yet yielded definitive results.

In an attempt to ascertain the location of the free radical of the air-stable semiquinone state of NADPH–cytochrome P-450 reductase and to probe the environments of the prosthetic flavins, FAD and FMN, ^{31}P NMR spectroscopy was applied to oxidized and one-electron-reduced samples of the flavoprotein. The effects of the addition of the paramagnetic ion Mn(II) on the ^{31}P resonances attributable to FAD, FMN, the reductant (NADPH), and residual 2′-AMP (used in affinity chromatography) were also examined. The results show that the free radical of the semiquinone state resides on the FMN prosthetic group. Line-broadening effects produced by Mn(II) were restricted to the residual 2′-AMP used in eluting the reductase from the 2′,5′-ADP–Sepharose 4B affinity

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