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Electron Spin Resonance of Calmodulin–Vanadyl Complexes†

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ABSTRACT: X-band (9.2 GHz) electron spin resonance spectroscopy was used to investigate the binding of vanadyl to calmodulin. Solution spectra, obtained at ambient temperature with various VO^{2+}:calmodulin molar ratios, suggested a binding stoichioimetry of 4 mol of VO^{2+}/mol of protein and the possibility of two classes of binding sites. The latter was confirmed by using frozen solutions of calmodulin–VO^{2+} complexes that gave splitting of the spectral bands corresponding to the parallel components, which was particularly pronounced with the three high-field peaks. Competition of Ca^{2+} for the VO^{2+} binding sites was investigated, and the results indicated that two of the VO^{2+} sites corresponded to two of the Ca^{2+} sites; the other two VO^{2+} binding sites may have a higher affinity for VO^{2+} than for Ca^{2+} or they may correspond to Ca^{2+}-independent sites. These results demonstrate that electron spin resonance spectroscopy can be used advantageously to probe subtle differences in the microenvironments of metal-binding sites in calmodulin.

Calmodulin is a ubiquitous and multifunctional regulatory protein involved in the regulation of many cellular functions (Cheung, 1980; Means et al., 1982; Klee & Vanaman, 1982). The protein binds four Ca^{2+} ions, two each in globular lobes that are connected by a long central helix (Babu et al., 1985). There is controversy regarding the relative affinities of the four sites, the order of binding, and whether or not cooperativity exists (Haiech et al., 1981; Potter et al., 1983; Burger et al., 1984; Iida & Potter, 1986). ^1H NMR studies are consistent with the presence of two high-affinity binding sites for Ca^{2+} in the carboxy-terminal domain and two low-affinity sites in the amino-terminal domain (Seamon, 1980; Ikura et al., 1983, 1984; Klevit et al., 1984; Thulin et al., 1984). Preferential ordering to Ca^{2+} binding has also been demonstrated by measuring rates of acetylation of the seven lysines in calmodulin; the results were consistent with Ca^{2+} binding first to the carboxy-terminal sites (Giedroc et al., 1987). It is also well recognized that monovalent and divalent metal cations other than Ca^{2+} bind to calmodulin (Forsen et al., 1980; Delville et al., 1980; Haiech et al., 1981; Potter et al., 1983; Cheung, 1984; Shimizu & Hatano, 1984; Wang et al., 1984; Mulqueen et al., 1985; Mills & Johnson, 1985).

Vanadium is an essential trace metal in experimental animals (Schwarz & Milne, 1971), and it is known that vanadate is rapidly and quantitatively converted to vanadyl (VO^{2+}) by glutathione and catechols (Cantley et al., 1978; Macara et al., 1980). Thus, VO^{2+} may have physiological significance. This cation forms strong complexes with a variety of ligands and binds to numerous metalloproteins (Chasteen, 1981, 1983); this ability to bind to diverse proteins may arise from the flexibility in the coordination geometry of VO^{2+} (Chasteen, 1981). In a preliminary paper we showed that VO^{2+} binds to calmodulin (Ahmed et al., 1985); herein we present ESR spectral data suggesting that VO^{2+} binds to the protein with a stoichiometry of 4:1 and demonstrate the existence of two classes of binding sites.

Materials and Methods

Materials. HEPES,[1] calcium chloride, and vanadyl sulfate were obtained from Sigma Chemical Co. (St. Louis, MO). Calmodulin was purified to homogeneity from bovine testes as described elsewhere (Jackson & Puett, 1984); special care

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[1] Abbreviations: HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; DPPH, diphenylpicrylhydrazyl.

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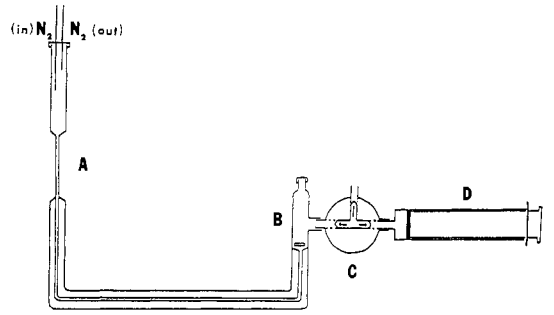

FIGURE 1: Modified quartz cell used for addition of $VOSO_4$ to calmodulin: (A) flat cell; (B) reaction compartment; (C) three-way stopcock; (D) gastight syringe.

was taken to minimize trace metal contamination. Dialysis of purified apocalmodulin against 0.1 M sodium perchlorate,[2] followed by dialysis against highly purified water, was also done to ensure reproducible ESR spectra.

ESR Spectroscopy. All X-band (9.2 GHz) ESR spectra were measured by using an IBM-BRUKER (ER-200 SRC) ESR spectrometer equipped with an IBM-9000 computer and a TE_{104} cavity. Repetitive scans were stored (typically five scans), and the data were subjected to standard signal averaging. Spectra were obtained on solutions at either room temperature (298 K) or frozen (150 K). The protein concentration used for the ESR experiments was 0.8 mM in 0.1 HEPES, pH 7.4. A stock solution of 0.3 M $VOSO_4$, pH 2, was prepared and standardized spectrophotometrically by using an extinction coefficient of 18 M^{-1} cm^{-1} at 750 nm (Fitzgerald & Chasteen, 1974a); this solution was stored at 278 K.

For the titration experiments at 298 K a modified quartz flat cell was designed (Figure 1) to allow additions to the protein solutions without removing the cell from the instrument, thus minimizing loss of sample, instrumental tuning errors, and oxidation of the sample. The cell is placed in the cavity and purged with nitrogen, the three-way stopcock being positioned to allow N_2 to escape (open position). The protein solution (350 μL) is then loaded into the reaction compartment with the stopcock maintained in the open position while the solution is stirred; the nitrogen flow purges the solution continuously, thus removing dissolved oxygen. Simultaneously, a standard flat cell, containing an aqueous 1 mM $VOSO_4$ solution at pH 2, is inserted in the second cavity. After 30 min, the stopcock is closed, and the gastight syringes and the reaction compartment are connected. A 0.3-μL aliquot of VO^{2+} or Ca^{2+} (from 0.3 M stock solutions of $VOSO_4$, pH 2, or $CaCl_2$ in 0.1 M HEPES, pH 7.4, respectively) is added slowly and stirred for 5 min, and the solution is then forced into the flat cell compartment with the syringe. After acquisition of the spectral data for the calmodulin–VO^{2+} complex, the spectrum for the standard 1 mM $VOSO_4$ solution in the dual cavity was obtained under the same conditions and used for intensity normalization. The solution is retrieved into the reaction compartment and the next aliquot added following the same procedure. All solutions were purged with nitrogen and maintained under a nitrogen atmosphere during the experiment.

For the frozen spectra, quartz capillaries (3-mm o.d.) were used, and the solutions were transferred under a nitrogen atmosphere. A solution of freshly prepared DPPH in benzene was used as a g factor standard ($g = 2.0036 \pm 0.0002$). By use of the double-cavity technique (Wertz & Bolton, 1972), matched standard samples were inserted into the dual-sample cavity and their spectra obtained. The separation of the centers of the two signals is a measure of the field difference at the two sample positions. The standard in the low-temperature insert was then replaced by the protein–VO^{2+} sample, and from the field difference, corrected for the standard sample field difference, the g value of the unknown was determined. These spectra were simulated by using a computer program kindly provided by Dr. N. D. Chasteen, and the simulated spectra could be superimposed on the experimental spectra with much less than 10% variation between the two.

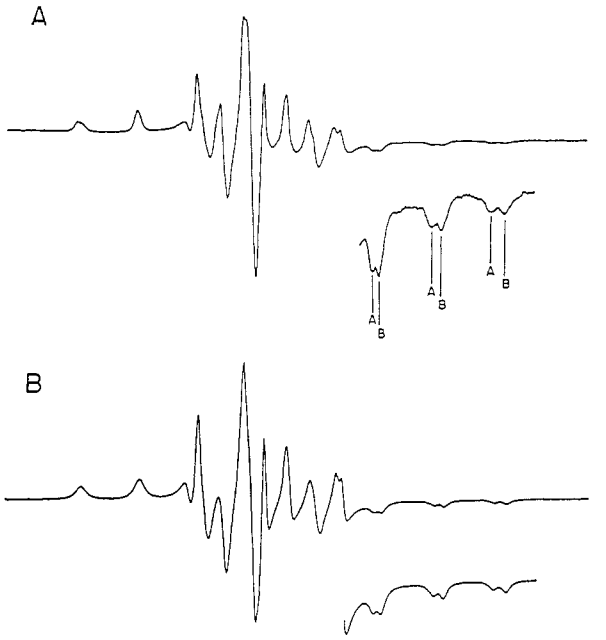

FIGURE 2: Frozen solution ESR spectrum of calmodulin–VO^{2+} (4 equiv of VO^{2+} to protein): (A) experimental spectrum; (B) computer-simulated spectrum.

RESULTS

The addition of VO^{2+} to apocalmodulin results in an ESR spectrum that differs considerably from the isotropic solution behavior of free VO^{2+} (Ahmed et al., 1985). The protein–VO^{2+} complex exhibits an anisotropic solution spectrum indicative of the VO^{2+} near "rigid limit" (Chasteen, 1981), clearly indicating that VO^{2+} assumes a correlation time similar to that of the protein. This observation, coupled with the knowledge that free VO^{2+} is ESR-silent at pH 7.4 (Chasteen, 1981), demonstrates that VO^{2+} binds to calmodulin.

In an effort to obtain more information on the nature of the protein–VO^{2+} complex, ESR spectra were determined on frozen solutions, where molecular motion is minimized (Figure 2A). The ESR spectrum shown was obtained at a VO^{2+}:calmodulin molar ratio of 4:1; under these conditions any free VO^{2+} would be ESR-silent. The eight-line spectrum arises from coupling of the unpaired electron with the ^{51}V nucleus. Since ^{51}V has a spin quantum number of $^7/_2$, there are $(2I + 1)$ or eight lines. The three high-field resonances of calmodulin-bound VO^{2+} are split, and the two components are denoted A and B. The simulated ESR spectrum is also shown (Figure 2B), and the parameters used to generate this spectrum are given in Table I. In addition to splitting of the high-field peaks, the low-field and center-field peaks are skewed, consistent with the contribution of two components to the ESR spectrum.

The observed splitting of the high-field bands is suggestive of two classes of binding sites. In order to obtain information on the stoichiometry of VO^{2+} binding at 298 K, a titration was

[2] We thank Dr. N. D. Chasteen for suggesting this important step.

Table I: ESR Parameters Used To Simulate the Frozen Solution Spectra of Calmodulin–VO^{2+} Complexes[a]

site	$10^4A_{\parallel}$ (cm^{-1})	$10^4A_{\perp}$ (cm^{-1})	$g_{\parallel}$	$g_{\perp}$
		VO^{2+} Only[b]		
A	171.2	60.51	1.940	1.976
B	176.3	64.63	1.930	1.974
		$VO^{2+} + Ca^{2+}$ [c]		
A	168.9	60.58	1.942	1.978
		Difference: $VO^{2+} - VO^{2+}/Ca^{2+}$ [d]		
B	174.7	64.21	1.936	1.976

[a] Solutions of apocalmodulin in 0.1 M HEPES, pH 7.4, containing either VO^{2+} or VO^{2+} and Ca^{2+} were frozen (150 K) and the ESR spectra measured. [b] 4:1 molar ratio of VO^{2+} to protein. [c] VO^{2+} and Ca^{2+} were each present at 2 molar equiv to protein. [d] The difference spectrum generated by subtracting the calmodulin–VO^{2+}/Ca^{2+} spectrum from the calmodulin–VO^{2+} spectrum.

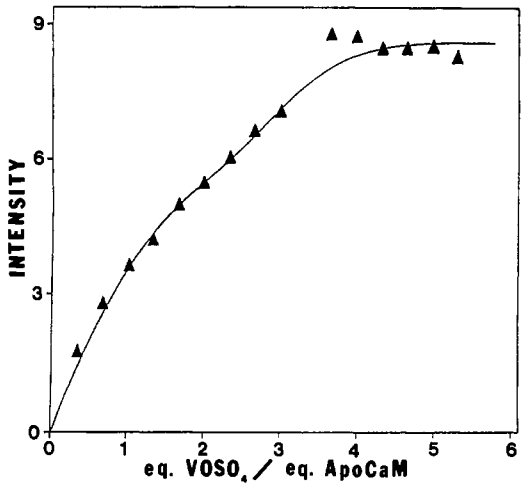

FIGURE 3: Normalized intensity (peak to peak) of the $-^1/_2$ line as a function of VO^{2+} concentration. These results are from solution ESR spectra at 298 K.

conducted in which VO^{2+} was added to a calmodulin solution in increments of 0.3 equiv to protein. The titration cell utilized was necessary to achieve the required accuracy in determining small spectral changes of the near rigid limit ESR spectrum. Figure 3 shows the variation in the normalized intensity (peak to peak) of the $-^1/_2$ line as a function of the VO^{2+}:calmodulin molar ratio. The results suggest a slight break in the titration curve at 2 equiv of VO^{2+}, and a plateau is reached at 4 equiv; the curve shown represents a typical titration. These results on solution spectra, although qualitative, are consistent with our findings on spectra of frozen solutions: namely, there appear to be two classes of VO^{2+} binding sites on calmodulin.

The competition of Ca^{2+} and VO^{2+} for possible common binding sites on the protein was examined by first adding 2 equiv of Ca^{2+}, followed in 5 min by 2 equiv of VO^{2+}. The solution was then frozen, and the experimental and simulated ESR spectra are shown in Figure 4; Table I gives the parameters used in spectral simulation. The three high-field peaks are principally characterized by "type A" components only (cf. Figure 2). In another experiment, 2 equiv of VO^{2+} was added to apocalmodulin and the frozen solution spectrum obtained (spectrum not shown). In this case the spectrum was similar to that in Figure 2, except that the line intensities from the A sites were somewhat higher than those from the B sites (e.g., the A:B intensity ratios were 1.04 with 2 equiv of VO^{2+} and 0.84 with 4 equiv). The result indicates that the A sites are of higher affinity than the B sites. Lastly, titration with additional Ca^{2+} (4 equiv) to a solution containing 2 equiv of Ca^{2+} and 2 equiv of VO^{2+} (cf. Figure 4) yielded a frozen

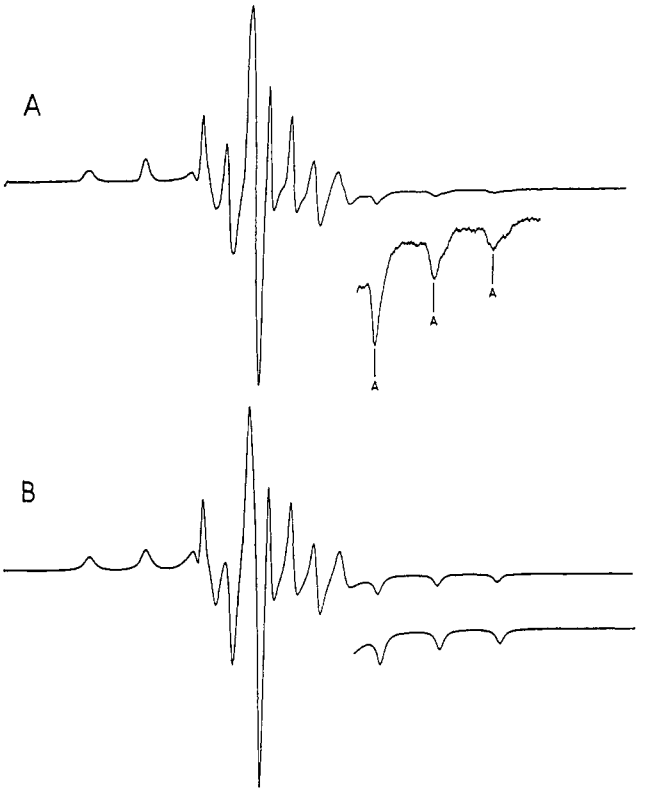

FIGURE 4: Frozen solution ESR spectrum of calmodulin–VO^{2+}/Ca^{2+} (2 equiv each of VO^{2+} and Ca^{2+} to protein): (A) experimental spectrum; (B) computer-simulated spectrum.

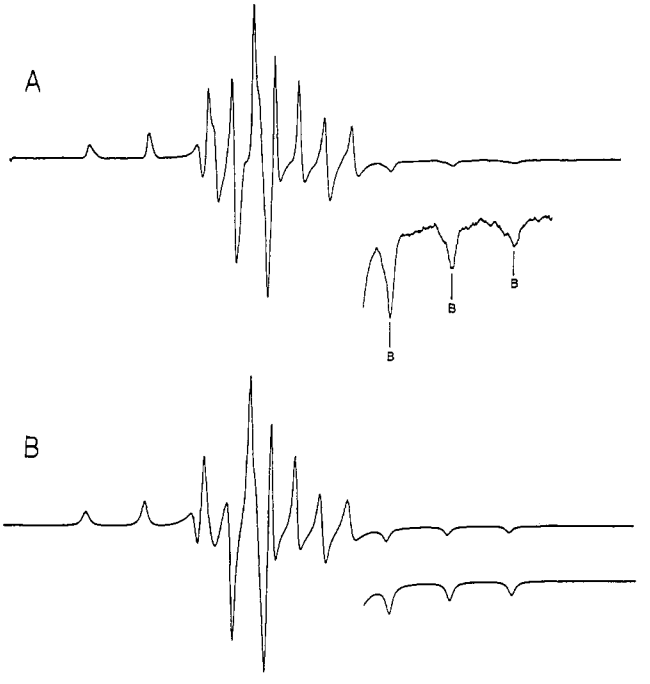

FIGURE 5: Frozen solution ESR computer-generated difference spectrum of calmodulin–VO^{2+} (cf. Figure 2) minus calmodulin–VO^{2+}/Ca^{2+} (cf. Figure 4): (A) experimental difference spectrum; (B) computer-simulated difference spectrum.

solution spectrum identical with that in Figure 4A (spectrum not shown), i.e., characterized by a VO^{2+} type A spectrum only.

Subtraction of the experimental ESR spectrum in Figure 4A from that in Figure 2A (i.e., with VO^{2+}-saturated CaM) yields the spectrum shown in Figure 5A. The corresponding simulated spectra is presented in Figure 5B, and the ESR parameters used for its simulation are listed in Table I. These

computer-generated difference spectra yield only a "type B" component of the high-field lines.

Discussion

The results presented herein demonstrate that VO^{2+} binds to calmodulin with an apparent stoichoimetry of 4:1, the same as that for Ca^{2+}. ESR spectra on liquid and frozen solutions of calmodulin–VO^{2+} suggest the presence of two classes of binding sites, denoted A and B. The present results do not enable us to conclude unequivocally that the A and B sites correspond to the four Ca^{2+}-binding sites, although it is tempting to consider that this may be the case. Clearly, Ca^{2+} competes with VO^{2+} for binding to the B sites, and the affinity for Ca^{2+} appears to be higher than that of VO^{2+}. The A sites may have a higher affinity for VO^{2+}, or they may represent Ca^{2+}-independent sites. It is important to emphasize that, for purposes of sensitivity, it is necessary to use relatively high protein concentrations, e.g., millimolar. Thus, it is not possible to make any conclusion about the affinity of VO^{2+} for the A sites; however, the contribution of the B sites to the VO^{2+} titration (cf. Figure 2) is suggestive of an affinity constant on the order of 10^3 M^{-1}. Thus, it is not surprising that millimolar concentrations of Ca^{2+} effectively compete with VO^{2+} if indeed the B sites correspond to two of the Ca^{2+}-binding sites. Interestingly, most of the ESR parameters for the A sites are not altered when Ca^{2+} is present, presumably in the B sites. This finding argues against any major interaction between these sites.

The use of multinuclear NMR and fluorescence has provided information on the binding of various metals and lanthanides to calmodulin (Delville et al., 1980; Forsen et al., 1980; Andersson et al., 1982; Siegel & Haug, 1983; Yazawa et al., 1984; Wang et al., 1984; Thulin et al., 1984; Mulqueen et al., 1985). Such measurements provide a unique opportunity to monitor the spectral properties of the metal ligands directly and can provide considerable information on subtle differences in the microenvironments of similar binding sites. In this context, Chasteen and co-workers (Fitzgerald & Chasteen, 1974b; DeKoch et al., 1974; Cannon & Chasteen, 1975; White & Chasteen, 1979; Chasteen, 1981, 1983) have shown that ESR spectroscopy of VO^{2+} complexes with other proteins can provide information on protein–metal binding sites that is not readily available when other techniques are used. The present study demonstrates that VO^{2+} binds to calmodulin and that ESR spectroscopy can be used to study competition with Ca^{2+}. Moreover, calmodulin–VO^{2+} complexes may prove useful in relaxation NMR spectroscopy of calmodulin, derivatives, and enzyme complexes, where the paramagnetic cation can be used as a probe for neighboring nuclei. Lastly, since vanadium is an essential trace metal that is rapidly converted to VO^{2+} in vivo, it will be of interest to ascertain if calmodulin–VO^{2+} complexes can act either to stimulate the activities of calmodulin-dependent enzymes or to serve as an antagonist to calmodulin binding.

Acknowledgments

It is a pleasure to thank Dr. N. D. Chasteen for providing us with the computer program used for ESR simulation and Dr. L. J. Bartolotti for adapting this program to the local university computer.

Registry No. VO^{2+}, 20644-97-7; Ca, 7440-70-2.

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Electron Paramagnetic Resonance Study of Ferrous Cytochrome P-450$_{scc}$–Nitric Oxide Complexes: Effects of Cholesterol and Its Analogues†

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ABSTRACT: Electron paramagnetic resonance (EPR) spectra of nitric oxide (NO) complexes of ferrous cytochrome P-450$_{scc}$ were measured at 77 K for the first time without using the rapid-mixing and freeze-quenching technique. Without substrate the EPR spectra were very similar to those of cytochrome P-450$_{cam}$ (from *Pseudomonas putida*) and cytochrome P-450$_{LM}$ (from rat liver microsomes) with rhombic symmetry; $g_x = 2.071$, $g_z = 2.001$, $g_y = 1.962$, and $A_z = 2.2$ mT for ^{14}NO complexes. Upon addition of substrates [such as cholesterol, 22(*S*)-hydroxycholesterol, 22(*R*)-hydroxycholesterol, 25-hydroxycholesterol, and 22-ketocholesterol], the EPR spectra exhibited many variations having rhombic symmetry in the major component and an additional minor component with less rhombic symmetry. Furthermore, addition of 20(*S*)-hydroxycholesterol caused a striking change in the EPR spectrum. The component with rhombic symmetry disappeared completely, and the component with less rhombic symmetry dominated ($g_x = 2.027$, $g_z = 2.007$, $g_y = 1.984$, and $A_z = 1.76$ mT for ^{14}NO complexes). These observations suggest the existence of the following physiologically important natures: (1) the conformational flexibility of the active site of the enzyme due to the steric interaction between the substrate and the heme-bound ligand molecule and (2) the importance of the hydroxylation of the cholesterol side chain at the 20*S* position to proceed the side-chain cleavage reaction in cytochrome P-450$_{scc}$.

Cytochrome P-450$_{scc}$,[1] which is located in the inner mitochondrial membrane of the adrenal cortex, catalyzes the cholesterol side-chain cleavage reaction. The cholesterol side-chain cleavage reaction involves three consecutive hydroxylation steps. The first hydroxylation occurs at the 22*R* position to yield 22(*R*)-hydroxycholesterol, the second occurs at the 20*S* position to give 20(*R*),22(*R*)-dihydroxycholesterol, and the third oxidative cleavage of the C20–C22 bond of the diol results in a formation of pregnenolone and isocaproic aldehyde (Burstein et al., 1975; Burstein & Gut, 1976; Hume et al., 1984). The successive conversion of cholesterol to pregnenolone is ensured by increased affinity of the hydroxylated intermediates to the enzyme and by increased stability of the ferrous dioxygen complex in each step (Tuckey & Kamin, 1982, 1983). These observations suggest the existence of strict stereochemical interactions among cholesterol side-chain, heme-bound dioxygen, and surrounding amino acid residues.

In order to elucidate the mechanism of the cytochrome P-450$_{scc}$ mediated side-chain cleavage reaction, it is necessary to have a detailed description of its active site. Since nitric oxide (NO) contains one unpaired electron, NO has been used as a paramagnetic probe of the ferrous–heme moiety in a variety of hemoproteins, particularly as a model for dioxygen-binding hemoproteins (Yonetani et al., 1972).

Several preliminary reports have suggested that nitric oxide combines with the microsomal cytochrome P-450 (Ullrich et al., 1968; Miyake et al., 1968). The observed EPR spectra of the ferrous–NO complexes prepared by NO-reduced and dithionite-reduced enzymes (Miyake et al., 1968, 1969), however, were very similar to the denatured ferrous–NO complex of hemoglobin (Kon, 1968, 1975). Ebel et al. (1975) found that the ferrous microsomal cytochrome P-450–NO complex was very labile and was converted quickly to a species which had an EPR spectrum similar to that of ferrous P-420–NO and/or denatured ferrous–NO complex of hemoglobin. Therefore, they had to measure the EPR spectra of the ferrous–NO complexes of cytochrome P-450$_{LM}$ and cytochrome P-450$_{cam}$ by rapidly mixing the dithionite-reduced

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[1] Abbreviations: EPR, electron paramagnetic resonance; cytochrome P-450$_{scc}$, cytochrome P-450 present in inner mitochondrial membranes of bovine adrenal cortex, which catalyzes the cholesterol side-chain cleavage reaction; cytochrome P-450$_{cam}$, cytochrome P-450 obtained from *Pseudomonas putida* grown on *d*-camphor; cytochrome P-450$_{LM}$, cytochrome P-450 present in microsomes isolated from livers of phenobarbital-treated male albino rats; EDTA, ethylenediaminetetraacetic acid.

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