NITRIC OXIDE COMPLEXES OF CYTOCHROME *P*-450

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1. Introduction

The use of nitric oxide as a spin label to probe the electronic structure of the heme prosthetic group in both native and structurally perturbed hemeproteins has received much attention recently. For example, it has been demonstrated that hemoglobin and myoglobin form ferrous NO complexes which exhibit EPR spectra having superhyperfine lines in the g_z signal, consistent with electron spin–nuclear spin interaction of the unpaired electron of the bound NO with a *trans* axial ligand, presumably due to one of the ^{14}N atoms of an imidazole moiety [1–4]. Perturbation of the protein structure either by a denaturing agent [1,5] or an allosteric effector [6] appears to result in either distortion or complete breakage of the bond between the heme iron and the *trans* ligand resulting in a pentacoordinate heme-NO species which has a sharp 3-line g_z EPR signal. Other hemeproteins, including horseradish peroxidase (HRP) and cytochrome *c* peroxidase (CCP), form stable ferric, as well as ferrous, NO complexes [7]. Their ferric-NO complexes appear diamagnetic and therefore have no EPR signals, while their ferrous-NO complexes have EPR spectra indicative of a rhombically-distorted field for the heme-bound NO. The distinctive superhyperfine lines in the g_z signal are even more readily apparent than in hemoglobin and myoglobin ferrous-NO complexes.

Several brief reports have suggested that microsomal and submicrosomal cytochrome *P*-450 form both ferric- and ferrous-NO complexes [8–12]. However, the observed EPR spectra of the ferric- and ferrous-NO complexes were identical [9,10], as well as being very similar to the denatured ferrous-NO complex of hemoglobin. In this preliminary communication we report that the ferrous microsomal cytochrome *P*-450–NO complex is unstable and is converted to a species which has an EPR spectrum similar to that of ferrous *P*-420–NO. We would also like to report some of the electronic and structural properties of the ferric- and ferrous-NO complexes of microsomal and bacterial cytochrome *P*-450, and to explain the differences between these properties and those previously reported in the literature.

2. Materials and methods

Microsomes were prepared, as described below, from the livers of 150–200 g male Sprague-Dawley rats supplied by Charles River Co. The animals received intraperitoneal injections of phenobarbital (80 mg/kg) for four days and were fasted for 18 hr prior to sacrific by decapitation. The livers were perfused with ice-cold 0.9% NaCl to remove most of the blood. They were then homogenized in 0.25 M sucrose (2 ml/g liver). The remainder of the microsomal preparation was as described by Franklin and Estabrook [13]. The microsomes were resuspended by homogenization in a small volume of potassium phosphate buffer (0.1 M, pH 7.4). The cytochrome *P*-450 content estimated by the procedure of Estabrook et al. [14] was approximately 3 nmol/mg protein. Cytochrome $P\text{-}450_{cam}$ was purified as previously described [15] from *P. putida* (ATCC 17453) grown on camphor as a carbon source. Nitric oxide (NO) was purchased from the Linde Division, Union Carbide Corp. All other reagents were of the highest quality commercially available and were used without further purification.

Optical absorption spectra were recorded with an Aminco DW-2 or an Aminco-Chance dual wavelength

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split beam spectrophotometer. Electron paramagnetic resonance (EPR) spectra were recorded with a Varian Model E-4 EPR spectrometer equipped with a variable temperature attachment. To enhance the signal to noise ratio of EPR spectra, a PDP 11 minicomputer was utilized to collect and process the data. A rapid quenching device designed after that of Ballou and Palmer [16] was used to prepare short reaction time EPR samples.

All samples for both optical absorption and EPR spectroscopy were prepared at 0°C in potassium phosphate buffer (1.0 M, pH 7.4) which had been deoxygenated with argon. Nitric oxide was passed through KOH pellets before use to remove any possible contamination by higher oxides of nitrogen. For the rapid quench EPR samples, microsomes (~50 mg/ml) or cytochrome $P\text{-}450_{cam}$ (200 μM), gently bubbled with argon at 0°C and reduced with excess sodium dithionite, were rapidly mixed with buffer saturated with NO and quenched according to the method of Ballou and Palmer [16]. The ferrous *P*-420–NO EPR sample was prepared by placing dithionite-reduced *P*-420 (20 mg/ml), prepared with sodium deoxycholate by the method of Omura and Sato [17], into an EPR tube which had been filled with NO gas before freezing in liquid nitrogen. Samples for optical absorption spectra were prepared by adding NO directly to a dilute microsomal suspension or cytochrome $P\text{-}450_{cam}$ solution.

3. Results and discussion

The difference spectrum of the stable ferric microsomal cytochrome *P*-450–NO complex (fig.1) has the Soret band maximum at 437 nm and α and β band maxima at 575 and 544 nm, respectively, as has been previously reported [8]. The absence of EPR signals suggests that this complex is probably diamagnetic and indicates spin-pairing of the unpaired electron of the bound NO with that of the hemin iron. Also shown in fig.1 is the spectrum of the complex formed immediately after the addition of NO to the ferrous microsomal cytochrome *P*-450. This spectrum is identical to that formed upon reduction of the ferric-NO complex. The difference spectrum of this ferrous-NO complex, which has the Soret band maximum at 443 nm and the visible band maximum at 586 nm, changes within minutes even at 0°C suggesting that this complex is unstable. However, at least partial stabilization of this complex is achieved by the addition of substrate (unpublished results). Similar spectra have been observed for cytochrome $P\text{-}450_{cam}$ (ferric-NO: Soret-431 nm, α-572.5 nm, β-541 nm, ferrous-NO: Soret-438 nm, visible-558.5 nm) (fig.2) with the exception that the ferrous-, as well as the ferric-, NO complex appears to be stable even at room temperature. As with the microsomal cytochrome *P*-450, the NO complex of ferric cytochrome $P\text{-}450_{cam}$ lacks EPR signals.

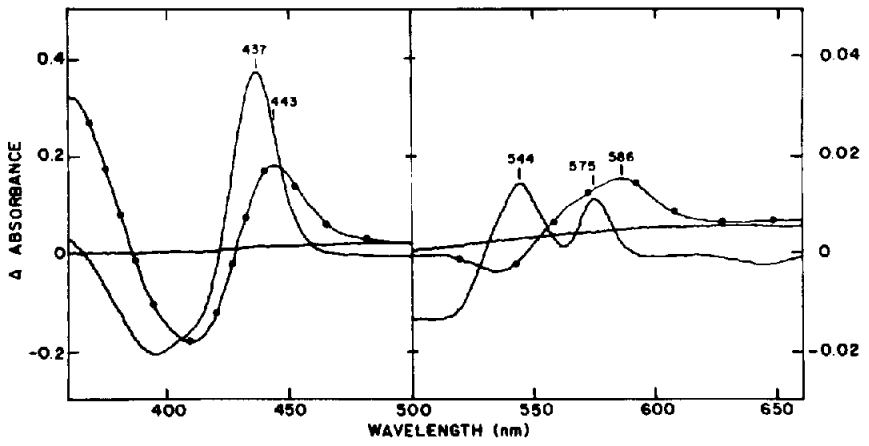


Fig.1. Difference spectra of microsomal cytochrome *P*-450–NO complexes using 2 mg microsomal protein per ml: ferric-NO versus ferric (——); ferrous-NO versus ferrous (—•—).

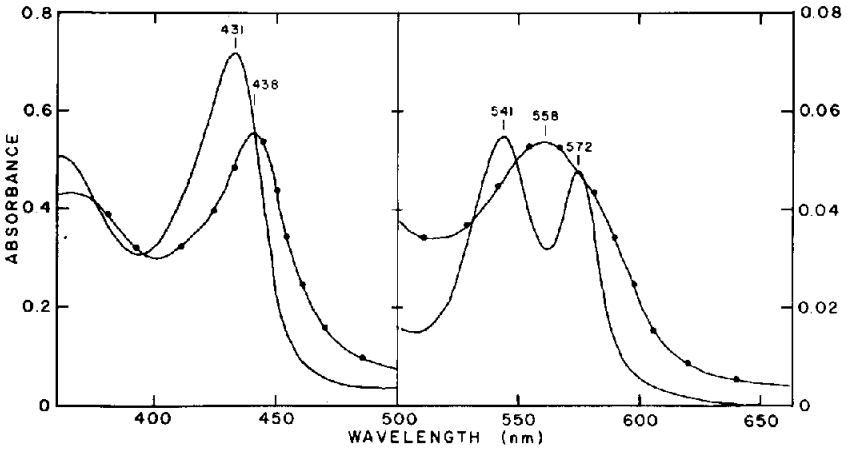
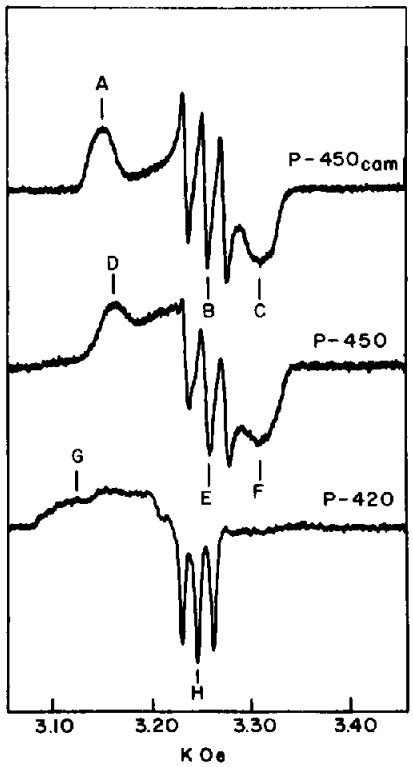


Fig.2. Optical absorption spectra of cytochrome $P\text{-}450_{cam}$–NO complexes (8 μM) in 50 mM potassium phosphate (pH 7.4), 100 mM potassium chloride, 4 mM camphor: ferric-NO (——); ferrous-NO (—•—).



In both the microsomal and bacterial cytochrome *P*-450 the intensities and positions of the absorbance band maxima observed for the ferric-NO complexes are similar to a low spin pyridine hemochromogen except that the β band is more intense than the α band. The spectrum of the ferrous-NO complex of cytochrome $P\text{-}450_{cam}$ resembles that of its ferrous-CO complex. Thus these cytochrome *P*-450 ferric- and ferrous-NO complexes have spectral characteristics similar to those to two other oxygen-activating hemeproteins, horseradish peroxidase (HRP) and cytochrome *c* peroxidase (CCP) [7].

The EPR spectra of the ferrous-NO complex resulting from the reaction of ferrous cytochrome $P\text{-}450_{cam}$ or microsomal cytochrome *P*-450 with NO, quenched 30 msec after mixing (see Methods), are shown in fig.3. In both cases an intense EPR signal centered around g = 2 with rhombic symmetry is observed. These spectra are quite similar with hyper-

Fig.3. EPR spectra of ferrous cytochrome *P*-450 and *P*-420–NO complexes. the EPR samples were prepared as described in Methods. The appropriate instrument parameters were: modulation amplitude, 2.0 Orsted; microwave frequency, 9.13 GHz; power, 10 mW and temperature, −170°C. The spectra represent the averaged signal for 32 scans which were collected by the minicomputer. The g values indicated in the figure are: A, 2.068; B, 2.002; C, 1.970; D, 2.062; E, 2.002; F, 1.970; G, 2.089; and H, 2.009. The points indicated by A, D and G are g_x; B, E and H are g_z; and C and F are g_y.

fine splitting for the g_z signal of 20 G. The origin of the signal at g = 2.03 in the microsomal cytochrome *P*-450 is unknown. The EPR spectrum of the ferrous microsomal cytochrome *P*-450–NO complex changes with time to that of the ferrous *P*-420–NO complex also shown in fig.3. The EPR spectrum of the *P*-420–NO complex resembles that of sodium dodecyl sulfate-modified hemoglobin [1] and, as has been proposed for the NO derivatives of structurally perturbed Fe(II) hemeproteins [5,6], may indicate that the bond to the axial ligand *trans* to the bound NO is severely distorted or broken. The EPR spectra previously reported for the ferrous-NO complex of cytochrome *P*-450 in microsomes [11,12] and in submicrosomal particles [9,10] resemble that of the ferrous *P*-420–NO complex and support the above suggestion that the ferrous microsomal cytochrome *P*-450–NO complex is unstable. This problem is currently under investigation.

We have also noted that the EPR signal assigned to the axial or z-absorption, especially in the ferrous cytochrome $P\text{-}450_{cam}$–NO complex, is significantly asymmetric although no distinct superhyperfine lines can be observed in contrast to similar spectra of HRP- and CCP-NO complexes.[7]. However, this asymmetry may indicate electron spin-nuclear spin interaction of the heme-bound NO with the *trans* axial ligand. Since sulfur lacks nuclear spin, the existence of an axial sulfhydryl (cysteine) lignad *trans* to bound NO or CO is questionable.

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