A MODEL COMPOUND FOR NITROSYL CYTOCHROME *P*-450; FURTHER EVIDENCE FOR MERCAPTIDE SULFUR LIGATION TO HEME*

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1. Introduction

Cytochromes *P*-450 comprise a ubiquitous class of hemoproteins, members of which are involved in binding and activation of molecular oxygen and subsequent mixed-function oxidation of a wide variety of substrates [1,2]. These enzymes were first distinguished by their unusual optical absorption spectrum when in the ferrous oxidation state with carbon monoxide bound as a heme ligand [3,4]. Whereas, carbon monoxide derivatives of heme proteins such as hemoglobin and myoglobin exhibit Soret absorptions near 420 nm, carbon monoxy cytochromes *P*-450 absorb near 450 nm. Previous e.p.r. studies have demonstrated that sulfur, presumably from cysteine, is a necessary axial ligand in all low-spin *ferric* cytochromes P-450 [5–8] but evidence that this ligand also occurs in the *ferrous* state has been nonexistent. Since oxygen binding to the ferrous proteins will be affected by the iron ligand it displaces and/or the ligand remaining which is *trans* to it, the identification of the unusual state of heme ligation found in ferrous cytochrome *P*-450 is essential to our understanding of enzyme function.

Recently, we have been able to reproduce the atypical optical spectrum of carbon monoxy cytochrome *P*-450 using models composed of mononuclear ferrous heme, CO and mercaptide anion (RS^-) [9,10]. These results, subsequently confirmed in different laboratories [11,12], strongly suggest the requirement for mercaptide anion as a ferrous heme ligand *trans* to CO in order to produce the unusual optical property that distinguishes cytochrome *P*-450 from other hemoproteins.

Not only can ferrous cytochrome *P*-450 bind to CO, but it is also capable of binding other exogenous ligands as well. One of these, nitric oxide, binds to cytochromes *P*-450 obtained from both liver microsomal and bacterial sources [13]. In a similar fashion that CO elicits an unusual Soret absorption from ferrous cytochrome *P*-450, NO does as well. Here, the Soret absorption is at 437 nm as compared to the usual absorption of 420 nm observed for nitrosyl myoglobin.

In this present communication, we describe the optical properties of a model compound for nitrosyl cytochrome *P*-450. We demonstrate that the requirement for mercaptide anion as a necessary heme ligand is a property of NO as well as CO ligation in these heme proteins.

2. Materials and methods

Dimethylsulfoxide (DMSO) (Burdick and Jackson, Inc., Muskegon, Michigan), deoxygenated by equilibration with argon, was saturated with sodium methyl mercaptide (0.30 M) followed by the anaerobic addition of dibenzo-18-crown-6 polyether (0.15 M) in order to enhance the activity of the mercaptide anion in the solvent [11]. Reagent grade crown ether was found to contain colored impurities which were removed by 2 × recrystallization from chloroform. Prior to adding nitric oxide, the solution of sodium methyl mercaptide and crown ether was mixed in a Thunberg optical cell with hemin chloride dissolved in DMSO (6–16 μM) either under argon or CO. Nitric oxide was scrubbed first through a concentrated

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solution of NaOH to remove higher oxides of nitrogen which causes precipitation and destruction of heme, followed by a column of NaOH pellets in order to remove water vapor. Hemin chloride was treated briefly before use with dry HCl gas in $CHCl_3$ in order to cleave any μ-oxo contaminants. In some cases the methyl mercaptide was replaced by an alkyl mercaptan (R = CH_3-,$CH_3(CH_2)_2$-,$OHCH_2CH_2$-, any of which yield similar results) of equivalent concentration.

3. Results

The typical absorption spectrum of heme CO is shown in fig.1 (curve a) resulting from mixing an alkyl mercaptan with ferric hemin chloride in DMSO under a CO atmosphere. This species exhibits absorption maxima at 332 nm (ϵ_{mM} = 40), 414 nm (ϵ_{mM} = 200), 535 nm (ϵ_{mM} = 13) and 567 nm (ϵ_{mM} = 16). As previously reported [9] the formation of this species is independent of the presence of mercaptan except as a source of reducing equivalents for the ferric heme. If this sample is now flushed with argon for a few minutes the spectral features of ferrous heme (fig.1, curve b) are seen at 424 nm (ϵ_{mM} = 205), 525 nm (ϵ_{mM} = 12) and 555 nm (ϵ_{mM} = 15). The same spectrum is observed in the absence of mercaptan if dithionite is used as a reductant. The introduction of nitric oxide at this point generates a heme NO species (fig.1, curve c) with absorption maxima at 402 nm (ϵ_{mM} = 88) and 556 nm (ϵ_{mM} = 11). Now adding pyridine shifts the Soret to 420 nm and the visible spectrum is that of a characteristic nitrosyl heme complex, similar to that seen for nitrosyl myoglobin (table 1).

The absorption spectrum obtained by mixing

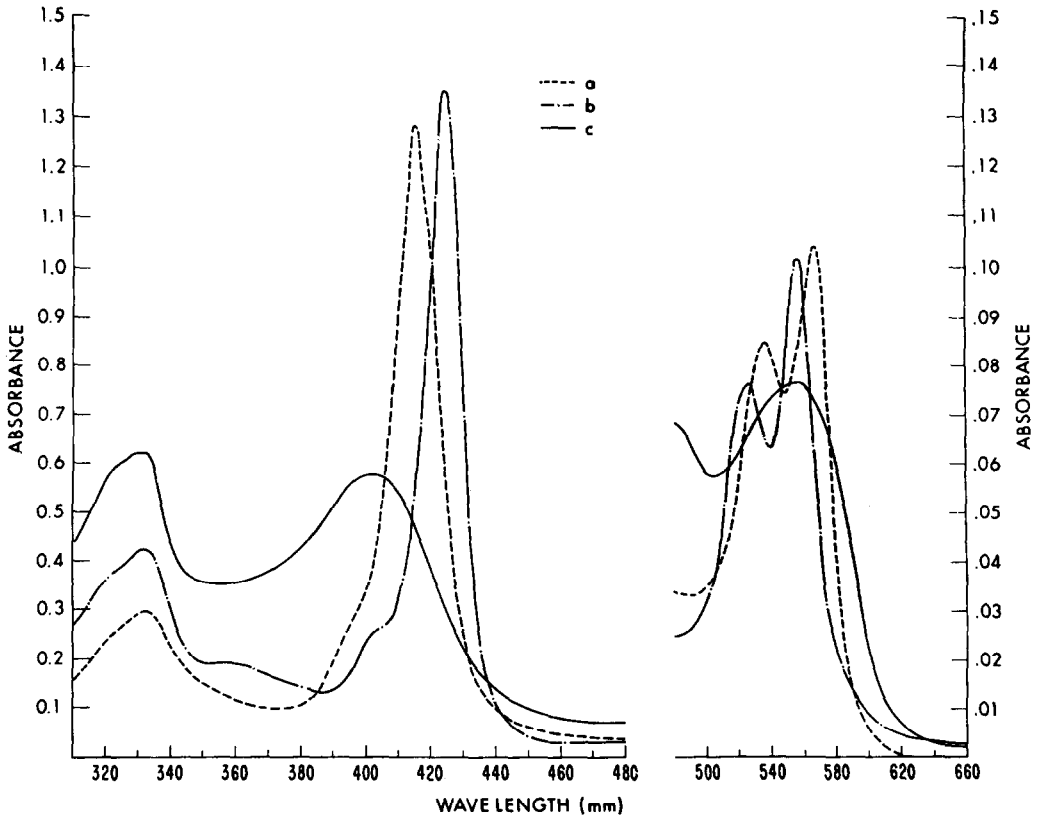

Fig.1. Optical absorption spectra of heme compounds prepared in the presence of mercaptoethanol. In (a) hemin chloride in dimethylsulfoxide was reacted with CO, in (b) CO was purged from the solution with argon, while in (c) NO was added afterwards. The concentration of heme (6.60×10^{-6} M) was the same in all three spectra.

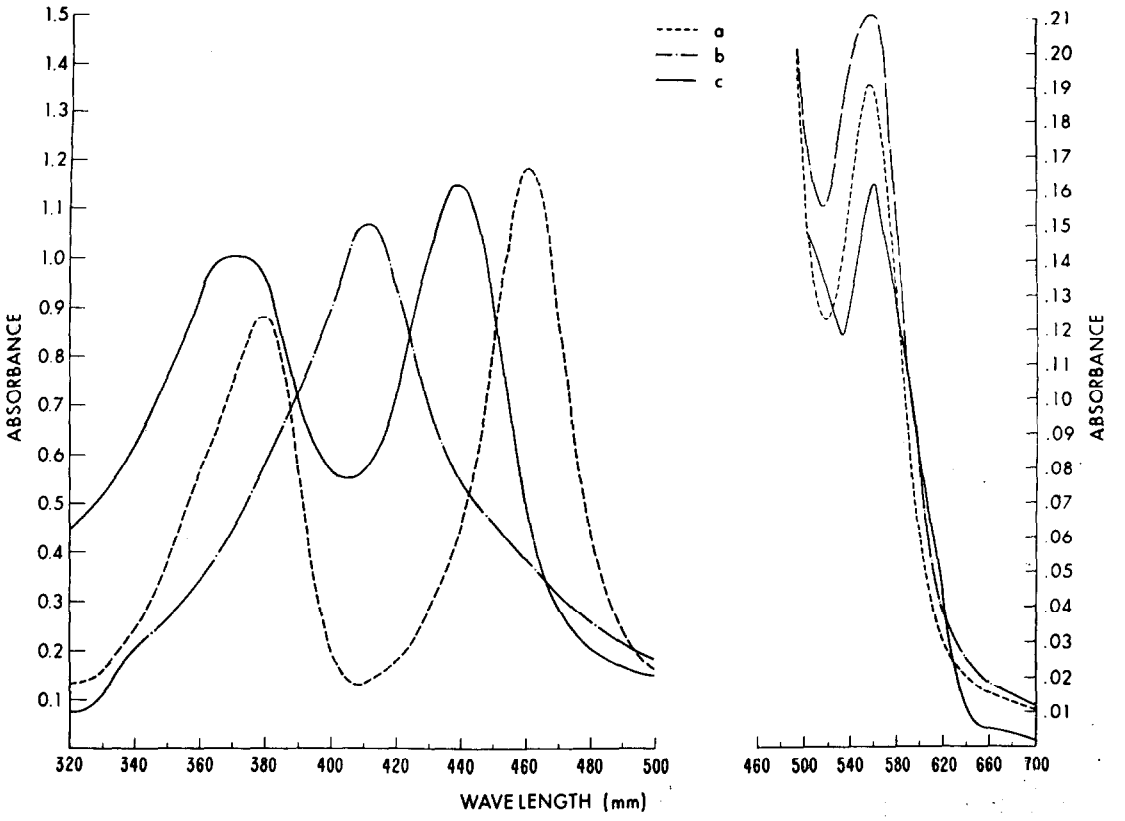

Fig.2. Optical absorption spectra of heme compounds prepared in the presence of sodium methyl mercaptide and dibenzo-18-crown-6-ether. In (a) hemin chloride in dimethylsulfoxide was reacted with CO, in (b) CO was purged from the solution with argon, and in (c) NO was added afterwards. The concentration of heme in (a) and (b) was 1.61×10^{-5} M and was 1.22×10^{-5} M in (c).

ferric hemin chloride, sodium methyl mercaptide and crown ether in DMSO under CO is shown in fig.2 (curve a). This *hyperporphyrin* spectrum [14] is characteristic of the CO adduct of ferrous cytochrome *P*-450. Absorption features are seen at 380 nm (ϵ_{mM} = 60), 460 nm (ϵ_{mM} = 73) and 555 nm (ϵ_{mM} = 12). The absorptions at 380 and 460 nm can be shifted by about 10 nm to higher energy (i.e., 370 (ϵ_{mM} = 70) and 450 nm (ϵ_{mM} = 85), respectively) remniscent of carbon monoxy cytochrome *P*-450, if a 1:1 DMSO–ethanol solvent mixture replaces the DMSO [9]. However, ethanolysis of sodium methyl mercaptide takes place in this solvent mixture and is therefore not routinely used*. If the carbon monoxide is removed from the model compound in DMSO by gassing with argon, a *normal-porphyrin* spectrum is seen (fig.2, curve b), with λ_{max} at 410 nm (ϵ_{mM} = 80) and 550 nm (ϵ_{mM} = 13), which differs markedly from the compound prepared where a thiol is used instead of a mercaptide (fig.1). Both of these ferrous heme species are stable and completely interconvertible to their respective CO complexes by cycling back and forth with argon and CO. When nitric oxide is added to the ferrous heme mercaptide complex in the presence of CO, precipitation takes place obscuring the optical spectrum. If, however, aliquots of NO are added after the removal of CO with argon, a new hyperporphyrin spectral species is formed (fig.2, curve c) which is identical to that seen for nitrosyl cytochrome *P*-450 [13], with absorption maxima at 370 nm (ϵ_{mM} = 84), 437 nm (ϵ_{mM} = 92) and 557 nm (ϵ_{mM} = 14) (table 1).

*As ethanolysis converts the sodium methyl mercaptide to methyl mercaptan, the 450 nm-absorbing species decreases and the 414 nm-absorbing species appears.

Table 1
Soret and visible absorption maxima of nitrosyl heme complexes

NO +	
Heme	402, 556
Heme + alkyl mercaptan	402, 556
Heme + sodium methyl mercaptide	437, 557
Heme + pyridine	420, 545, 576
Cytochrome *P*-450$_{cam}$[a]	438, 558
Sperm whale myoglobin[b]	420, 543, 577

[a] After [12].
[b] Prepared by the NO displacement of CO in sperm whale carbo myoglobin (Seravac).

4. Discussion

The intensive investigations by our group [9,10] and others [11,12] regarding the spectral properties of heme-mercaptide complexes strongly imply that mercaptide anion is ligated to heme in CO complexes of cytochromes *P*-450. The ability of mercaptide anion to generate these unique cytochrome *P*-450 spectra is now firmly based on theoretical grounds which has provided a molecular orbital explanation for these types of hyperporphyrin spectra [14]. From the spectral similarities of the model compound prepared from heme, mercaptide anion and NO compared with the nitrosyl derivative of cytochrome *P*-450$_{cam}$ (table 1), we conclude that the same mercaptide ligation occurs for the NO complexes as well.

Based on the spectral differences observed for ferrous heme prepared both in the presence and absence of mercaptide (figs.1 and 2), it is suggested that mercaptide anion binds to heme even in the absence of CO or NO. It is unlikely that undissociated thiol binds to heme [15] as its absence has little effect on the optical spectrum of ferrous heme as well as the CO- and NO-ligated forms.

Recently, Chang and Dolphin [12] have synthesized and oxygenated mercaptide heme complex which also displays a hyperporphyrin absorption spectrum. In this case, the Soret absorption is at 428 nm (as compared to oxygenated complexes of hemoglobin and myoglobin which absorb near 418 nm) and also shows a strong absorption in the ultraviolet. Curiously, the oxygenated complex of cytochrome *P*-450$_{cam}$ [16] exhibits a normal porphyrin spectrum with a weak absorption in the ultraviolet, a Soret absorption centered at 418 nm and a visible absorption very similar to that for oxymyoglobin, oxyhemoglobin or a complex of imidazole, ferrous heme and oxygen [17]. Whereas NO or CO binding to cytochrome *P*-450 can be demonstrated either in the presence or absence of substrate, oxygen binding can only be demonstrated when substrate is present [18,19]. It is suggested, therefore, that in oxy cytochrome *P*-450, mercaptide is no longer bound to heme, being displaced by the oxygen molecule [20]. The mechanism of control of binding neutral diatomic molecules has been speculated upon [21] for hemoglobin, but this differentiation of 0_2- as compared to NO- and CO-ligation is unique in hemoprotein chemistry and will undoubtedly provide insight into the activation of molecular oxygen as related to drug metabolism.

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