Formation of Prostaglandin Synthase–Iron–Nitrosoalkane Inhibitory Complexes upon in Situ Oxidation of N-Substituted Hydroxylamines

J. P. Mahy and D. Mansuy*

Laboratoire de Chimie et Biochimie Pharmacologiques et Toxicologiques, URA 400 CNRS, Université René Descartes, 45 rue des Saints-Pères, 75270 Paris Cedex 06, France

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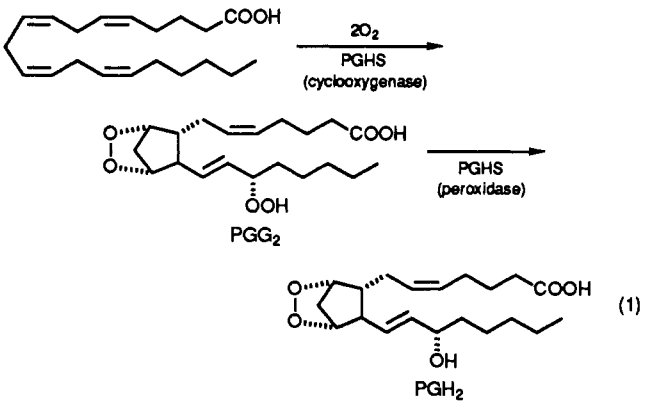
ABSTRACT: Various *N*-alkylhydroxylamines such as *N*-hydroxyamphetamine react with prostaglandin synthase (PGHS) from sheep seminal vesicles, with the formation of new complexes characterized by a Soret peak around 421 nm. These complexes are very stable toward O_2 or dithionite but are destroyed upon oxidation by $Fe(CN)_6K_3$ with regeneration of starting PGHS–Fe^{III}. Their spectral characteristics, chemical properties, and routes of formation (either by direct oxidation of RNHOH or by in situ reduction of RNO_2 in the presence of dithionite) are very similar to those previously reported for nitrosoalkane complexes of hemoglobin–, myoglobin–, and cytochrome P-450–Fe^{II}. Their Fe^{II}–N(O)R structure was completely confirmed in the case of *N*-hydroxyamphetamine, both by extraction of the heme complex by butanone and by identification to authentic protoporphyrin IX–Fe^{II}–N(O)-amphetamine, and by insertion of this authentic complex into apoPGHS. Phenylhydroxylamine also reacts with PGHS–Fe^{III} to give a PGHS–Fe^{II}–N(O)Ph complex which is not stable in the presence of dithionite because of its weaker PGHS–Fe^{II}–N(O)R bond when compared to PGHS–Fe^{II}–nitrosoalkane complexes. The ability of various *N*-alkylhydroxylamines to form PGHS–Fe^{II}–N(O)R complexes greatly depends upon their hydrophobicity. Actually, CH_3NHOH and C_2H_5NHOH are totally inactive whereas about 10 molar excess of *N*-hydroxyamphetamine and C_6H_5NHOH already lead to 50% complex formation. This is in favor of an hydrophobic environment of the heme in PGHS. Finally, PGHS engaged in such Fe^{II}–nitrosoalkane complexes completely loses its dioxygenase activity, suggesting that N-substituted hydroxylamines or compounds that can be metabolized in vivo to give such hydroxylamines could act as strong PGHS inhibitors.

PGH synthase (PGHS) catalyses the first two steps of the biosynthesis of prostaglandins, thromboxanes, and prostacyclin (Hemler et al., 1976; Miyamoto et al., 1976; Van Der Ouderaa et al., 1977). It first acts as a dioxygenase (cyclooxygenase) which oxidizes arachidonic acid to prostaglandin G_2 (PGG_2) and then as a peroxidase which reduces the hydroperoxide PGG_2 to prostaglandin H_2 (PGH_2) (Hamberg et al., 1974; Ohki et al., 1979) (eq 1). PGH synthase has been purified



(1)

to apparent electrophoretic homogeneity from bovine (Miyamoto et al., 1976) and sheep (Hemler et al., 1976; Marnett et al., 1984; Nastainczyk et al., 1984; Roth et al., 1981; Van Der Ouderaa et al., 1977) vesicular glands and sheep platelets (Boopathy & Balasubramanian, 1986), and it has been characterized as a homodimer of two 70-kDa polypeptide subunits (Boopathy & Balasubramanian, 1986; Hemler et al., 1976; Marnett et al., 1984; Miyamoto et al., 1976; Nastainczyk et al., 1984; Roth et al., 1981; Van Der Ouderaa et al., 1977). The amino acid sequence of the polypeptide has recently been deduced from the sequence of the cDNA encoding for sheep (De Witt & Smith, 1988; Merlie et al., 1988; Yokoyama et al., 1988) and human (Yakoyama et al., 1989) PGHS. Moreover, it has been shown that this polypeptide required heme for both cyclooxygenase and peroxidase activities (Hemler et al., 1976; Roth et al., 1981; Van Der Ouderaa et al., 1977). EPR titrations clearly showed that one heme is bound per subunit (Karthein et al., 1987). However, the nature of the endogenous axial ligand of the heme iron atom remains unclear. The presence of a tyrosinate ligand has been proposed on the basis of an EPR study (Karthein et al., 1987), whereas the great analogy of the UV–visible spectral properties of PGHS with those of horseradish peroxidase suggests the presence of a histidine as a proximal axial ligand (Karthein et al., 1987; Kulmacz et al., 1987; Lambeir et al., 1985; Mac Donald & Dunford, 1989; Markey et al., 1987; Marnett et al., 1979; Plé & Marnett, 1989; Van Der Ouderaa et al., 1979).

Only a few complexes of PGHS iron with exogenous ligands have been reported so far. They include the Fe^{III}–CN (Kulmacz et al., 1987; Mac Donald & Dunford, 1989; Van Der Ouderaa et al., 1979) complex and complexes of PGHS–Fe^{II} with NO (Karthein et al., 1987), ^-CN (Van Der Ouderaa et al., 1979), CO (Marnett et al., 1979), and O_2 (Lambeir et al., 1985). Moreover, as yet, the formation of PGHS–iron–metabolite complexes derived from the in situ oxidation of exogenous compounds by PGHS has never been reported, although PGHS was found to be able to oxidize a wide variety of such compounds thanks to its peroxidase activity (Markey et al., 1987; Plé & Marnett, 1989). In order to explore the possibility of formation of such PGHS–iron–metabolite complexes and to further define the coordination

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chemistry of PGHS, we have studied the ability of this heme protein to form Fe^{II}–nitrosoalkane complexes. It has been previously reported that very stable porphyrin–Fe(II)–nitrosoalkane complexes were easily formed upon oxidation of alkylhydroxylamines in the presence of ferric porphyrins (Mansuy et al., 1977c, 1983). Several heme proteins including hemoglobin and myoglobin (Antonini & Brunori, 1971; Gibson, 1960; Mansuy et al., 1976, 1977b; Murayama, 1960) led to Fe(II)–RNO complexes upon in situ oxidation of alkylhydroxylamines. Such complexes of cytochrome P-450 (Mansuy et al., 1976, 1977a, 1978) have been observed not only upon metabolic oxidation of alkylhydroxylamines but also upon that of a wide variety of amines both in vitro and in vivo (Mansuy et al., 1976, 1977a, 1978).

This paper shows that PGHS is able to oxidize various alkyl- or arylhydroxylamines with the formation of very stable Fe^{II}–nitrosoalkane (or nitrosoarene) complexes which exhibit characteristics similar to those already described in the case of cytochrome P-450, hemoglobin, and myoglobin.

Experimental Procedures

Physical Measurements. Absolute UV–visible spectra were recorded on an Aminco-DW2 spectrophotometer. Differential UV–visible spectra were recorded on a Uvikon 820 spectrophotometer. Dioxygen consumption was measured on a Gilson 516H oxygraph fitted with a Clark electrode.

Chemicals. Arachidonic acid, hemin chloride, horseradish peroxidase, bovine liver catalase, and human serum albumin were purchased from Sigma. Nitromethane, nitroethane, 2-nitropropane, and nitrobenzene were purchased from Janssen. Methylhydroxylamine, ethylhydroxylamine, isopropylhydroxylamine (Beckmann, 1909; Ryer & Smith, 1951), and phenylhydroxylamine (Coleman et al., 1955) were obtained by reduction of the corresponding nitro compounds by Zn/NH_4Cl. *N*-Hydroxyamphetamine (**1**) was prepared by reduction of 2-nitro-1-phenylpropene (Gildsdorf & Nord, 1952) by $LiAlH_4$. Sodium dithionite was purchased from Merck and potassium ferricyanide from Prolabo.

Biological Materials. Sheep seminal vesicles were prepared at the slaughterhouse and frozen in liquid nitrogen. They were then homogenized and microsomes were prepared by differential centrifugation according to an already described procedure (Nastainczyk et al., 1984). The microsomes were suspended in 0.1 M Tris-HCl buffer, pH 8.1, with 30% glycerol and stored at −80 °C.

Purification of PGHS from Sheep Seminal Vesicles. Sheep seminal vesicle microsomes suspended in 20 mM phosphate buffer, pH 7.4, containing 0.1 mM flufenamic acid and 1 mM EDTA, were solubilized at 0 °C by addition of 2% Tween 20. The solubilized enzyme was purified after two DEAE-Sephacel columns as described previously (Nastainczyk et al., 1984). The obtained purified enzyme was concentrated and stored at −80 °C in 0.5 M Tris-HCl buffer, pH 8.1, containing 30% glycerol. The pure enzyme showed a band at 70 kDa in SDS gel electrophoresis which was performed as described by Laemmli (1970). Heme incorporation into apoPGHS was performed at 22 °C by progressive addition of apoPGHS to a 4 μM hemin solution obtained by dilution of 10 μL of 0.4 mM hemin (in 0.1 M NaOH) in 1 mL of 0.1 M Tris-HCl buffer, pH 8.1. Addition was done until the absorbance of PGHS–Fe^{III} at 410 nm was maximum. This was obtained after addition of an excess of apoPGHS (between 10 and 16 μM). The ε value of the 410-nm Soret peak of PGHS–Fe^{III} was 120 mM^{-1} cm^{-1}. The dioxygenase activity of the obtained enzyme, measured at 22 °C as described in the following paragraph, was 16.6 μmol of O_2 consumed·min^{-1}·(mg of protein)$^{-1}$ (Van Der Ouderaa et al., 1977).

Protein determination of microsomes and purified PGH synthase was carried out as described by Lowry et al. (1951).

Determination of the Cyclooxygenase Activity. Cyclooxygenase activity was measured at 22 °C by monitoring dioxygen consumption in a 1.3-mL incubation cell equipped with a Clark electrode. For routine determinations of the activity of the different fractions eluted from the DEAE-Sephacel columns, 0.3-mL aliquots of each fraction were pipetted into the cell. A 1 mM solution of hemin in 0.1 M NaOH (3.25 μL; final concentration 2.5 μM) and a 1 M solution of phenol in EtOH (1.3 μL; final concentration 1 mM) were added, and the volume was completed to 1.3 mL with 0.1 M Tris-HCl buffer, pH 8.1. The reaction was started by the addition of 5.2 μL of 0.1 M arachidonic acid in EtOH (final concentration 400 μM). The activity of the enzyme in nmol of O_2·min^{-1}·(mg of protein)$^{-1}$ was calculated by assuming that at 22 °C 0.26 μmol of O_2 was dissolved per milliliter of buffer (Nastainczyk et al., 1984).

For the studies concerning the inhibition of the cyclooxygenase activity by N-substituted hydroxylamines, a slightly different procedure was employed. The cell was filled with 1.3 mL of either 16 or 1 μM purified apoPGHS in 0.1 M Tris-HCl buffer, pH 8.1. Final hemin concentrations of either 4 or 0.25 μM were obtained by addition of either 13 μL of 0.4 mM hemin or 5 μL of 0.065 mM hemin in 0.1 M NaOH. The corresponding solutions were then incubated 15 min with increasing amounts of RNHOH in EtOH. After that time, the reaction was started by addition of 1.3 μL of 0.1 M arachidonic acid in EtOH (final concentration 100 μM).

Spectral Measurements. (A) Reaction of Sheep Seminal Vesicle Microsomes with N-Hydroxyamphetamine (**1**). Microsomes were suspended in 0.1 M Tris-HCl buffer, pH 8.1, so as to have 2 mL of a 4 mg of protein/mL suspension. This final suspension was equally divided between two cuvettes, and a base line of equal light absorbance was recorded. *N*-Hydroxyamphetamine (**1**; final concentration 1 mM) was then added to the sample cuvette, and the difference spectra were recorded after 1, 5, and 9 min. All spectral measurements were done at 22 °C.

(B) Extraction of the Heme of the PGHS–Fe^{II}–N(O)Amph Complex (**2**). A 1-mL aliquot of a solution of 32 μM complex **2** in 0.1 M Tris-HCl buffer, pH 8.1, was prepared by reaction of 1 mM *N*-hydroxyamphetamine (**1**) with a 32 μM solution of PGHS–Fe^{III} prepared as described above. After the formation of complex **2** was complete, as shown by UV–visible spectroscopy, the solution was extracted with 3 mL of ethyl methyl ketone at 0 °C (Teale, 1969). The UV–visible spectrum of the organic layer was then recorded.

(C) Reinsertion of (PPIX)Fe^{II}–N(O)Amph in ApoPGHS. A 5-μL aliquot of 0.8 mM hemin in 0.1 M NaOH was diluted in 1 mL of 0.1 M Tris-HCl buffer, pH 8.1. (PPIX)Fe^{II}–N-(O)Amph (4 μM) was then prepared by addition of 1 mM *N*-hydroxyamphetamine to that solution. When the formation of that complex was complete, 16 μM purified apoPGHS was added progressively until the evolution of the UV–visible spectrum had stopped.

Results

Reaction of N-Hydroxyamphetamine (**1**) *(AmphNHOH) with Microsomal and Purified PGH Synthase.* The addition, under aerobic conditions, of 1 mM *N*-hydroxyamphetamine (**1**) to a microsomal suspension of sheep seminal vesicles in 0.1 M Tris-HCl buffer, pH 8.1, led to the gradual appearance, with isosbestic points at 384, 410, 456, and 511 nm, of a difference spectrum with peaks at 424, 532, and 560 nm and

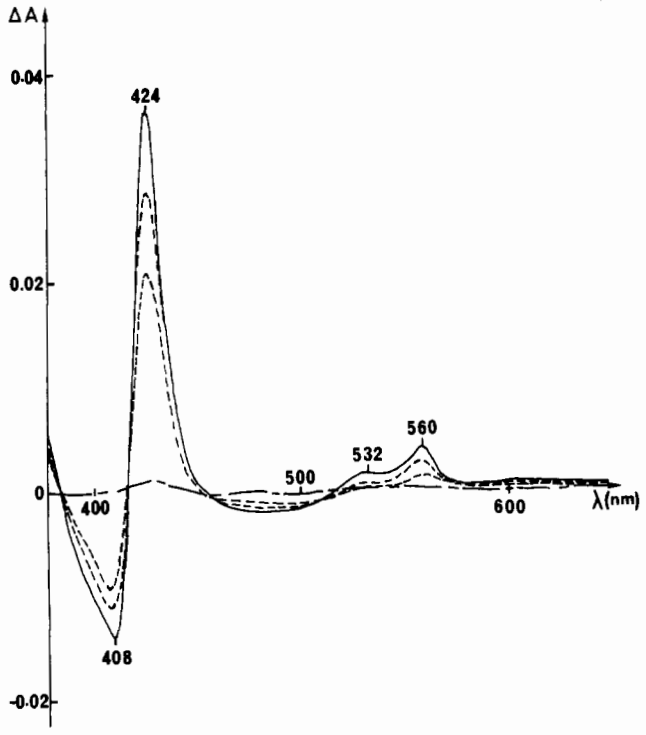


FIGURE 1: Difference spectra of microsomes from sheep seminal vesicles obtained after the addition of *N*-hydroxyamphetamine. The two cuvettes contained 4 mg of protein in 1 mL of 0.1 M Tris-HCl buffer, pH 8.1. *N*-Hydroxyamphetamine (1 mM) was added to the sample cuvette, and the spectra were recorded after 1, 5, and 9 min.

a trough at 408 nm (Figure 1). The same reaction was done with purified PGHS from sheep seminal vesicles (Nastainczyk et al., 1984). For that, a solution of 4 μM iron(III)–PGHS was prepared by progressive addition of purified apoPGHS to a 4 μM solution of iron(III)–protoporphyrin IX in 0.1 M Tris-HCl buffer, pH 8.1, until the absorption at 410 nm was optimal to ensure that no more free hemin remained in the cuvette. This was obtained with a 2.5–4 molar excess of apoPGHS relative to hemin. When this solution was then treated with 1 mM *N*-hydroxyamphetamine (**1**), the absorption spectrum of PGHS–Fe^{III} was replaced within 15 min, with isosbestic points at 412, 453, 513, and 599 nm, by a new spectrum with maxima at 421, 537, and 564 nm (Figure 2). Thus, the difference spectrum observed upon reaction of microsomes with excess AmphNHOH (**1**) can be explained by the formation in the sample cuvette of a new complex of PGHS, **2**, absorbing around 424 nm, the trough at 408 nm being characteristic of unchanged PGHS–Fe^{III} in the reference cuvette.

Nature of Complex **2**. The similarity between the absorption spectrum of complex **2** and those of nitrosoalkane complexes of hemoglobin and myoglobin, Hb– and Mb–Fe^{II}–N(O)R, obtained upon reaction of alkylhydroxylamines with hemoglobin and myoglobin (Antonini & Brunori, 1971; Gibson, 1960; Mansuy et al., 1976, 1977b; Murayama, 1960), strongly suggested a PGHS–Fe^{II}–N(O)Amph structure for complex **2**. This hypothesis was further supported by the following properties of complex **2** which were identical with those previously reported for Fe^{II}–nitrosoalkane complexes of Hb, Mb (Antonini & Brunori, 1971; Gibson, 1960; Mansuy et al., 1976, 1977b; Murayama, 1960), and cytochrome P-450 (Mansuy et al., 1977c, 1983). First, its formation only occurred in the presence of O_2; no spectral change was observed upon addition of 1 mM AmphNHOH to PGHS under strictly anaerobic conditions. Second, complex **2** was stable in 0.1 M

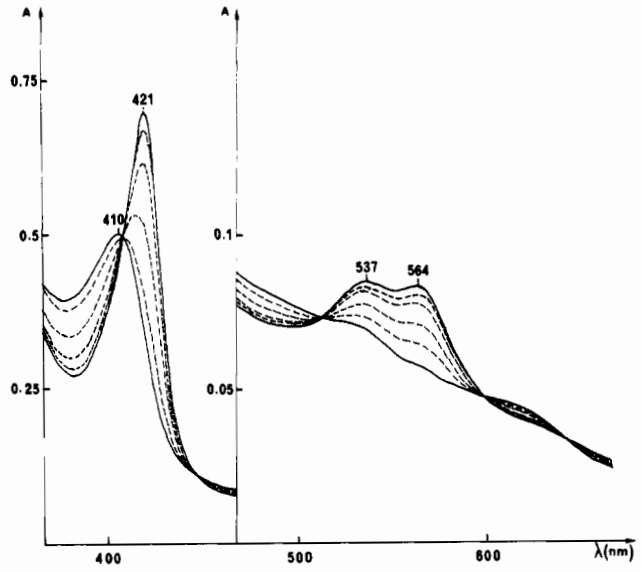


FIGURE 2: Absolute spectra of purified PGH synthase after the addition of *N*-hydroxyamphetamine. Spectra were recorded at t = 0, 1, 5, 9, 13, and 17 min after the addition of 1 mM AmphNHOH to 4 μM PGHS–Fe^{III} obtained upon addition of 4 μM hemin to 16 μM apoPGHS.

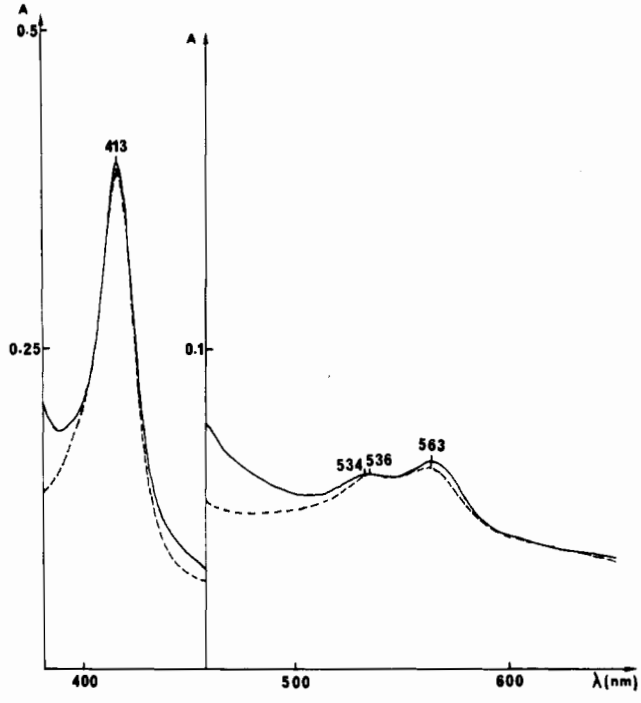


FIGURE 3: Extraction of the heme of complex **2** by ethyl methyl ketone. (—) Visible spectrum of the organic layer obtained upon extraction of the heme of complex **2** (32 μM in 0.1 M Tris-HCl buffer, pH 8.1) by ethyl methyl ketone at 0 °C. (---) Visible spectrum of the (PPIX)Fe^{II}–N(O)Amph complex obtained by reaction of 1 mM *N*-hydroxyamphetamine with 4.05 μM (PPIX)Fe^{III} in 0.1 M Tris-HCl buffer, pH 8.1.

Tris-HCl buffer, pH 8.1, for several hours, either alone or in the presence of a reducing agent (10 mM sodium dithionite), but was rapidly destroyed upon addition of an oxidant [0.2 mM $Fe(CN)_6K_3$], giving back the starting PGHS–Fe^{III} spectrum (data not shown).

Two additional results strongly confirmed the PGHS–Fe^{II}–N(O)Amph structure for complex **2**. Extraction of complex **2** heme by ethyl methyl ketone around 0 °C (Teale, 1969) led to a protoporphyrin IX–iron complex, the visible spectrum of which was identical with that of the penta-

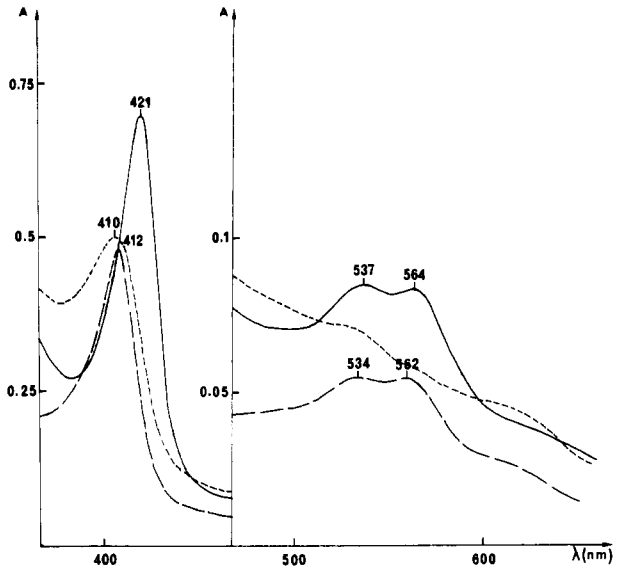


FIGURE 4: Insertion of the (PPIX)Fe^{II}–N(O)Amph complex into apoPGHS. (--) Electronic spectrum of (PPIX)Fe^{II}–N(O)Amph, obtained upon addition of 25 μM AmphNHOH to 4 μM (PPIX)Fe^{III} in 0.1 M Tris-HCl buffer, pH 8.1. (—) Spectrum obtained after addition of 16 μM apoPGHS. (---) Spectrum observed after further addition of 30 μM ferricyanide.

coordinate protoporphyrin IX–Fe^{II}–N(O)Amph complex prepared by direct reaction of AmphNHOH with protoporphyrin IX–Fe^{III}–OH (Mansuy et al., 1983) (Figure 3). Since ethyl methyl ketone is known to cleave the heme–protein bond and to extract the heme from various heme proteins like Mb, Hb, horseradish peroxidase, and catalase (Teale, 1969), these data indicated that complex **2** contained the protoporphyrin IX–Fe^{II}–N(O)Amph complex. Accordingly, addition of 16 μM purified apoPGHS to a solution of (PPIX)-Fe^{II}–N(O)Amph, prepared by reaction of 0.2 mM AmphNHOH with 4 μM Fe^{III}(PPIX)(OH) in 0.1 M Tris-HCl buffer, pH 8.1, led to the almost immediate replacement of the spectrum of the starting complex by that of complex **2** (Figure 4). In order to prove that this insertion of the heme–nitrosoalkane complex into apoPGHS really occurred in the active site of PGHS, the so-formed complex **2** was treated with 0.25 mM $Fe(CN)_6K_3$ to give resting PGHS–Fe^{III} (as shown by visible spectroscopy, Figure 4). The cyclooxygenase activity of this regenerated PGHS was measured (O_2 consumption) and found almost equal to that of purified PGHS at the same concentration and under identical conditions.

Influence of the Concentration of N-Hydroxyamphetamine on the Formation of the PGHS–Fe^{II}–N(O)Amph Complex and on Cyclooxygenase Activity. Figure 5 shows the amount of complex **2** (determined by UV–visible spectroscopy) formed after the addition of increasing concentrations of AmphNHOH (**1**) to a solution of 4 μM PGHS–Fe^{III} in 0.1 M Tris-HCl buffer, pH 8.1. This amount increased with the AmphNHOH concentration, and for concentrations higher than 3 mM, PGHS was totally converted into complex **2**. The AmphNHOH concentration necessary to convert 50% of PGHS–Fe^{III} (4 μM) into complex **2** was 68 μM.

Measurements of PGHS activity [O_2 consumption in the presence of 100 μM arachidonic acid (Nastainczyk et al., 1984)] showed that complex **2** formation was accompanied by a total inactivation of the cyclooxygenase activity of PGHS (Figure 6). However, after treatment of complex **2** by 0.2 mM $Fe(CN)_6K_3$, which regenerates PGHS–Fe^{III}, at least 80% of the cyclooxygenase activity could be recovered (Figure 6). Measurements of O_2 consumption by 4 μM PGHS–Fe^{III} in the presence of 100 μM arachidonic acid, after 15-min preincubation of PGHS–Fe^{III} at 20 °C with increasing amounts of AmphNHOH (**1**), showed that a 240 μM concentration of **1** was necessary to inhibit half of the cyclooxygenase activity of PGHS. These experiments were done with an unusually high PGHS concentration (4 μM) for the sake of comparison since this concentration was previously used for studies of nitrosoalkane complex formation. When identical experiments were done with 0.25 μM PGHS–Fe^{III}, the

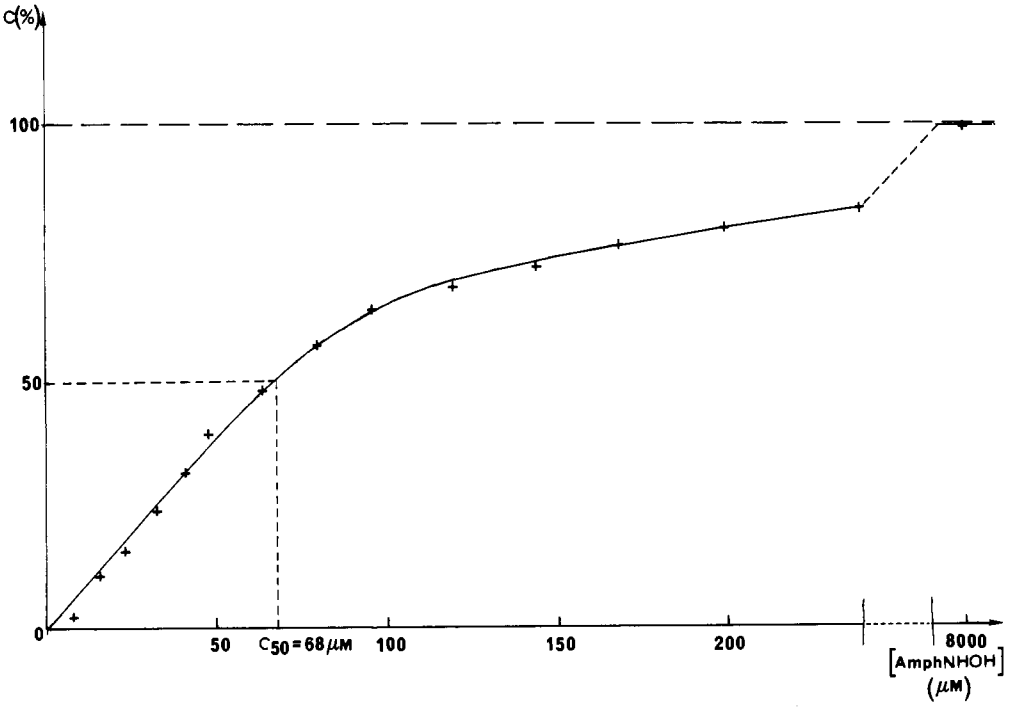


FIGURE 5: Formation of the PGHS–Fe^{II}–N(O)Amph complex as a function of AmphNHOH concentration. The percentage of PGHS having formed complex **2** was determined as a function of AmphNHOH concentration: starting PGHS–Fe^{III} 4 μM in 0.1 M Tris-HCl buffer, pH 8.1.

Table I: Formation of PGHS-Fe^{II}-RNO Complexes by Reaction between PGHS and N-Substituted Hydroxylamines: UV-Visible Characteristics and Compared Formation and Reactivity

PGHS-Fe^{II}-N(O)R, R	UV-visible λ_{max} (nm) [ϵ (mM^{-1} cm^{-1})]			max complex level (%)[a]	C_{50} (μM)[b]	stability to $Fe(CN)_6K_3$[c]	stability to $Na_2S_2O_4$[d]
CH_3[e] (4)	420	532	557	0		-	+
C_2H_5[e] (5)	421	534	559	0		-	+
$(CH_3)_2CH$[f] (3)	421 (162)	536 (20)	564 (19)	47[f]	$>10^4$	-	+
$PhCH_2CHCH_3$ (2)	421 (167)	537 (20)	564 (20)	100	68	-	+
Ph (6)	422 (164)	540 (21)	561 (20)	100	32	-	-
1-naphthyl				0		-	-

[a] Calculated from the following ratio: absorbance at 421 nm after reaction of 1000 equiv of RNHOH with 4 μM PGHS-Fe^{III} in 0.1 M Tris-HCl buffer, pH 8.1, versus theoretical absorbance at 421 nm of 4 μM PGHS-Fe^{II}-N(O)R. [b] RNHOH concentration leading to the conversion of 50% of 4 μM PGHS-Fe^{III} into a nitrosoalkane complex. [c] (-) Fast destruction of the PGHS-RNO complex in the presence of 20 μM $Fe(CN)_6K_3$ with regeneration of PGHS-Fe^{III}. [d] (+) PGHS-RNO complex stable for more than 1 h in the presence of 1 mM $Na_2S_2O_4$; (-) complex destroyed rapidly under identical conditions. [e] In those cases, the PGHS-Fe^{II}-N(O)R complexes were only partially formed upon reduction of the corresponding nitro compounds, and the ϵ values could not be calculated. [f] Total conversion of PGHS-Fe^{III} into the corresponding complex 3 was only possible upon reduction of 2-nitropropane.

concentration of **1** necessary to inhibit half the cyclooxygenase activity was 45 μM.

Reactions of Purified PGHS with Various N-Substituted Hydroxylamines RNHOH. N-Monosubstituted hydroxylamines different from **1**, like isopropyl- and phenylhydroxylamine, were found capable to react with purified PGHS, with the formation of complexes exhibiting spectral properties similar to those of complex **2** (Table I). However, the various studied hydroxylamines exhibited different abilities to form these complexes. In particular, the RNHOH concentration leading to a 50% conversion of PGHS into PGHS-Fe^{II}-RNO (C_{50} value) and the maximum amount of PGHS converted into a nitrosoalkane complex at the highest possible RNHOH concentration varied very much as a function of R in RNHOH (Table I). For instance, under conditions identical with those used previously for **1**, $(CH_3)_2CHNHOH$ reacted with purified PGHS with the formation of a complex exhibiting UV-visible characteristics and reactivities toward sodium dithionite or $Fe(CN)_6K_3$ almost identical with those of complex **2**. However, the ability of $(CH_3)_2CHNHOH$ to react with PGHS was lower than that of **1** since only 47% of the enzyme was converted into a nitrosoalkane complex even when high hydroxylamine concentrations (10 mM) were used. Meanwhile, it is noteworthy that PGHS was completely converted into this complex upon reaction with 2-nitropropane in the presence of 1 mM sodium dithionite (eq 2). The formation of nitrosoalkane complexes

$$\text{PGHS-Fe}^{III} + (CH_3)_2CHNO_2 \xrightarrow{Na_2S_2O_4} \text{PGHS-Fe}^{II}\text{-N(O)CH(CH}_3)_2 \quad (2)$$

of Hb, Mb (Mansuy et al., 1976, 1977b), and cytochromes P-450 (Mansuy et al., 1976, 1977a) by in situ reduction of nitroalkanes was previously reported. This allowed us to calculate the ϵ values of the visible spectrum peaks of the PGHS-Fe^{II}-N(O)$CH(CH_3)_2$ complex **3** (Table I). The formation of complex **3** both by $(CH_3)_2CHNO_2$ reduction and by $(CH_3)_2CHNHOH$ oxidation was a further evidence in favor of its PGHS-Fe^{II}-N(O)$CH(CH_3)_2$ structure.

The most hydrophilic hydroxylamines, CH_3NHOH and C_2H_5NHOH, failed to react with PGHS in the presence of O_2, even though they were used at high concentrations (10^{-2} M). However, the CH_3NO and C_2H_5NO complexes of PGHS, **4** and **5**, could be prepared by reaction of the corresponding nitroalkanes (10^{-2} M) with PGHS (4 μM) in the presence of sodium dithionite (1 mM). Their visible spectra and reactivity toward $Na_2S_2O_4$ and ferricyanide were similar

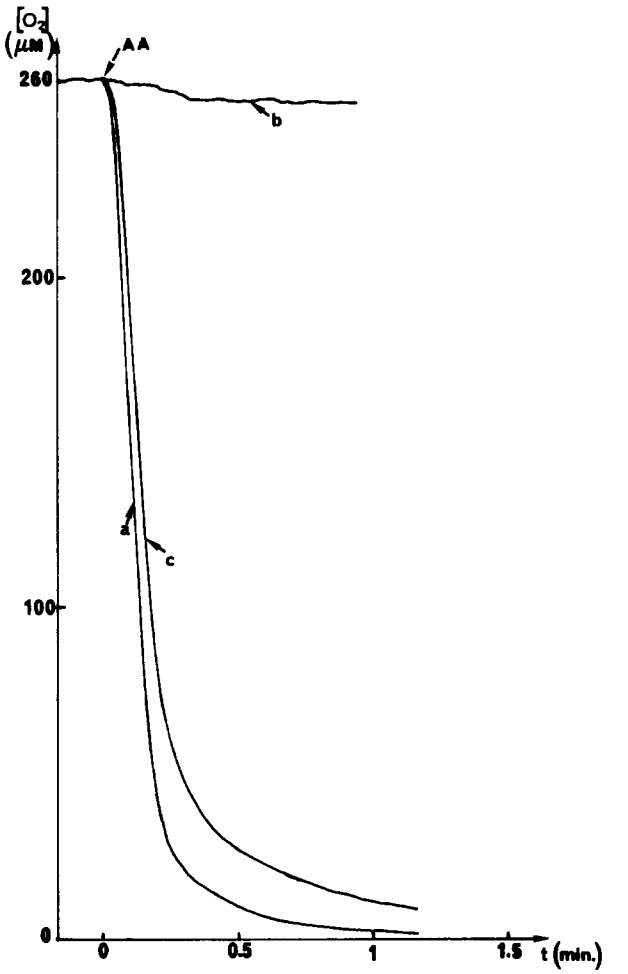


FIGURE 6: Inhibition of the cyclooxygenase activity of PGHS after formation of the PGHS-Fe^{II}-N(O)Amph complex. Trace a: O_2 consumption by 4 μM PGHS-Fe^{III} in the presence of 100 μM arachidonic acid. Trace b: Identical experiments performed with the PGHS-Fe^{II}-N(O)Amph complex instead of PGHS-Fe^{III}. Trace c: O_2 consumption of the solution used for trace b but after addition of 0.2 mM $Fe(CN)_6K_3$ to regenerate PGHS-Fe^{III}.

to those of complex **2** (Table I). Formation of PGHS-nitrosoalkane complexes from reduction of CH_3NO_2 and $C_2H_5NO_2$ but not from oxidation of CH_3NHOH and C_2H_5-NHOH is presumably due to the greater hydrophobicity of the nitroalkanes and their relative better affinity for PGHS.

Table I also shows the results observed upon reaction of arylhydroxylamines with PGHS under the same conditions. Whereas 1-naphthylhydroxylamine did not react even at high

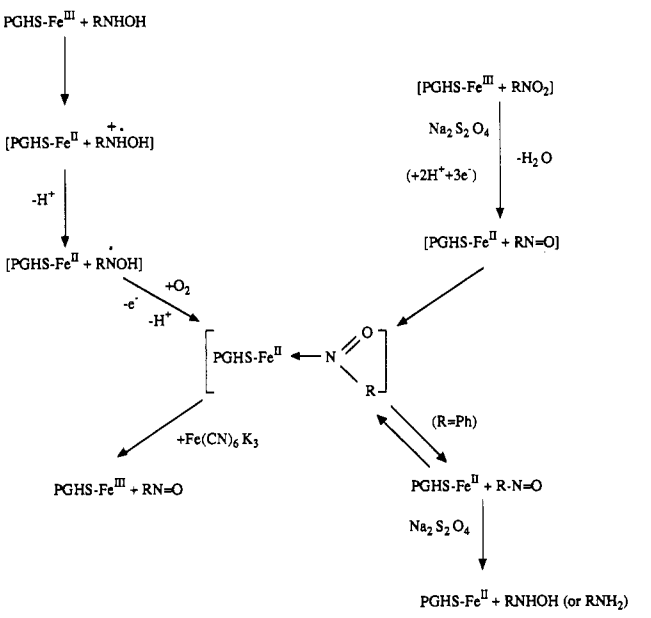
concentrations ($>10^{-2}$ M), 1 mM phenylhydroxylamine, PhNHOH, reacted within 10 min with PGHS-Fe^{III} to yield a complex absorbing at 422, 540, and 561 mn (Table I). As do the aforementioned PGHS-Fe^{II}-nitrosoalkane complexes, this complex, **6**, was transformed into PGHS-Fe^{III} when treated with ferricyanide. However, contrary to the PGHS-Fe^{II}-nitrosoalkane complexes, it was also destroyed by treatment with dithionite (1 mM). Those properties are typical of heme protein-Fe^{II}-nitrosoarene complexes (Antonini & Brunori, 1971; Gibson, 1960; Mansuy et al., 1977a, 1978; Murayama, 1960). The spectral and reactivity characteristics of the PhNHOH-derived PGHS complex, which were identical with those of previously described Hb- and Mb-Fe^{II}-PhNO complexes (Antonini & Brunori, 1971; Gibson, 1960; Mansuy et al., 1977a, 1978; Murayama, 1960), strongly suggest a PGHS-Fe^{II}-PhNO structure. Strikingly, PhNHOH appeared as a very good substrate for complex formation since the concentration necessary to convert PGHS into complex **6** was only 32 μM (less than an 8-fold excess relative to PGHS), the lowest value observed for the studied N-substituted hydroxylamines (Table I). Accordingly, PhNHOH was also the best inhibitor of PGHS, its concentration necessary to inhibit half of the PGHS cyclooxygenase activity being much lower (8 μM for 4 μM PGHS and 0.6 μM for 0.25 μM PGHS) than those observed with AmphNHOH (240 μM for 4 μM PGHS and 45 μM for 0.25 μM PGHS) and $(CH_3)_2CHNHOH$ (>4 mM).

Discussion

Structure of PGHS Complexes Formed by Oxidation of N-Substituted Hydroxylamines RNHOH or Reduction of Nitroalkanes RNO_2. The aforementioned results show the formation of new complexes of PGHS absorbing around 421 nm, upon reaction of alkyl- or arylhydroxylamines with microsomal and purified iron(III)-PGHS under aerobic conditions. A PGHS-Fe^{II}-N(O)R structure is strongly suggested for those complexes by the following characteristics which are almost identical with those previously found for the nitrosoalkane (or arene) complexes of Hb and Mb (Antonini & Brunori, 1971; Gibson, 1960; Mansuy et al., 1976, 1977b; Murayama, 1960): (i) a visible spectrum with peaks around 420, 540, and 560 nm, (ii) a great stability of the alkylhydroxylamine-derived complexes, but not of those derived from arylhydroxylamines, in the presence of an excess of sodium dithionite, (iii) a fast regeneration of the resting ferric heme protein upon treatment by ferricyanide, and (iv) the formation of the Fe^{II}-nitrosoalkane complexes either by in situ oxidation of alkylhydroxylamines or by reduction of the corresponding nitroalkanes in the presence of dithionite. The PGHS-Fe^{II}-N(O)R structure of the AmphNHOH-derived complex was completely confirmed by the following: (i) the identification of its heme, after extraction by ethyl methyl ketone, with a sample of protoporphyrin IX-Fe^{II}-N(O)Amph prepared by direct reaction between AmphNHOH and protoporphyrin IX-Fe^{III} and (ii) the preparation of complex **2** by incorporation of the protoporphyrin IX-Fe^{II}-N(O)Amph complex into purified apoPGHS.

The following results indicate that the RNHOH-derived complexes are really formed in the active site of PGHS and are not heme-RNO complexes bound in an unspecific manner to apoPGHS: (i) they are formed not only by reconstituted purified PGHS (even obtained after addition of a 4 molar excess of apoPGHS to hemin to ensure that no free hemin remains in the solution) but also in seminal vesicle microsomes which contain original PGHS with its endogenous heme; (ii) destruction of complex **2** by ferricyanide regenerates PGHS-Fe^{III} with a cyclooxygenase activity almost equal to that of purified resting PGHS under identical conditions; (iii) whereas 4 μM PGHS-Fe^{III} is converted into complex **2** in the presence of 1 mM AmphNHOH within less than 15 min, the conversion of protoporphyrin-Fe^{III} bound unspecifically to bovine serum albumin into the corresponding Fe^{II}-RNO complex is very slow and requires at least 3 h under identical conditions; and (iv) the hydrophilic hydroxylamines CH_3NHOH and C_2H_5NHOH fail to react with PGHS whereas they are easily oxidized by protoporphyrin IX-Fe^{III} and Hb- or Mb-Fe^{III} to give the corresponding iron^{II}-nitrosoalkane complexes (Mansuy et al., 1977c, 1983). This very different behavior of AmphNHOH, CH_3NHOH, and C_2H_5NHOH toward PGHS and heme, either alone or unspecifically bound to albumin, is presumably due to an efficient recognition of hydrophobic AmphNHOH by the active site of PGHS.

Scheme I



Mechanism of Formation of the PGHS-Fe^{II}-RNO Complexes. As the formation and properties of these complexes are very similar to those previously described for other heme protein-Fe^{II}-RNO complexes, their formation mechanisms (Antonini & Brunori, 1971; Gibson, 1960; Mansuy et al., 1976, 1977a-c, 1978, 1983; Murayama, 1960) should also be very similar.

As far as nitrosoalkane complexes are concerned, and according to what was shown for the reactions between $(CH_3)_2CHNHOH$ with iron(III)-porphyrins (Mansuy et al., 1983), the first step of the reaction between PGHS-Fe^{III} and an alkylhydroxylamine RNHOH should be a one-electron transfer leading to PGHS-Fe^{II} and the radical cation $[RNHOH]^{\cdot +}$ (Scheme I). The second one-electron oxidation necessary to give RNO could then be achieved by O_2. The nitrosoalkane species formed after the loss of two protons should bind to PGHS-Fe^{II}. It is noteworthy that both the RNO species, which are known to be unstable as monomers in the reaction medium (Mansuy et al., 1976, 1977b), and PGHS-Fe^{II}, which is oxidized into PGHS-Fe^{III} by O_2 (Kulmacz et al., 1987), are stabilized by formation of a Fe^{II}-N(O)R bond since PGHS-RNO complexes are stable for days in aerobic buffer. This stabilization is due to the exceptional strength of the porphyrin-Fe^{II}-nitrosoalkane bond (Mansuy et al., 1977c, 1983). On the contrary, porphyrin-Fe^{III}-RNO bonds are very weak (Mansuy et al., 1977c, 1983), explaining why, upon oxidation of PGHS-Fe^{II}-N(O)R complexes by ferricyanide, the RNO ligand not enough stabilized

by PGHS-Fe^{III} is released from the active site and undergoes almost irreversible dimerization or tautomerization to the corresponding oxime (Antonini & Brunori, 1971; Gibson, 1960; Mansuy et al., 1976, 1977a–c, 1978, 1983; Murayama, 1960). Thus, as in the case of Hb (Mansuy et al., 1977b) or cytochrome P-450 (Mansuy et al., 1976, 1977a, 1978), starting PGHS-Fe^{III} is regenerated with an almost intact activity.

The formation of PGHS-Fe^{II}-RNO complexes in the presence of nitroalkanes and dithionite should be due to the concomitant reduction of PGHS-Fe^{III} into PGHS-Fe^{II} and of RNO_2 into RNO (may be via PGHS-Fe^{II}) by sodium dithionite, followed by the strong binding between RNO and PGHS-Fe^{II} (Scheme I, route b). The great stability of the Fe^{II}–nitrosoalkane bond explains the stability of the obtained complexes in the presence of dithionite in excess. On the contrary, the heme protein-Fe^{II}–nitrosoarene complexes are not stable in the presence of dithionite (Antonini & Brunori, 1971; Gibson, 1960; Mansuy et al., 1976, 1977a–c, 1978, 1983; Murayama, 1960). This is due to the strength of the Fe^{II}–nitrosoarene bonds which was found much smaller than that of the Fe^{II}–nitrosoalkane bonds in the case of Hb, Mb (Mansuy et al., 1977b), or cytochrome P-450 (Mansuy et al., 1978). Actually, whereas Fe^{II}–nitrosoalkane complexes involve an almost irreversible bond, Fe^{II}–nitrosoarene complexes are in equilibrium with free nitrosoarene (Mansuy et al., 1977b, 1978). Dithionite reduces free nitrosoarenes irreversibly (Aloy & Rabaut, 1905; Audrieth & Sveda, 1944), displacing completely the equilibrium toward heme protein-Fe^{II}. This explains why the PGHS-Fe^{II}–N(O)Ph complex is not stable in the presence of dithionite.

Comparison between the Different N-Substituted Hydroxylamines. The reactivity of N-substituted hydroxylamines RNHOH toward PGHS is highly dependent on the nature of the R substitutent (Table I). Methyl- and ethylhydroxylamine do not give PGHS–nitrosoalkane complexes, presumably because they are not hydrophobic enough to interact with the PGHS active site. The more hydrophobic isopropylhydroxylamine reacts with PGHS-Fe^{III} but is only able to convert half of the enzyme into complex **3** at very high concentrations ($C_{50} \approx 10$ mM). Replacement of one hydrogen atom of a methyl group of $(CH_3)_2CHNHOH$ by a phenyl group strikingly increases its reactivity since AmphNHOH is able to convert all the starting PGHS into complex **2** and a much lower concentration ($C_{50} = 68$ μM) is sufficient to convert half of the starting PGHS (Table I). Another hydroxylamine bearing a phenyl substituent, PhNHOH, totally and rapidly converts PGHS into complex **6**, the concentration necessary to convert 50% of the enzyme being only 32 μM in that case (Table I). The very hydrophobic 1-naphthylhydroxylamine fails to give a PGHS-Fe^{II}–RNO complex, presumably because the steric hindrance of the naphthyl substituent of the nitrosoarene ligand prevents its binding to the iron atom.

The easier formation of PGHS-Fe^{II}–N(O)R complexes by reduction of CH_3NO_2 or $C_2H_5NO_2$ than from the oxidation of the corresponding hydroxylamines could be explained by the greater hydrophobicity of the nitroalkanes.

Inhibition of the cyclooxygenase activity of PGHS is also very dependent on the nature of RNHOH. Isopropylhydroxylamine and *N*-hydroxyamphetamine are poor inhibitors since 240 μM AmphNHOH and more than 4 mM $(CH_3)_2CHNHOH$ concentrations are necessary to inhibit 50% of the starting cyclooxygenase (4 μM) activity. The same effect is produced by only 8 μM PhNHOH, which appears as the best inhibitor among the studied hydroxylamines. Since these values are different from the above-mentioned C_{50} values (Table I), it is clear that the formation of inactive PGHS-Fe^{II}–N(O)R complexes is not the only phenomenon responsible for the inhibition of PGHS. It is noteworthy that, with 0.25 μM PGHS-Fe^{III}, the AmphNHOH and PhNHOH concentrations necessary to inhibit 50% activity were only 45 and 0.6 μM. Further work is needed to determine the mechanism of these inhibitory effects.

Table II: Compared UV–Visible Characteristics and Proximal Axial Ligands of N-Substituted Nitroso Complexes of Heme Proteins

complex[a]	UV-visible characteristics λ_{max} (nm)			axial ligand of Fe	ref
PGHS-Fe^{II}-N(O)-Amph (**2**)	421	537	564	?	this work
P-450-Fe^{II}-N(O)-Amph	455			cysteinate	Mansuy et al., 1976
Mb-Fe^{II}-N-(O)C_5H_{11}	425	547	576	imidazole	Mansuy et al., 1977b
Hb-Fe^{II}-N(O)Ph	422	542	562	imidazole	Antonini & Brunori, 1971
Cat-Fe^{II}-N(O)Ph	421	540	563	tyrosine	this work

[a] For each hemoprotein the complex described here is the one bearing the biggest ligand able to bind the iron.

Comparison of the Iron(II)–RNO Complexes Formed from Various Heme Proteins. (A) Analogy of Their Spectral and Chemical Properties. The formation of iron(II)–RNO complexes upon N-substituted hydroxylamine oxidation or nitroalkane reduction has been observed with various heme proteins including Hb, Mb (Antonini & Brunori, 1971; Gibson, 1960; Mansuy et al., 1976, 1977b, Murayama, 1960), cytochromes P-450 (Mansuy et al., 1976, 1977a, 1978), and PGHS (this work). We have also observed the formation of a catalase–Fe^{II}–PhNO complex upon reaction of bovine liver catalase with PhNHOH (data not shown). The properties of those complexes—i.e., stability versus O_2, dithionite, and ferricyanide—are very similar as well as their visible spectra (Table II). Except for cytochrome P-450, which involves a proximal cysteinate ligand of the iron and whose nitrosoalkane complexes exhibit a highly red-shifted Soret peak around 455 nm (Mansuy et al., 1976), all the other studied heme protein-Fe^{II}–RNO complexes display a Soret peak around 420 nm (Table II). Some of the mentioned heme proteins (Hb and Mb) have a proximal imidazole ligand in trans position to the RNO ligand while catalase has a tyrosine proximal ligand of iron. Thus, it is not possible to conclude the nature of the PGHS proximal ligand (histidine or tyrosine) from these data.

(B) Different Abilities of Heme Proteins To Bind More or Less Bulky and Hydrophobic Nitrosoalkanes. On the contrary, the studied heme proteins exhibit very different abilities to bind RNO ligands of different size and hydrophobicity. PGHS and rat liver cytochromes P-450 are able to bind bulky hydrophobic ligands such as nitrosoamphetamine but are almost unable to form RNO complexes from the highly hydrophilic CH_3NHOH and C_2H_5NHOH. This is in agreement with a wide hydrophobic active site for these cytochromes P-450 and suggests a PGHS active site hydrophobic in nature and capable of accommodating large and bulky ligands for the iron. At the opposite extreme, Hb, Mb, and catalase are not able to form Fe^{II}–nitrosoamphetamine complexes whereas Hb and Mb easily form CH_3NO or C_2H_5NO complexes from the corresponding hydroxylamines. This is in agreement with smaller active sites unable to bind ligands more bulky than nitrosobenzene. From all the studied hydroxylamines, only PhNHOH was found able to give a catalase-Fe^{II}–RNO complex; none of them led to new complexes with horseradish

peroxidase (data not shown), which is known to be able to bind only very small iron ligands.

The aforementioned results show that PGHS, like liver cytochromes P-450 and contrary to Mb, Hb, or horseradish peroxidase, is able to bind relatively large iron ligands which could be formed in its active site by in situ oxidation. This is in agreement with a previous suggestion that the active site of PGHS is intermediate in size between those of cytochrome P-450 and horseradish peroxidase (Plë & Marnett, 1989). The wide variety of substrates that can be oxidized by PGHS and this easy accessibility of ligands to PGHS iron should lead to a rich coordination chemistry of PGHS.

As the strong binding of nitrosoalkanes to PGHS–Fe^{II} leads to a severe inhibition of PGHS activity, N-substituted hydroxylamines or compounds that can be metabolized to give such hydroxylamines could act as PGHS inhibitors in vitro or in vivo.

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