Phenylhydrazones as New Good Substrates for the Dioxygenase and Peroxidase Reactions of Prostaglandin Synthase: Formation of Iron(III)–σ-Phenyl Complexes

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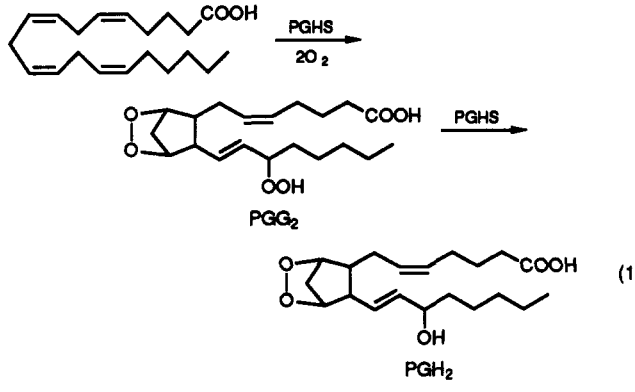
Received December 3, 1992; Revised Manuscript Received January 27, 1993

ABSTRACT: Phenylhydrazones of various aromatic and aliphatic aldehydes or ketones act as good substrates of the dioxygenase reaction of prostaglandin synthase (PGHS). Corresponding α-azo hydroperoxides are formed as intermediates with maximum initial rates of O_2 consumption between 8 and 230 mol (mol of PGHS)$^{-1}$ s^{-1} for benzophenone and hexanal phenylhydrazone, respectively. The K_m values for these reactions vary from 100 to 300 μM. These α-azo hydroperoxides are then converted to the corresponding α-azo alcohols by the peroxidase reaction of PGHS. During such oxidations of phenylhydrazones by PGHS, a new complex of this hemeprotein characterized by peaks at 438 and 556 nm is formed. This complex was obtained both by direct reaction of PGHS Fe(III) with phenyldiazene and by reaction of PGHS Fe(III) with phenylhydrazine in the presence of O_2. By analogy to results previously reported for hemoglobin, myoglobin, catalase, and cytochrome P450, this species should be a σ-phenyl PGHS Fe^{III}–Ph complex. The PGHS Fe^{III}–Ph complex should derive from an oxidation of the intermediate α-azo alcohol by PGHS Fe(III), cleavage of the resulting alkoxy radical with formation of a ketone (or aldehyde) and Ph$^\bullet$, and combination of PGHS Fe(II) with Ph$^\bullet$. Such an oxidation of α-azo alcohols by lipoxygenase–Fe^{III} with formation of Ph$^\bullet$ was reported previously. The formation of Ph$^\bullet$ and of PGHS Fe^{III}–Ph is likely the cause of the inhibitory effects previously reported for arylhydrazones toward PGHS.

Prostaglandin H synthase (PGHS)[1] is an enzyme tightly associated with the endoplasmic reticulum membrane (Rollins & Smith, 1980; De Witt et al., 1981), which catalyzes the first two steps of the biotransformation of arachidonic acid into prostaglandins and thromboxanes (Nugteren & Hazelhof, 1973; Hamberg et al., 1974; Miyamoto et al., 1976; Van der Ouderaa et al., 1977; Ohki et al., 1979). It thus expresses two activities: a cycloxygenase (dioxygenase) activity which converts arachidonic acid into prostaglandin G_2 (PGG_2), and a peroxidase activity which reduces PGG_2 into prostaglandin H_2 (PGH_2) (eq 1) (Ohki et al., 1979; Hemler & Lands, 1980; Kulmacz et al., 1985; Markey et al., 1987). It can be released upon extraction in the presence of detergents and purified from bovine (Miyamoto et al., 1976; Hemler et al., 1976) and sheep seminal vesicles (Van der Ouderaa et al., 1977; Roth et al., 1981; Jahnke et al., 1986; Kulmacz & Lands, 1987; Marnett et al., 1984) and from sheep platelets (Boopathy & Balasubramanian, 1986).

The purified protein is a homodimer of 70-kDa subunits (Miyamoto et al., 1976; Hemler et al., 1976; Van der Ouderaa et al., 1977; Roth et al., 1981) containing 3.5% (by mass) carbohydrates of the high-mannose type (Van der Ouderaa et al., 1977; Mutsaers et al., 1985). Although the enzyme has been crystallized (Jahnke et al., 1990), its tertiary structure remains unknown. However, the amino acid sequence of the polypeptide has recently been deduced from the sequence of cDNA encoding for sheep (De Witt & Smith, 1988; Merlie et al., 1988; Yokoyama et al., 1988), mouse (De Witt et al., 1990), and human (Yokoyama & Tanabe, 1989) PGHS. Heme is the prosthetic group of PGHS (Ogino et al., 1978)



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and is required for both cyclooxygenase and peroxidase activities (Hemler et al., 1976; Roth et al., 1981; Van der Ouderaa et al., 1977). The two activities are fully reconstituted with one heme per subunit (Karthein et al., 1987; Kulmacz & Lands, 1984; Roth et al., 1981; Ruf et al., 1984; Ueno et al., 1982), although the protein is able to bind higher amounts of heme which do not play a role in the catalysis (Van der Ouderaa et al., 1977). On the basis of an EPR study (Karthein et al., 1987), a tyrosinate has been proposed as the proximal axial ligand of the heme iron atom, whereas the great analogy of the UV–visible properties of PGHS with those of horseradish peroxidase suggests a histidine as axial ligand (Karthein et al., 1987; Kulmacz et al., 1987; Lambeir et al., 1985; MacDonald & Dunford, 1989; Markey et al., 1987; Marnett et al., 1979; Plé & Marnett, 1989; Van der Ouderaa et al., 1979). Recent site-directed mutagenesis studies have shown that it is the histidine 309 which binds the heme iron to the protein (Shimokawa & Smith, 1991).

A mechanism for the oxidation of arachidonic acid catalyzed by PGHS has been proposed (Karthein et al., 1987). PGHS is activated by hydroperoxides, especially lipid hydroperoxides and PGG_2 (Hemler et al., 1978; Ogino et al., 1978; Pap-

[1] Abbreviations: PGHS, prostaglandin H synthase; PGG_2, prostaglandin G_2; PGH_2, prostaglandin H_2; EPR, electron paramagnetic resonance; HPLC, high-pressure liquid chromatography; GC, gas chromatography; NMR, nuclear magnetic resonance; EDTA, ethylenediaminetetraacetate; DDC, diethyldithiocarbamate; PPIX, protoporphyrin IX.

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theofanis & Lands, 1985; Smith & Marnett, 1991; Smith et al., 1992), which undergo a heterolytic cleavage of their O–O bond (Dietz et al., 1988; Kulmacz et al., 1985, 1987) with the formation of an iron–oxo species analogous to compound I of horseradish peroxidase (Dietz et al., 1988; Karthein et al., 1988; Kulmacz et al., 1987; Lambeir et al., 1985; Nastainczyk et al., 1984). This complex is able to abstract an electron from a tyrosine residue to lead to a tyrosyl radical and an iron(IV)–oxo complex analogous to complex II of horseradish peroxidase (Dietz et al., 1988; Kulmacz, 1986; Lambeir et al., 1985; Schreiber et al., 1986). Site-directed mutagenesis experiments have recently shown that this tyrosine could be tyrosine 385 (Shimokawa et al., 1990). The tyrosine radical, or another protein-derived radical (Smith et al., 1992), would then be able to abstract a bis-allylic hydrogen atom of arachidonic acid (Dietz et al., 1988), leading to a carbon radical centered at C_{13} (Hemler et al., 1979; Kulmacz et al., 1983; Mason et al., 1980; Schreiber, et al., 1986). However, it has recently been proposed that the PGHS iron–oxo species analogous to compound I of horseradish peroxidase might be able to perform this hydrogen abstraction (Hsuanyu & Dunford, 1992). The reaction of cyclooxygenation then proceeds by addition of two dioxygen molecules to that radical (Samuelsson & Hamberg, 1967; Smith & Marnett, 1991). Eventually, the C_{15} peroxy radical formed in this reaction abstracts a hydrogen from tyrosine 385 to regenerate the tyrosyl radical (Karthein et al., 1987).

A large number of hydroperoxides are reduced by the peroxidase function of PGHS (Eling et al., 1990; Ohki et al., 1979; Smith & Marnett, 1991). This is the case not only of PGG_2 (K_m = 2 μM) and lipid hydroperoxides (2 $\leq K_m \leq$ 20 μM) (Kulmacz & Lands, 1983) but also of hydrogen peroxide and primary and secondary alkyl hydroperoxides. Furthermore, a large variety of chemicals can be used as cosubstrates for the peroxidase activity of PGHS in order to reduce the intermediate iron–oxo species into resting iron(III)–PGHS either by two one-electron-transfer reactions with phenols, aromatic amines, and β-dicarbonyl compounds (Dietz et al., 1988; Eling et al., 1990; Karthein et al., 1985; Lambeir et al., 1985; Markey et al., 1987) or by oxene transfer to sulfides (Egan et al., 1980, 1981; Plé & Marnett, 1989).

On the contrary, the only substrates described until now for the cyclooxygenase (dioxygenase) function of PGHS are unsaturated fatty acids (Hamberg et al., 1974; Smith & Marnett, 1991) such as arachidonic acid (K_m = 5 μM), 8,11,14-eicosatrienoic acid, 5,8,11,14,17-eicosapentaenoic acid, and 10,13,16-docosatrienoic acid. The research of new substrates for the cyclooxygenase function of PGHS was thus of interest and could provide an access to the characteristics of the active site of this enzyme. This article shows that phenylhydrazones constitute a new class of good substrates for the dioxygenase function of PGHS and that the hydroperoxides derived from this reaction act as substrates for the peroxidase function of PGHS. It also shows that iron(III)–σ-phenyl complexes of PGHS are formed during this oxidation of phenylhydrazones catalyzed by PGHS, a phenomenon which could be at the origin of the inhibitory effects previously described for these phenylhydrazones (Ghiglieri-Bertez et al., 1987).

MATERIALS AND METHODS

Physical Measurements. Absolute and difference UV–visible spectra were performed on Uvikon 820 and Aminco DW2 spectrophotometers. Dioxygen consumption was measured on a Gilson 316M oxygraph fitted with a Clark electrode. HPLC analyses were performed on a spherosorb ODS2 column (5 μm, 25 cm) with a Kontron Analytical HPLC system, a Beckman 165 variable-wavelength detector, and a D2000 Merck chromatointegrator. GC analyses were performed on an Intersmat IGC 120 FL with a FFAP 5% column. ^{1}H NMR spectra were recorded with a Brucker 250 spectrometer operating at 250 MHz.

Chemicals. Arachidonic acid and hemin chloride were purchased from Sigma. Acetophenone, benzophenone, benzaldehyde, and phenol were purchased from Prolabo; epinephrine, hydroquinone, chlorobenzene, diphenyl sulfide, benzyl phenyl sulfide, and thioanisole were from Janssen, hexanal was from Merck, and methyl chloroformate was from Aldrich. Phenylhydrazine from Prolabo was distilled over NaOH pellets under reduced pressure.

Hexanal phenylhydrazone was prepared by reacting 10 mmol of phenylhydrazine with 10 mmol of hexanal in 50 mL of methanol under argon, as previously described by Galey et al. (1988). The final product was characterized by UV (λ_{max} = 275 nm, ϵ = 17 000). ^{1}H NMR showed that a mixture of *Z* (30%) and *E* (70%) isomers was obtained: δ (N=CH) (ppm/TMS) in $CDCl_3$, δ_Z = 6.5 (0.3 H, t, J = 5 Hz) and δ_E = 6.8 (0.7 H, t, J = 8 Hz) (Karabatsos & Taller, 1963). Phenylhydrazones of acetone, benzaldehyde, acetophenone, and benzophenone were prepared by the same procedure, and their UV and ^{1}H NMR characteristics were found to be identical to those previously described by Yao and Resnick (1965).

The α-azo hydroperoxide, $C_5H_{11}CH(OOH)N{=}NC_6H_5$, was prepared by oxidation of hexanal phenylhydrazone by dioxygen in benzene (Bellamy & Guthrie, 1965) and characterized by UV (in EtOH: λ_{max} = 268 nm, ϵ = 10 500; λ_{max} = 385 nm, ϵ = 500) and ^{1}H NMR (in C_6D_6, δ(ppm/TMS): 0.8–1.0 (m, 3 H), 1.0–1.6 (m, 6 H), 1.7–1.9 (m, 2 H), 5.3 (t, J = 5 Hz, 1 H), 7.0–7.7 (m, 5 H), 9.1 (s, 1 H)). $C_6H_5CH(OOH)N{=}NC_6H_5$ (**3**) was prepared by the same procedure; its UV and ^{1}H NMR characteristics were found to be identical to those previously described by Yao and Resnick (1965).

The α-azo alcohol, $C_5H_{11}CH(OH)N{=}NC_6H_5$ (**9**), was synthesized as described by Bombard (1990). The corresponding α-azo hydroperoxide (8 mg) was dissolved in 18 mL of ether at 0 °C, and 2 mL of a 1 M ethanol solution of sodium borohydride was added. The formation of the α-azo alcohol **9** was followed by HPLC on a spherisorb ODS2 column (gradient from 80% CH_3OH in H_2O to 100% CH_3OH as eluent). **9** was then identified by its retention time in those conditions and by its UV–visible spectrum in EtOH (λ_{max} = 268 nm, ϵ = 10 000), which was identical to that already described (Bombard, 1990). When the reaction was complete, ether was evaporated and the final solution of **9** in EtOH was used for the reactions with PGHS without further purification.

Phenyldiazene was synthesized as previously described by Kosower et al. (1968). (Methylphenyl)diazenecarboxylate was first prepared by reacting 11.7 g of phenylhydrazine with 5 g of methyl chloroformate. Phenyldiazene was then obtained by decarboxylation of (methylphenyl)diazenecarboxylate in 0.6 N NaOH. The ^{1}H NMR characteristics of the intermediates and the final product were found to be identical to those previously described (Kosower et al., 1968).

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

Purification of PGHS from Sheep Seminal Vesicles. Sheep seminal vesicle microsomes suspended in 10 mM Tris-HCl

buffer (pH 8) containing 0.5 mM EDTA and 0.1 mM DDC were solubilized at 0 °C by addition of 1% Tween 20. The solubilized enzyme was purified after two DE53 columns as previously described (Marnett et al., 1984). The purified enzyme was stored at −80 °C. Its dioxygenase activity measured at 37 °C as described in the following paragraph was 20 μmol of O_2 consumed min^{-1} (mg of protein)$^{-1}$, in the absence of exogenous hemin. Those preparations contained 25% holoenzyme as shown by a comparison of the cyclooxygenase activity measured in the presence and in the absence of 1 μM hemin. The concentration of PGHS Fe(III) was confirmed by a UV–visible spectroscopy analysis using an ε value of 120 mM^{-1} cm^{-1} at 410 nm (Kulmacz & Lands, 1984). The dioxygenase activity was thus 91 mol of O_2 consumed (mol of PGHS Fe(III))$^{-1}$ s^{-1}.

Cyclooxygenase Activity Assay. Cyclooxygenase activity was measured at 37 °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 DE53 columns, 0.3-mL aliquots of each fraction were pipeted into the cell, and 3.25 μL of a 1 mM solution of hemin in 0.1 M NaOH (final concentration 2.5 μM) and 1.3 μL of a 1 M solution of phenol in EtOH (final concentration 1 mM) were added. The volume was completed to 1.3 mL with 0.1 M Tris-HCl buffer (pH 8.1), and the reaction was started by addition of 5.2 μL of 0.1 M arachidonic acid in EtOH (final concentration 400 μM). The activity of the enzyme in μmol of O_2 min^{-1} (mg of protein)$^{-1}$ was calculated by assuming that at 37 °C 0.26 μmol of O_2 was dissolved per milliliter of buffer (Nastainczyk et al., 1984).

O_2 Consumption in the Presence of Phenylhydrazones. Dioxygen consumption was measured at 37 °C. The oxygraph cell was filled with 1.3 mL of 125 nM PGHS Fe(III) in 0.1 M Tris-HCl buffer, pH 8.1, without exogenous hemin, and the reaction was started by the addition of various amounts of hydrazone. When dioxygen consumption was measured in the presence of a cosubstrate, 250 μM cosubstrate was added to the solution of PGHS Fe(III) before the reaction was started by the addition of hydrazone.

For all of the reactions, the ionic strength was mainly due to 0.05 M Tris-H^+ and 0.05 M Cl^- provided by the 0.1 M Tris-HCl buffer (pH 8.1) and was almost constant since all of the other reactants were used in much lower concentrations ([PGHS] < 1 μM, [hydrazones] < 250 μM, [cosubstrates] ≤ 250 μM).

Spectral Measurements. A 1 μM solution of PGHS Fe(III) (2 mL) in deaerated 0.1 M Tris-HCl buffer (pH 8.1) was equally divided between two deaerated cuvettes, and a base line of equal absorbance was recorded. Then, the substrate studied and (or) O_2 were added to the sample cuvette.

Extraction of the Heme of the PGHS Fe^{III}–Ph Complex. A 1-mL aliquot of 40 μM complex **2** in 0.1 M Tris-HCl buffer, pH 8.1, was prepared by reaction of 30 equiv of phenyldiazene with a 40 μM deaerated solution of PGHS Fe(III). After the formation of the PGHS Fe^{III}–Ph complex was complete as shown by UV–visible spectroscopy, the solution was extracted with 1 mL of ethyl methyl ketone at 0 °C (Teale, 1969), and the UV visible spectrum of the organic layer was then recorded. It was found to be identical to that of an authentic sample of PPIX Fe^{III}–Ph (Delaforge et al., 1986).

Study of the Reaction of **8** *with O_2 and PGHS*

GC Analysis. PGHS Fe(III) (250 nM) was incubated with 100 μM hexanal phenylhydrazone in 1.3 mL of 0.1 M Tris-HCl, pH 8.1. Every minute (from 1 to 10 min) after the beginning of the reaction a 1-mL aliquot was removed from the reaction mixture and extracted with 4 mL of ether. The organic products were then analyzed by GC on a 5% FFAP column (temperature of the oven was increased from 80 to 270 °C at 10 °C/min). Hexanal was identified by comparison with an authentic sample and quantified using chlorobenzene as a standard.

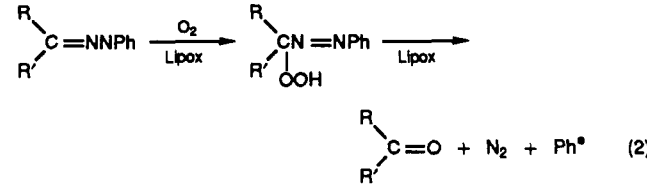
HPLC Analysis. A reaction between 250 nM PGHS and 100 μM hexanal phenylhydrazone in 1.3 mL of 0.1 M Tris-HCl (pH 8.1) was monitored by oxygen uptake. Aliquots (100 μL) were taken after different incubation times (1–10 min), treated with 100 μL of CH_3OH, and analyzed by HPLC on a spherisorb ODS2 column (5 μm, 25 cm) using a gradient from 80% CH_3OH in H_2O to 100% CH_3OH over 20 min as eluent. Hexanal phenylhydrazone and the corresponding α-azo hydroperoxide and α-azo alcohol were identified by comparison with authentic samples and quantified after integration of the surface of the corresponding peaks.

RESULTS

Reaction of Benzaldehyde Phenylhydrazone (**1**, *PhCH=NNHPh*) *with Purified PGHS Fe(III).* Reaction of benzaldehyde phenylhydrazone (**1**) with PGHS Fe(III), purified according to Marnett et al. (1984) and used without additional exogenous heme, was followed by difference visible spectroscopy. Addition of 100 μM **1** to 1 μM PGHS Fe(III) under strictly anaerobic conditions did not lead to a new spectrum, whereas the same reaction performed in the presence of dioxygen led to the gradual appearance of a difference spectrum characterized by peaks at 438 and 556 nm and a trough at 410 nm (Figure 1). This difference spectrum can be explained by the formation of a new PGHS complex **2**, exhibiting a Soret peak around 438 nm in the sample cuvette, the trough at 410 nm being due to unchanged PGHS Fe(III) in the reference cuvette.

The occurrence of a reaction between PGHS and **1** only in the presence of dioxygen suggested that **1** could act as a substrate of the dioxygenase function. Dioxygen consumption measurements completely confirmed this hypothesis, since the addition of 100 μM **1** to a 125 nM solution of PGHS Fe(III) led to fast consumption of O_2 (initial rate of 520 nmol of O_2 consumed (nmol of PGHS Fe(III))$^{-1}$ min^{-1}) (Figure 2). Dioxygen was consumed (0.4 mol per mol of **1**); the reaction ended after about 2 min, presumably because of the inactivation of PGHS (see the following paragraphs). It is noteworthy that **1** alone or **1** in the presence of 125 nM hemin (protoporphyrin XI-Fe(III)) did not lead to any significant O_2 consumption under identical conditions (Figure 2). The catalytic role of PGHS Fe(III) in the observed reaction of **1** with O_2 was also shown by the linear increase of the initial rate of O_2 consumption as a function of the increase of the starting PGHS Fe(III) concentration, at least between 100 and 250 nM (data not shown).

Possible products of dioxygenation of **1** by O_2 catalyzed by PGHS were the corresponding α-azo hydroperoxide and benzaldehyde by analogy to what was previously found for the oxidation of phenylhydrazones by O_2 catalyzed by other dioxygenases, the lipoxygenases (eq 2) (Galey et al., 1988).



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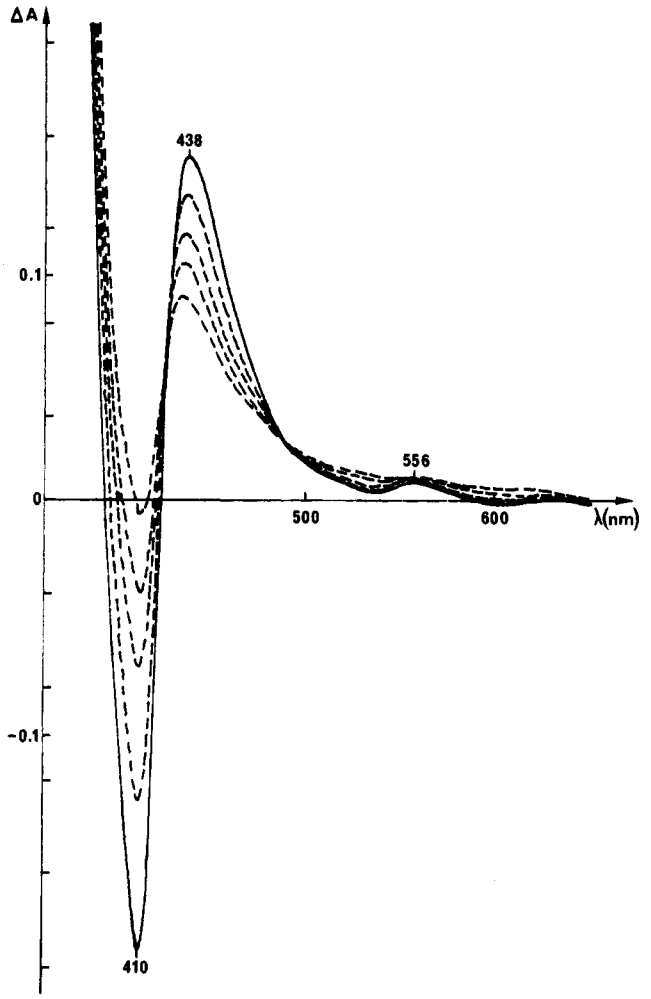


FIGURE 1: Formation of complex **2** upon reaction of benzaldehyde phenylhydrazone (**1**) with PGHS Fe(III) at 37 °C. Both sample and reference cuvette initially contained 1 mL of 1 μM PGHS Fe(III) in 0.1 M Tris-HCl buffer (pH 8.1). UV–visible difference spectra were recorded 1, 4, 7, 10, and 15 min after the addition of 100 μM **1** to the sample cuvette.

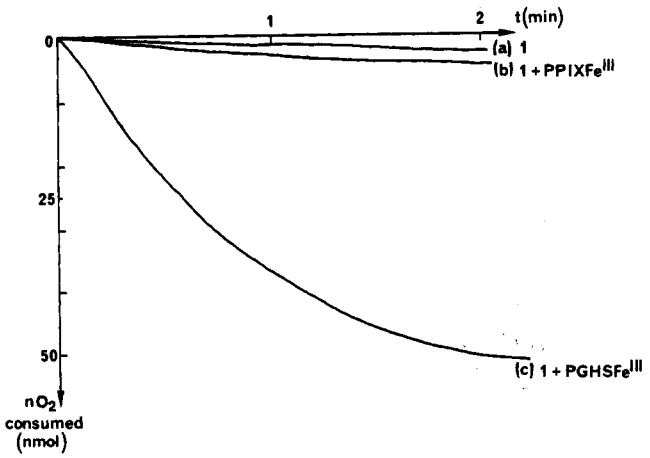


FIGURE 2: Dioxygen consumption of **1** as a function of time at 37 °C: catalysis by PGHS: (a) 100 μM benzaldehyde phenylhydrazone (**1**) in 0.1 M Tris-HCl buffer (pH 8.1); (b) 100 μM **1** in the presence of 125 nM hemin; (c) 100 μM **1** in the presence of 125 nM PGHS Fe(III).

Therefore, the reaction of **1** with PGHS was followed by UV spectroscopy between 200 and 350 nm, a region where both α-azo hydroperoxides and benzaldehyde are known to absorb. Addition of 150 μM **1** to 1 μM PGHS Fe(III) in 0.1 M Tris-HCl buffer, pH 8.1, led to the fast appearance of a species absorbing at 277 nm. Authentic samples of the α-azo hydroperoxide **3** (PhCH(OOH)N=NPh) or of the corresponding α-azo alcohol **4** (PhCH(OH)N=NPh) also exhibit UV spectra characterized by a 277-nm peak (Yao & Resnick, 1965). The peak at 277 nm then progressively disappeared to give rise to a peak at 248 nm characteristic of benzaldehyde (Figure 3). Kinetic studies showed that the absorbance at 277 nm first rapidly increased with time to reach a maximum value after approximately 2 min and then decreased slowly. The initial rate of formation of **3** was calculated assuming an extinction coefficient of 11 300 M^{-1} cm^{-1} at 277 nm (Yao & Resnick, 1965). The value obtained (550 ± 80 nmol (SEM from three determinations) of **3** formed (nmol of PGHS Fe(III))$^{-1}$ min^{-1}) was found to be in good agreement with that of the initial rate of O_2 consumption observed when 150 μM phenylhydrazone **1** was added to 1 μM PGHS Fe(III) (650 ± 30 nmol of O_2 (nmol of PGHS)$^{-1}$ min^{-1}).

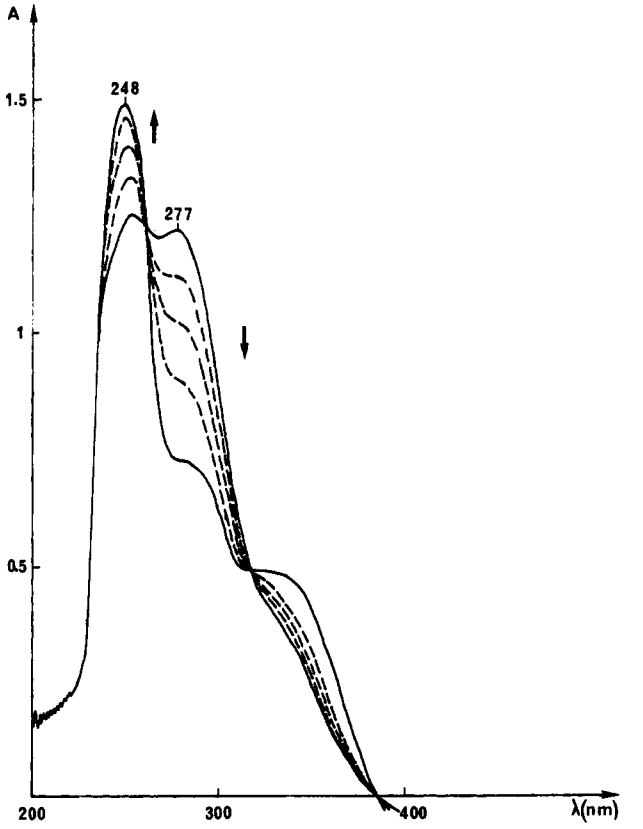
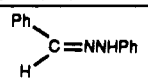
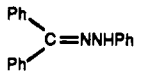
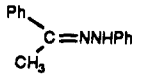
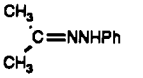
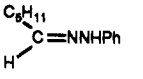


FIGURE 3: Difference spectra obtained after reaction of benzaldehyde phenylhydrazone (**1**) and PGHS Fe(III). Both cuvettes initially contained 1 μM PGHS Fe(III) in 0.1 M Tris-HCl, pH 8.1. Spectra were recorded between 200 and 400 nm, 1, 4, 7, 10, and 15 min after the addition of 150 μM **1** to the sample cuvette at 37 °C.

Reactions of Various Phenylhydrazones with Purified PGHS Fe(III). The other phenylhydrazones studied, **5**–**8** (Table I), also acted as substrates of the dioxygenase function of PGHS. O_2 consumption was measured at 37 °C for the reaction between each hydrazone and 125 nM purified PGHS Fe(III) in 0.1 M Tris-HCl buffer, pH 8.1. A double reciprocal plot of the initial rate of O_2 consumption versus the concentration of phenylhydrazone allowed us to determine the V_m and K_m values for each hydrazone (Table I). V_m values for the dioxygenation of the phenylhydrazones studied varied between 8 and 230 turnovers s^{-1}, the highest value observed with **8** being about 2 times larger than the V_{max} observed for arachidonic acid dioxygenation by PGHS under identical conditions. In a general manner, phenylhydrazones of aliphatic aldehydes or ketones led to larger V_m values than phenylhydrazones of aromatic aldehydes or ketones (68 and 230 mol of O_2 (mol of PGHS)$^{-1}$ s^{-1}, respectively, for **7** and

Table I: V_m and K_m Values Found for the Dioxygenation of Various Phenylhydrazones by PGHS[a]

phenylhydrazone	V_m (mol of O_2 (mol of PGHS)$^{-1}$ s^{-1})	K_m (μM)
 **1**	19 ± 1	100 ± 5
 **5**	8 ± 2	153 ± 10
 **6**	20 ± 1	300 ± 20
 **7**	68 ± 5	300 ± 30
 **8**	230 ± 20	205 ± 20

[a] Conditions are described in Materials and Methods; results represent means ● SEM from triplicate experiments.

8 compared to 19 and 8, respectively, for **1** and **5**). However, the hydrazones of aromatic aldehydes or ketones exhibited a slightly better affinity for PGHS than those derived from aliphatic aldehydes or ketones (K_m = 100 and 150 μM for **1** and **5** instead of 200 and 300 μM for **8** and **7**, respectively).

Effects of Reducing Cosubstrates on the Reactions of Phenylhydrazones with PGHS. Reaction of PGHS Fe(III) with the alkyl hydroperoxides formed in the dioxygenase reaction leads to a high-valent iron–oxo species, formally a Fe^{V}=O or a (porphyrin radical cation) Fe^{IV}=O species. This species is responsible for the oxidation of various reducing substrates of the peroxidase function of PGHS (Egan et al., 1980, 1981; Karthein et al., 1985; Lambeir et al., 1985; Markey et al., 1987; Dietz et al., 1988; Plé & Marnett, 1989; Eling et al., 1991). In the absence of such reducing cosubstrates, oxidation of polyunsaturated fatty acids by PGHS is accompanied by a denaturation of the enzyme, presumably due to an autodestruction of the enzyme by the high-valent iron–oxo species (Lassman et al., 1991). The relatively fast decrease of the activity of PGHS during the oxidation of **1** could be due to this kind of autodestruction. In order to avoid it, we have studied the reaction of 100 μM benzaldehyde phenylhydrazone (**1**) with 125 nM PGHS Fe(III) in 0.1 M Tris-HCl buffer, pH 8.1, in the presence of several compounds reported as reducing cosubstrates for the peroxidase function of PGHS (Markey et al., 1987). As shown in Figure 4, hydroquinone, L-epinephrine, and thioanisole were not good reducing cosubstrates for that reaction since they only led to a slight increase of the total amount of O_2 consumed before inactivation of the enzyme (from 0.4 mol of O_2 (mol of starting **1**)$^{-1}$ in the absence of cosubstrate to, respectively, 0.46, 0.47, and 0.51 mol of O_2 consumed (mol of starting **1**)$^{-1}$). On the contrary, benzyl phenyl sulfide, diphenyl sulfide, and phenol appeared as good reducing cosubstrates for the studied reaction as they respectively led to a total consumption of 0.66, 0.70, and 0.76 mol of O_2 (mol of starting **1**)$^{-1}$ and to an increase of the initial rates of O_2 consumption.

Study of the Reactions of Hexanal Phenylhydrazone (**8**) *with Purified PGHS Fe(III).* The reaction of 130 nmol of hexanal phenylhydrazone (**8**), the best substrate for the

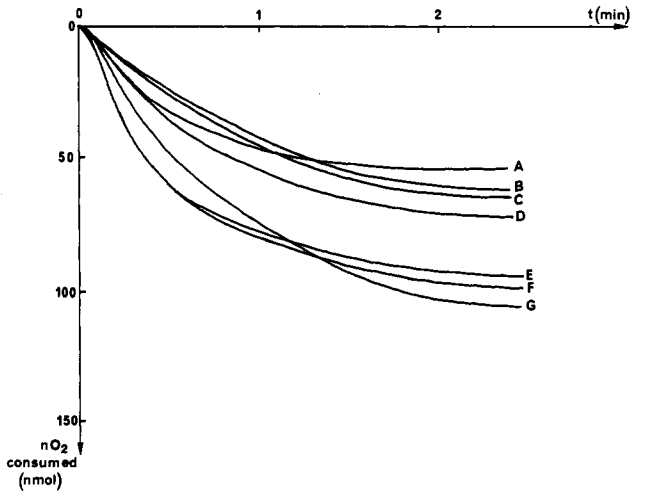


FIGURE 4: Dioxygen consumption as a function of time for the reactions of benzaldehyde phenylhydrazone (**1**) with PGHS Fe(III) in the presence of various reducing cosubstrates. Conditions as in Figure 2c, but with 250 μM cosubstrate. Curve A, **1** alone, curves B–G, **1** + cosubstrate: (B) hydroquinone; (C) L-epinephrine; (D) thioanisole; (E) benzyl phenyl sulfide; (F) diphenyl sulfide; (G) phenol.

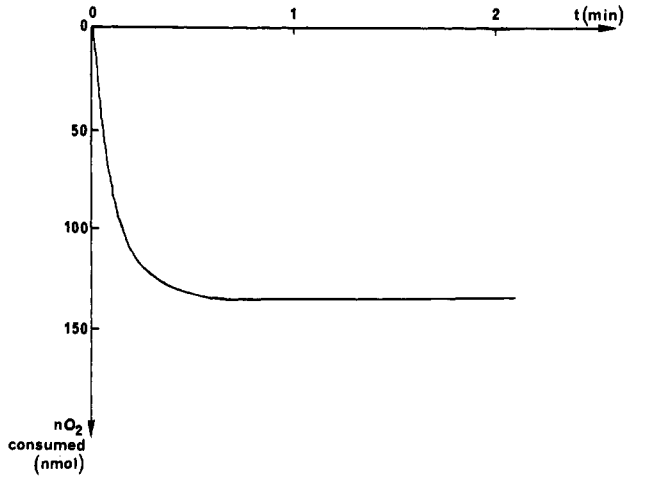


FIGURE 5: Reaction of hexanal phenylhydrazone (**8**) with PGHS, dioxygen consumption as a function of time: 100 μM **8** with 125 nM PGHS Fe(III) at 37 °C in 0.1 M Tris-HCl buffer, pH 8.1.

cyclooxygenase function of PGHS, with 125 nM PGHS Fe(III) was performed at 37 °C. A study of the reaction with an oxygraph fitted with a Clark electrode first showed a fast disappearance of O_2 ($V_i \approx$ 81 mol of O_2 (mol of PGHS)$^{-1}$ s^{-1}), leading to a final consumption of 135 nmol of O_2 approximately 30 s after the beginning of the reaction (Figure 5). Thus 1 mol of dioxygen was consumed per mole of starting phenylhydrazone **8**. The same stoichiometry was found for starting concentrations of **8** ranging between 0 and 100 μM (data not shown).

Aliquots of the reaction mixture, taken at different times after the beginning of the reaction, were analyzed by reversed-phase HPLC after precipitation of the proteins with CH_3OH (Materials and Methods). The only product detected by this method was the α-azo alcohol **9**, which exhibited an HPLC retention time and a UV spectrum identical to those of an authentic sample of **9** (Bombard, 1990). Its concentration reached a maximum value of 40 μM (when 100 μM **8** was used) 4 min after the beginning of the reaction and then decreased slowly (Figure 6). Extraction of the organic products from the reaction mixture with diethyl ether followed by gas chromatography analysis allowed the detection of hexanal. The concentration of that product rapidly reached

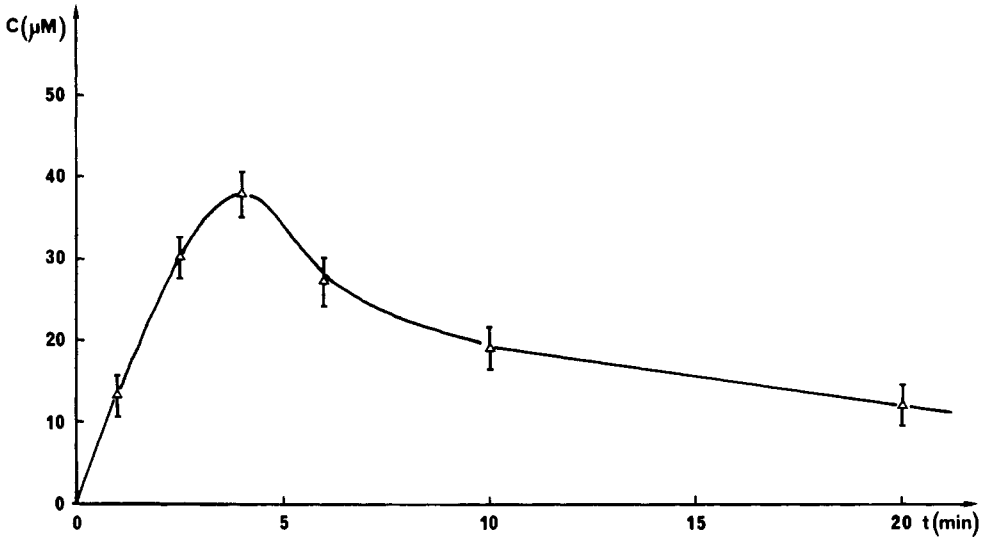


FIGURE 6: Time course of formation of **9** during reaction of **8** with PGHS: 100 μM hexanal phenylhydrazone (**8**) with 125 nM PGHS Fe(III) at 37 °C in 0.1 M Tris-HCl buffer, pH 8.1, at 37 °C.

40 μM in 2 min and then slowly increased to 85 μM. Hexanal and the azo alcohol **9**, the two products so far detected in the oxidation of **8** by O_2 and PGHS, were thus formed with a total yield of 85%. These results show that oxidation of **8** with PGHS led to the consumption of 1 mol of O_2 (mol of **8**)$^{-1}$ with the almost complete conversion of **8** into **9** and hexanal.

The Nature of Complex **2**. Complex **2**, was formed not only during the reaction of 100 μM phenylhydrazone **1** with 1 μM PGHS in the presence of O_2 (Figure 1) but also during the reaction of 150 μM α-azo hydroperoxide **3** with 1 μM PGHS Fe(III) under anaerobic conditions. However, in the latter case, the reaction was complete in less than 1 min as shown by visible spectroscopy. Parallel kinetic studies showed a concomitant fast decrease in the absorbance at 277 nm of the α-azo hydroperoxide **3** and increase in the absorbance at 248 nm (data not shown) due to the partial transformation (in less than 10 s) of the α-azo hydroperoxide **3** into benzaldehyde. Reaction of 150 μM α-azo alcohol **4** with 125 nM PGHS Fe(III) also led to the immediate formation of complex **2** under the same anaerobic conditions.

Since it had already been reported that the oxidation of various phenylhydrazones by cytochrome P450 led to σ-phenyl complexes of this cytochrome, e.g., P450 Fe^{III}–Ph (Mansuy et al., 1985), a σ-phenyl PGHS Fe^{III}–Ph structure seemed possible for complex **2**. As far as phenylhydrazine, Ph-$NHNH_2$, and phenyldiazene, PhN=NH, were known to react with hemeproteins such as hemoglobin, myoglobin (Mansuy et al., 1982; Kunze & Ortiz de Montellano, 1983; Ringe et al., 1984), catalase (Ortiz de Montellano & Keer, 1983), chloroperoxidase (Samokyszyn & Ortiz de Montellano, 1991), and cytochrome P450 (Jonen et al., 1982; Battioni et al., 1983; Raag et al., 1990) to yield σ-phenyl Hp–Fe^{III}–Ph complexes, reaction of those two products with PGHS Fe(III) was examined. The reaction of 10 μM $PhNHNH_2$ in the presence of O_2 with 1 μM PGHS Fe(III) led to a difference spectrum with a trough at 410 mn and maxima at 438 and 556 mn, identical to that obtained upon reaction of phenylhydrazone **1** with 1 μM PGHS Fe(III) and O_2 (Figure 1). The same spectrum was obtained upon reaction of excess PhN=NH with PGHS Fe(III) under anaerobic conditions (Figure 7).

Extraction of the heme of complex **2** by butanone at 0 °C, which is known to cleave the heme–protein bond in hemeproteins such as hemoglobin (Teale, 1969), yielded a solution absorbing at 399 and 560 nm. This spectrum was characteristic of the σ-phenyl protoporphyrin–Fe^{III}–Ph complex

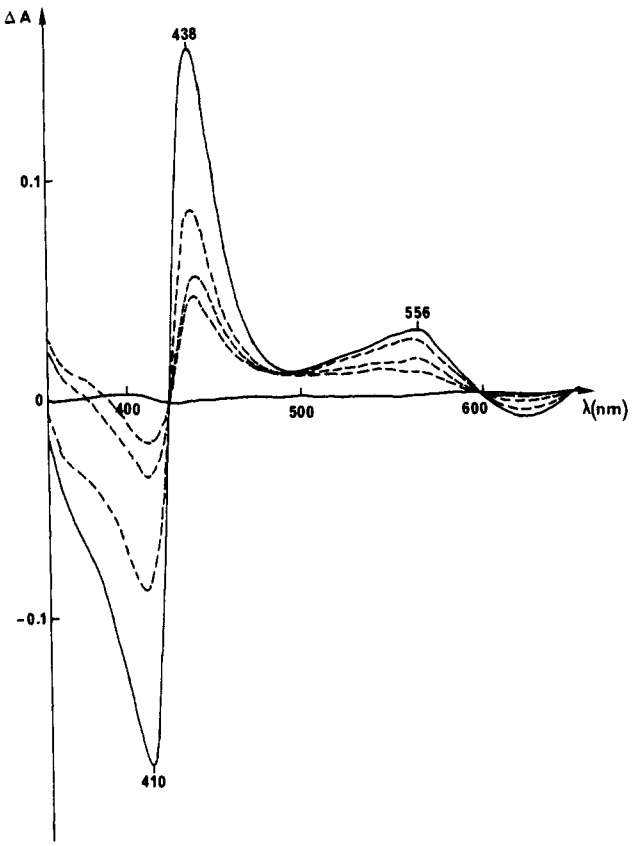


FIGURE 7: Reaction of phenyldiazene with PGHS Fe(III). Both sample and reference cuvette contained 1 μM PGHS Fe(III) in 0.1 M Tris-HCl buffer, pH 8.1. Difference spectra were recorded after the addition of 1, 2, 4, and 6 molar equiv of phenyldiazene.

(Delaforge et al., 1986). A σ-phenyl PGHS Fe^{III}–Ph structure was thus reasonable for complex **2**.

DISCUSSION

The aforementioned results show that phenylhydrazones constitute a new class of substrates for the dioxygenase activity of PGHS. This is consistent with the very fast consumptions of O_2 by these compounds in the presence of PGHS and with the intermediate formation of the α-azo hydroperoxide **3** in the case of benzaldehyde phenylhydrazone (**1**). Such a dioxygenation of phenylhydrazones with formation of the

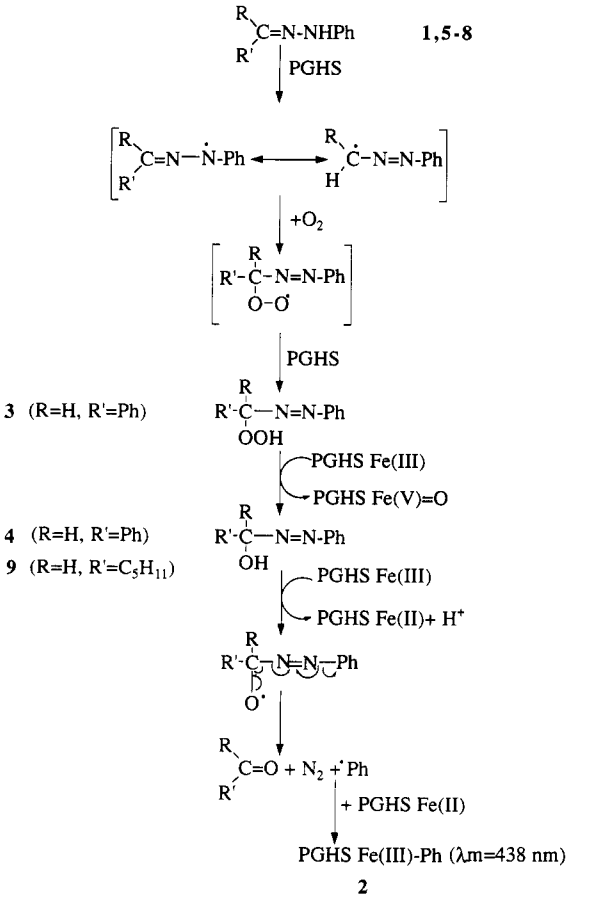


FIGURE 8: Mechanism proposed for the reactions of phenylhydrazones with PGHS.

corresponding α-azo hydroperoxides was found to be catalyzed by lipoxygenases (Galey et al., 1988). Its mechanism should be similar to that proposed for the dioxygenation of unsaturated fatty acids, except that the hydrogen abstracted by the PGHS active species is at a benzylic-allylic NH site instead of a bis-allylic CH_2 site. Binding of O_2 should occur at the carbon atom of the [N=NĊ<] radical (Figure 8) and lead to an α-azo hydroperoxide after reduction of the intermediate alkylperoxy radical. This PGHS-dependent dioxygenation of phenylhydrazones is in agreement with (i) the stoichiometry of the reaction of hexanal phenylhydrazone **8** with O_2 and PGHS (1 mol of O_2 consumed per mole of **8**) and (ii) the initial rate of O_2 consumption in the reaction of **1** with PGHS, which is equal to the initial rate of formation of the α-azo hydroperoxide **3**.

As the peroxidase function of PGHS applies to a large number of alkyl hydroperoxides (Eling et al., 1990), α-azo hydroperoxides like **3** should be easily reduced to the corresponding α-azo alcohols like **4** by PGHS Fe(III) with the formation of a PGHS high-valent iron–oxo complex (Dietz et al., 1988; Karthein et al., 1988; Kulmacz et al., 1987; Lambeir et al., 1985; Nastainczyk et al., 1984). This complex may either irreversibly oxidize the protein or react with reducing cosubstrates like phenol or aromatic sulfides that were added to the reaction medium. This transformation of phenylhydrazones into α-azo alcohols in two steps (dioxygenase and peroxidase reactions) (Figure 8) is similar to the transformation of arachidonic acid into PGH_2, except that 1 mol of O_2 instead of 2 is inserted into the substrate. It is remarkable that hydrazone **8** is oxidized by PGHS with a V_m 2 times higher than that of arachidonic acid. However, the K_m value found for **8** (200 μM) is markedly higher than that reported for arachidonic acid (5 μM) (Hamberg et al., 1974).

Contrary to PGH_2, which is not reactive toward PGHS, α-azo alcohols like **4** are strong reducing agents and are able to react with PGHS Fe(III). It was previously reported that they were oxidized by lipoxygenase Fe(III) with formation of the corresponding alkoxy radical which underwent a β C–C bond cleavage, leading to the corresponding aldehyde (or ketone), N_2, and Ph$^\bullet$ (Galey et al., 1988) (eq 2). A similar reaction between **4** and PGHS Fe(III) should lead to benzaldehyde, N_2, Ph$^\bullet$, and PGHS Fe(II) (Figure 8). Combination of Ph$^\bullet$ with PGHS Fe(II) then should give the σ-phenyl PGHS Fe^{III}–Ph complex, which is characterized by a 438-nm Soret peak (Figure 1). This mechanism is in complete agreement with the formation of benzaldehyde and hexanal, which were observed, respectively, upon reaction of PGHS with phenylhydrazones **1** and **8**. Such a formation of σ-phenyl–Fe(III) hemeprotein complexes, characterized by a red-shifted Soret peak, upon reaction of hemeprotein Fe(II) with Ph$^\bullet$ produced by oxidation of $PhNHNH_2$, PhN=NH, or phenylhydrazones was reported in the case of myoglobin, hemoglobin (Mansuy et al., 1982; Kunze & Ortiz de Montellano, 1983; Ringe et al., 1984), cytochrome P450 (Jonen et al., 1982; Battioni et al., 1983; Raag et al., 1990), catalase (Ortiz de Montellano & Kerr, 1983), and chloroperoxidase (Samoskyszyn & Ortiz de Montellano, 1991).

The net result of the oxidation of phenylhydrazones by O_2 catalyzed by PGHS is shown on Figure 8. It involves the consumption of 1 mol of phenylhydrazone and 1 mol of O_2 for the final formation of 1 mol of ketone (or aldehyde) and 1 mol of Ph$^\bullet$, with the intermediate formation of 1 mol of α-azo hydroperoxide. The phenyl radical may either combine with PGHS Fe(II) leading to PGHS Fe^{III}–Ph, an inactive form of PGHS, or bind covalently to an amino acid residue of the protein. These two fates of the phenyl radical could be at the origin of the inhibition of PGHS by arylhydrazones previously reported (Ghiglieri-Bertez et al., 1987). A third reaction should be responsible for PGHS inactivation during oxidation of phenylhydrazones or arachidonic acid: the irreversible oxidation of the protein by the high-valent iron–oxo PGHS complex involved as an intermediate (Lassmann et al., 1991).

The different results observed for the hydrazones as a function of the ketone (or aldehyde) substituents appear to be related to the different rates of the first step of their PGHS-dependent oxidation. Those like **8**, which are very rapidly dioxygenated (V_m = 230 mol of O_2 (mol of PGHS)$^{-1}$ s^{-1}), undergo a complete transformation into products before PGHS inactivation, explaining the complete consumption of **8** with that of O_2 (1 mol per mole) and the good yields of products like **9** and hexanal (final yield of hexanal of 85%). On the contrary, those like **1**, which are less rapidly dioxygenated, are not completely consumed before PGHS inactivation (40% consumed before PGHS total inactivation). In the presence of reducing agents like phenol or thioethers, which reduce the active PGHS–iron–oxo species and limit PGHS autoinactivation, a greater proportion of **1** (70%) was dioxygenated. The greater V_m values observed for aliphatic hydrazones (Table I) than for aromatic hydrazones could be due to the greater reactivity of the corresponding RR'C$^\bullet$N=NPh radical toward O_2 when R and R' are alkyl groups than when R (or R') is an aryl group (benzylic radical in this case).

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