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Formation of Iron(II)–Nitrosoalkane Complexes: A New Activity of Microperoxidase 8

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Microperoxidase 8 (MP8) is a heme octapeptide, obtained by enzymatic hydrolysis of heart cytochrome c, in which a histidine is axially coordinated to the heme iron, and acts as its fifth ligand. It exhibits two kinds of activities: a peroxidase-like activity and a cytochrome P450-like activity. We here show that MP8 is not only able to oxidize various aliphatic and aromatic hydroxylamines with the formation of MP8-Fe(II)-nitrosoalkane or -arene complexes absorbing around 414 nm, but also that these complexes can be obtained by reduction of nitroalkanes. This is the first example of fully characterized iron(II)-metabolite complexes of MP8. Such complexes constitute good models for those obtained upon oxidation of amphetamine or macrolids by cytochromes P450. In addition, this is a new catalytic activity of MP8, which validates the use of this mini-enzyme as a convenient model for hemoproteins of interest in toxicology and pharmacology such as cytochromes P450 and peroxidases.

Key Words: **microperoxidase; hemoprotein; mini-enzyme; N-substituted hydroxylamine; nitroalkane; nitrosoalkane complexes.**

Microperoxidase 8 (MP8) is obtained by controlled proteolytic digestion of horse heart cytochrome c (1). It consists of an iron(III)-protoporphyrin IX covalently bound to an octapeptide which contains the Cys-$(Xaa)_2$-Cys-His sequence. The protoporphyrin IX is covalently bound through thioether links to the cysteine side chains and the histidine is axially coordinated to the heme iron, and acts as its fifth ligand. MP8 has an open active site, which leads to broad substrate specificity in two types of catalytic reactions. First of all, MP8 shows a peroxidase-like activity and is able to perform the oxidation of several typical peroxidase cosubstrates like *o*-dianisine (2) and ABTS (3). Second, MP8 also shows a cytochrome P450-type like activity and catalyzes the *para*-hydroxylation of aniline (4), the monooxygenation of polycyclic aromatic compounds (5), the S-oxidation of sulfides (6), and the N-demethylation of *N*-methylaniline (7).

Several hemoproteins including hemoglobin, myoglobin (8), cytochrome P450 (9), and PGH synthase (10), have been reported to form Fe(II)-nitrosoalkane (RNO) complexes. Such complexes are not only obtained *in vitro*, either by oxidation of hydroxylamines or by reduction of nitroalkanes in the presence of a reducing agent but also *in vivo* during the oxidative metabolism of several drugs or exogenous compounds containing an amine or an hydroxylamine function (11), or during the reduction of the corresponding nitroalkanes (12). The RNO ligand binds tightly to the iron (II) of the hepatic detoxifying cytochromes P450, causing a severe inhibition of the catalytic functions of these cytochromes (9).

$$(P)Fe^{III} \xrightarrow{RNHOH} (P)Fe^{II} \leftarrow N(=O)R \xleftarrow[\text{Reductant}]{RNO_2} (P)Fe^{III}$$

P = hemoglobin, myoglobin,

cytochrome P450, PGH synthase.

This paper shows that MP8 is not only able to oxidize various aliphatic and aromatic hydroxylamines with the formation of Fe(II)-nitrosoalkane or -nitrosoarene complexes, but also that these complexes can be obtained by reduction of nitroalkanes in the presence of a reducing agent. This constitutes a new activity for MP8 and further validates the use of this heme-octapeptide as a model for hemoproteins such as cytochromes P450 and peroxidases.

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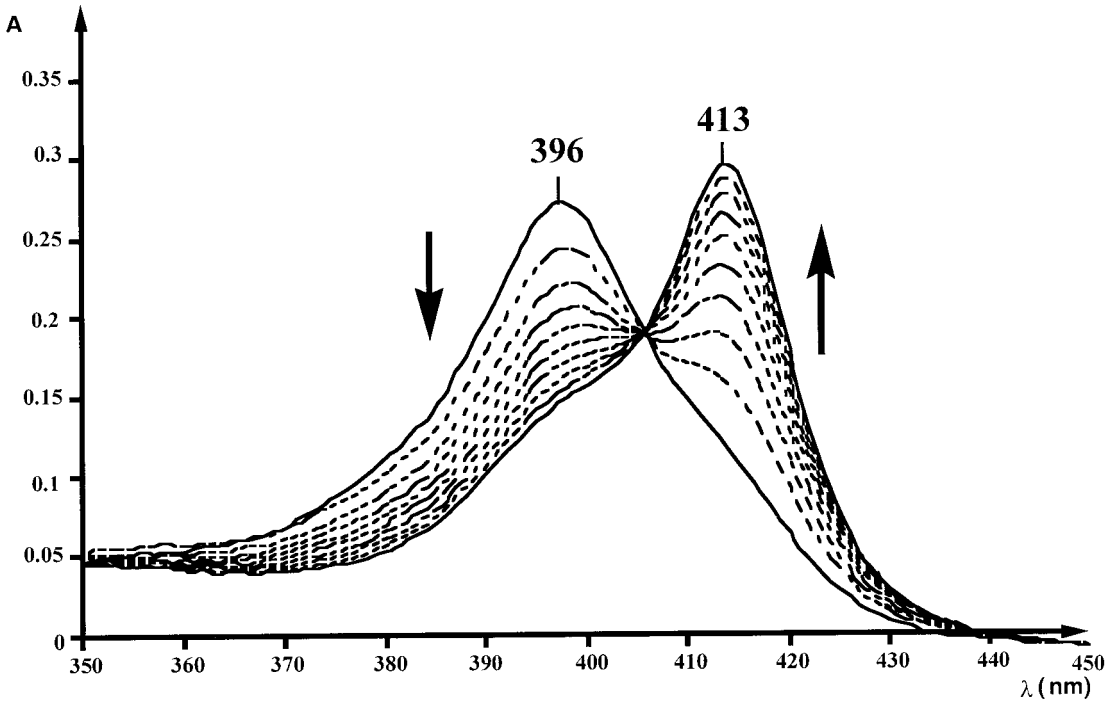


FIG. 1. Evolution of the absolute spectrum of MP8 after the addition of *N*-isopropylhydroxylamine. Spectra were recorded every 2 min after the addition of 400 μM iPrNHOH.

MATERIALS AND METHODS

Purification of MP8. Microperoxidase 8 (MP8) was prepared by sequential peptic and tryptic digestion of horse-heart cytochrome c (sigma) as previously described (1). The heme content was determined by the pyridine chromogen method (1). The purity of the sample was over 97% based on MALDI-TOF mass spectroscopy analysis.

Physical measurements. Absolute and differential UV-visible spectra were recorded at room temperature (20°C) on a Kontron 942 spectrophotometer. For all reactions, 2 μM MP8 Fe (III) in 0.1 M PBS pH 7.4 was used. When the reactions were followed by difference spectroscopy, both sample and reference cuvette were filled with 1 ml of this solution and a baseline of equal absorbance was recorded. The reactions were then performed in the sample cuvette and difference spectra were recorded.

Chemicals. Nitromethane, nitroethane, nitropropane, 2-nitropropane, nitrobenzene were purchased from Jansen. Nitrohexane, nitrocylohexane and 2-methyl-2-nitropropane were purchased from Aldrich. Sodium dithionite was purchased from Merck and potassium ferricyanide was purchased from Prolabo.

Hydroxylamines. *N*-methylhydroxylamine **1** was purchased from Sigma. *N*-propyl- and *N*-isopropylhydroxylamine **2** and **3** were prepared by reduction of the corresponding 1- and 2-nitropropane by Zn in the presence of ammonium chloride according to an already described procedure (13). The characteristics of the two products were found identical to those reported in the literature (14). *N*-(1-phenylpropyl) hydroxylamine (*N*-hydroxyamphetamine) **4** and *N*-(1-*p*-chlorophenylpropyl) hydroxylamine **5** were prepared in two steps. First the condensation of benzaldehyde and *p*-chlorobenzaldehyde with nitroethane under acidic conditions afforded the corresponding 1-phenyl- and 1-*p*-chlorophenyl-2-nitropropene (15) which were then reduced into N-substituted hydroxylamines **4** and **5** by lithium-aluminium hydride as described previously (16).

2-Nitrosopropane **6** was prepared by oxidation of *N*-isopropylhydroxylamine **3** by silver carbonate precipitated on celite and its characteristics were found identical to those already reported (17).

Reactions of MP8 with N-monosubstituted hydroxylamines. Forty microliters of a 10^{-2} M solution of N-monosubstituted hydroxylamine in CH_3OH were added in a cuvette containing 1 ml of 2 μM MP8-Fe(III) in 0.1 M PBS pH 7.4. The evolution of the UV-visible spectrum of the solution was then monitored as a function of time between 350 and 550 nm. Kinetic data were then obtained by measuring the absorbance at the maximum of absorption of the MP8-Fe(II)-RNO complex as a function of time.

Reactions of MP8 with aliphatic and aromatic nitroakanes. The reference cuvette of the spectrophotometer was filled with 1 ml of a 2 μM solution of MP8 in 0.1 M PBS, pH 7.4 and a large excess of sodium dithionite (1 mM) was added. Increasing volumes of a 0.1 M solution of nitro compound in 0.1 M PBS, pH 7.4 (or in $(CH_3)_2SO$ in the case of 1-nitrohexane and nitrocylohexane, maximum amount 1% v/v) were then added and the absolute absorption spectra were recorded between 350 at 650 nm at a rate of 200 nm/min.

Difference spectra were recorded to determine the ϵ value corresponding to the maximum of absorption of the MP8-Fe(II)-RNO complexes as follows. Both reference and sample cuvettes were filled with 1 ml of a 2 μM solution of MP8 in 0.1 M PBS, pH 7.4 and a large excess of sodium dithionite (1 mM) was added in both cuvettes. Increasing volumes of a 0.1 M solution of nitro compound in 0.1 M PBS, pH 7.4 or in $(CH_3)_2SO$ were then added to the sample cuvette and difference spectra were recorded between 350 at 650 nm. The ϵ value at 413 nm for the MP8-Fe(II)-RNO complexes was then calculated using an ϵ value of 157 mM^{-1} cm^{-1} at 396 nm for MP8-Fe(III) as follows: $\epsilon_{413} = \epsilon_{396} \times \Delta A_{413\text{-}550}/\Delta A_{550\text{-}396}$.

RESULTS

*Reaction of N-Isopropylhydroxylamine (iPrNHOH) **3** with MP8*

The addition, under aerobic conditions, of 400 μM *N*-isopropylhydroxylamine **3** to a 2 μM solution of MP8

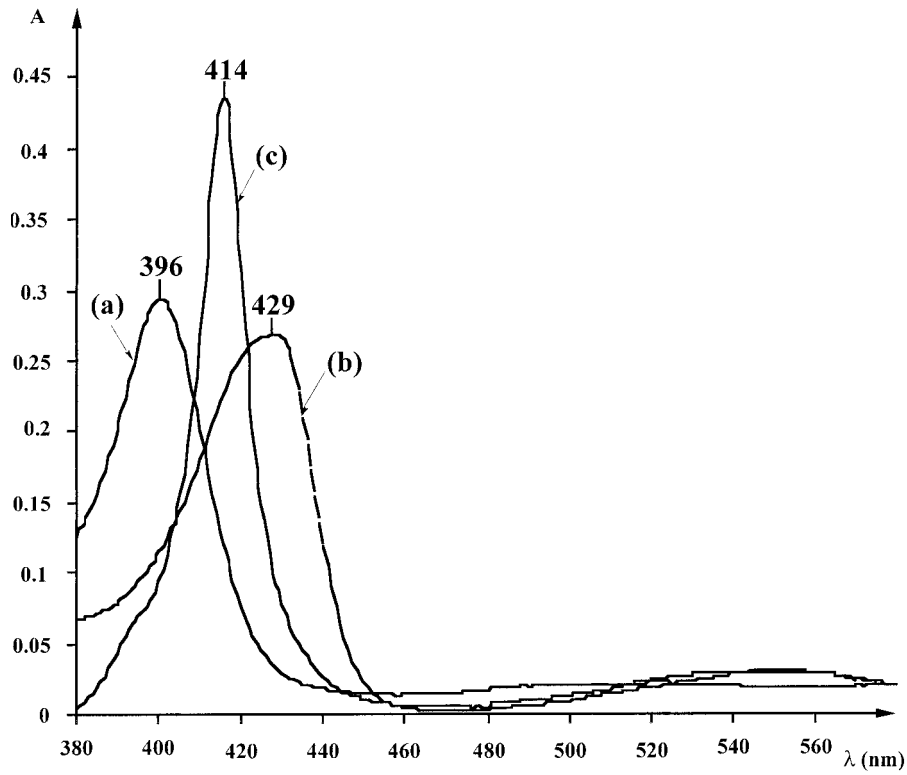


FIG. 2. Reaction of MP8 with 2-nitropropane in presence of dithionite. (a) Spectrum of 2 μM MP8-Fe(III) in 0.1 M PBS pH 7.4 containing 2% of CTABr. (b) New spectrum obtained after addition of 1 mM of dithionite. (c) Spectrum obtained 5 min after addition of 5 mM RNO_2.

in 0.1 M PBS, pH 7.4 led to the gradual replacement, with an isobestic point at 405 nm, of the soret band characteristic of iron(III)MP8 at 396 nm by a new spectrum with a soret band at 413 nm (Fig. 1). This new spectrum can be explained by the formation of a new complex of MP8 **7** absorbing at 413 nm.

Nature of Complex ***7***

The similarity between the reaction of *N*-isopropylhydroxylamine **3** with iron(III)MP8 with those of N-substituted hydroxylamines with hemoproteins such as hemoglobin, myoglobin (8), cytochrome P450 (9), and PGH synthase (10) or with iron(III)meso-tetraphenylporphyrin (18) which lead to (P)Fe(II)N(O)R complexes, strongly suggested a MP8Fe(II)N(O)iPr structure for complex **7.** This hypothesis was further supported by the following properties of complex **7** which were identical to those already reported for Fe(II)-nitrosoalkane complexes of hemoglobin, myoglobin (8), cytochrome P450 (9), and PGH synthase (10). First, its formation only occurred in the presence of O_2, no spectral change being observed upon addition of 400 μM *N*-isopropyl-hydroxylamine **3** to MP8 under strictly anaerobic conditions. Second, complex **7** was stable for hours in 0.1 M PBS, pH 7.4 either alone or in the presence of 1 mM sodium dithionite as a reductant but was rapidly destroyed in the presence of an oxidant like $Fe(CN)_6K_3$ (0.2 mM).

Three other results confirmed the MP8Fe(II)N(O)iPr structure of complex **7.** First, complex **7** could also be obtained by reduction of nitro-2-propane by MP8 in the presence of sodium dithionite. Indeed, when 1 mM sodium dithionite was added to a 2 μM solution of iron(III)MP8 in 0.1 M PBS pH 7.4 containing 2% CATBr, the absorption spectrum of iron(III)MP8 was immediately replaced by a new one with a maximum of absorption at 429 nm characteristic of iron(II)MP8 (Fig. 2). Further addition of 5 mM 2-nitropropane led after 5 min to a spectrum with maxima of absorption at 416 and 533 nm very similar to that of complex **7** (Fig. 2). Second, complex **7** could also be obtained directly by addition of an excess of 2-nitrosopropane to a 2 μM solution of iron(II)MP8 in 0.1 M PBS pH 7.4 containing 2% CATBr, previously prepared by reduction of iron(III)MP8 by 1 mM sodium dithionite (Fig. 3). Third, when complex **7** was analyzed by ^{1}H NMR spectroscopy in CD_3OD, a spectrum characteristic of a diamagnetic species was obtained (data not shown) and two signals corresponding to the protons of the iPrN = O ligand of the iron atom were observed at −0.80 ppm

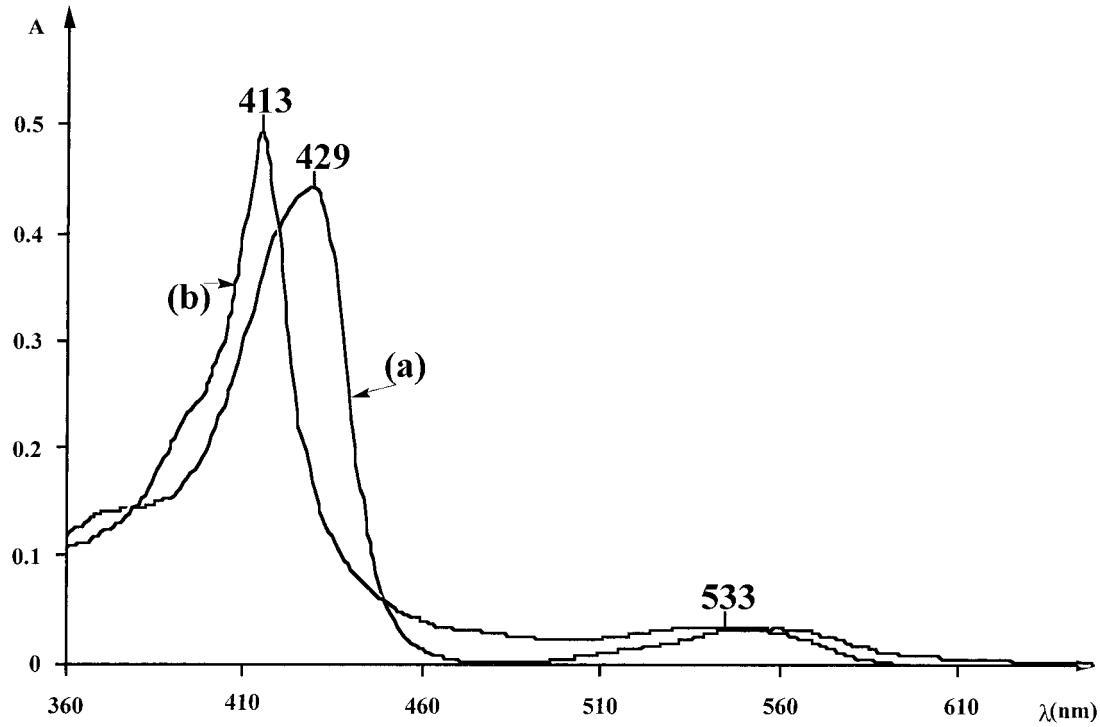


FIG. 3. Reaction of iron(II)MP8 with 2-nitrosopropane. (a) Spectrum of 2 μM MP8-Fe(II) in 0.1 M PBS pH 7.4 containing 2% of CTABr. (b) New spectrum obtained after addition of excess iPrNO.

(m, 1H, C-H) and −2.05 ppm (d, 6H, -CH_3). Those signals were identical to those already reported for the protons of the iPrN = O ligand of the iron atom in diamagnetic iron(II) (iPrNO) (tetraarylporphyrin) complexes (18).

Reactions of MP8 with Various N-Substituted Hydroxylamines RNHOH

N-monosubstituted hydroxylamines other than **3,** like *N*-methyl- (**1**), *N*-propyl- (**2**), *N*-(1-phenylpropyl)- (**4**) (*N*-hydroxyamphetamine) and *N*-(1-*p*-chlorophenylpropyl)- (**5**) hydroxylamine were found able to react with MP8 to form complexes exhibiting properties similar to those of complex **7** (Table 1). All were rapidly destroyed in the presence of 50 μM $Fe(CN)_6K_3$ and exhibited visible spectra with maxima of absorption around 415 and 530 nm. However, the ϵ value corresponding to the soret band, calculated from difference spectra as explained in the experimental section, was greatly dependent on the nature of the R substituent of the RNO ligand. With alkyl substituents an ϵ value of about 60 to 80 $mM^{-1}cm^{-1}$ was found whereas with substituents bearing an aryl group a higher ϵ value of about 100 $mM^{-1}cm^{-1}$ was found.

The kinetics of the reaction was also dependent on the nature of R (Fig. 4). The highest initial rate was observed with the hydroxylamine **3** bearing a branched alkyl group (R = CH_3-CH-CH_3), followed by hydroxylamines substituted with a linear alkyl group **1** (R = CH_3) and **2** (R = n-C_3H_7). Substitution of one of the hydrogen atoms of the isopropyl group of **3** by an aryl group, like in **4** (R = Ph-CH_2-CH-CH_3) and **5** (R = pCl-Ph-CH_2-CH-CH_3), led to a lower value of the initial rate of the reaction.

On the contrary, as can be seen from Table 1, the proportion of MP8 converted into MP8-Fe(II)-RNO complex at the equilibrium, using a concentration of 200 μM RNHOH, was not greatly dependent on the

TABLE 1

UV-Visible Characteristics and Reactivity of MP8-Fe(II)-RNO Complexes Formed by Reaction of Microperoxidase 8 with N-Substituted Hydroxylamine

MP8-Fe(II)-RNO R =	UV-visible λ_{max} (nm), ϵ (mM^{-1} cm^{-1})	Complex level[a] (%)	Stability to $Fe(CN)_6K_3$[b]
Ph—CH_2—CH—CH_3	415 (105), 532	63	—
Cl—Ph—CH_2—CH—CH_3	414 (96), 532	73	—
CH_3—	413, 531		—
CH_3—CH—CH_3	413 (60), 530	67	—
CH_3—CH_2—CH_2—	413 (77), 530	57	—

[a] Calculated from the absorbance at 413 nm after reaction of 200 equiv of RNHOH with 2 μM MP8 Fe(III) in 0.1 M PBS Buffer pH 7.4.

[b] Fast destruction of the MP8-RNO complex in presence of 50 μM $Fe(CN)_6K_3$ with regeneration of MP8-Fe(III).

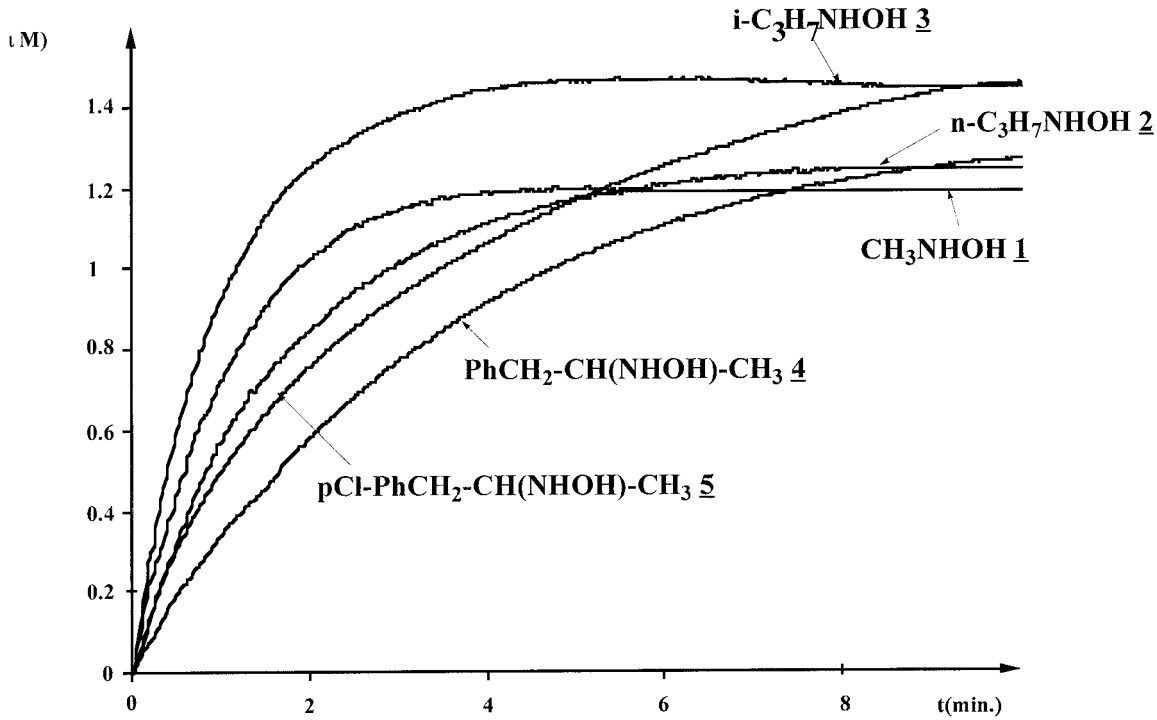


FIG. 4. Time dependence of the formation of MP8-iron(II)-RNO complexes for the reactions of 2 μM MP8 with 400 μM RNHOH in 100 mM PBS, pH 7.4: concentration of MP8-iron(II)-RNO complex (C) as a function of time. The initial rate of the reaction is obtained from the slope at the origin of this curve.

nature of R and values ranging between 57 and 73% could be calculated from the e value at 413–415 nm.

Reactions of MP8 with Various Nitroalkanes RNO_2

In addition to complex **7,** nitrosoalkane complexes of iron(II)MP8 could be prepared by reaction, in the presence of 1 mM sodium dithionite, of 2 μM MP8 in 100 mM PBS, pH 7.4, with a range of nitroalkanes (5 mM) such as nitromethane, nitroethane, 1-nitropropane, nitrobenzene, nitrohexane, and nitrocyclohexane. Their visible spectra were similar to that of complex **7** (Table 2) as well as their reactivity toward $Fe(CN)_6K_3$.

TABLE 2

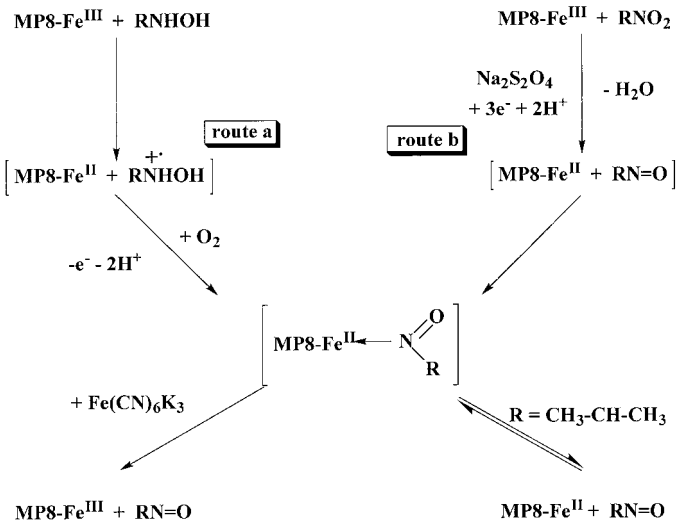
Formation of MP8-Fe(II)-RNO Complexes after Reaction of MP8-Fe(III) with Nitroalkanes RNO_2 and Dithionite

RNO_2	UV-visible λ_{max} (nm)
nitromethane	416, 533
nitroethane	415, 532
1-nitropropane	416, 533
2-nitropropane	416, 533
nitrobenzene	416, 533
nitrohexane	416, 534
nitrocyclohexane	416, 534

DISCUSSION

Structure of MP8 Complexes Formed upon Oxidation of N-Monosubstituted Hydroxylamines or Reduction of Nitroalkanes

The aforementioned results show the formation of new complexes of MP8 absorbing around 414 nm, upon reaction of N-monosubstituted hydroxylamines with iron(III)-MP8 under aerobic conditions (Fig. 1). A MP8-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 complexes of hemoproteins such as hemoglobin, myoglobin (8), cytochrome P450 (9), and PGH synthase (10) or with iron(III)meso-tetraphenylporphyrin (18): (i) a great stability in the presence of an excess of sodium dithionite, (ii) an immediate regeneration of ferric MP8 upon treatment by ferricyanide, and (iii) the formation of the Fe(II)-nitrosoalkane complexes either by in situ oxidation of *N*-alkylhydroxylamines or by reduction of the corresponding nitroalkanes in the presence of dithionite (Fig. 2). This MP8-Fe(II)-N(O)R structure was completely confirmed for the iPrNHOH-derived complex by the following: (i) the direct formation of complex **7** by coordination of 2-nitrosopropane on the iron(II) of MP8 previously reduced by sodium dithionite (Fig. 3), (ii) the ^{1}H NMR spectrum of complex **7** which is characteristic of an



SCHEME 1

iron(II) diamagnetic species with signals due to the protons of the nitroso-2-propane ligand almost identical to those already reported for the diamagnetic iron(II) (iPrNO) (tetraarylporphyrin) complexes (18).

Mechanism of Formation of the Various MP8-Fe(II)-RNO Complexes

Since the reactions leading to the formation of MP8-Fe(II)-N(O)R complexes are very similar to those already described for the formation of other hemoprotein- and tetraarylporphyrin-Fe(II)-N(O)R complexes (8–10, 18), their mechanisms should also be very similar. According to what was shown for the reactions of *N*-isopropylhydroxylamine **3** with iron(III)-porphyrins (18), the first step for the reaction between a *N*-alkylhydroxylamine RNHOH and iron(III)-MP8 should be a one electron oxidation of the hydroxylamine leading to the radical cation $[RNHOH]^{+\cdot}$ and to the reduced iron(II)-MP8 (Scheme 1, route a). This is consistent with our findings that the more reactive N-substituted hydroxylamines were those bearing the more electron donating substituent and consequently those which were the best reductants (Fig. 4). A second one electron oxidation could then be achieved by O_2 to give, after loss of two protons, the nitrosoalkane RNO which should bind to MP8-Fe(II). Accordingly, the formation of MP8-Fe(II)-N(O)R complexes, starting from MP8-Fe(III) and RNHOH only occurred under aerobic conditions. Once formed those complexes are remarkably stable under aerobic conditions, which is due to the great strength of the porphyrin-Fe(II)-nitrosoalkane bond (18). On the contrary the porphyrin-Fe(III)-nitrosoalkane bond is very weak (18), this explains why upon oxidation of MP8-Fe(II)-N(O)R complexes by $Fe(CN)_6K_3$ the RNO ligand is released and native MP8-Fe(III) recovered (Scheme 1).

The formation of MP8-Fe(II)-N(O)R complexes from nitroalkanes or -arenes and dithionite should be due to the concomitant reduction of MP8-Fe(III) into MP8-Fe(II) and RNO_2 into RNO by sodium dithionite (or MP8-Fe(II)) followed by the strong binding between RNO and MP8-Fe(II) (Scheme 1, route b). The great strength of the MP8-Fe(II)-nitrosoalkane bond explains the stability of the obtained complexes in the presence of excess dithionite.

Comparison between the Different N-monosubstituted Hydroxylamines and Comparison of the Ability of MP8 and Various Hemoproteins to Bind More or Less Bulky and Hydrophobic Nitrosoalkanes

As we already mentioned above, the reactivity of the various N-monosubstituted hydroxylamines RNHOH was dependent on the nature of the R substituent. The most reactive N-monosubstituted hydroxylamine was hydroxylamine **3** bearing a branched alkyl group (R = CH_3-CH-CH_3) followed by those substituted with a linear alkyl group **1** (R = CH_3) and **2** (R = n-C_3H_7) (Fig. 4). This correlated with the higher donating effect of the isopropyl substituent in **3** than that of the linear alkyl group in **1** and **2** and could be explained by the fact that the first step of the reaction of N-monosubstituted hydroxylamines with MP8 was the reduction of MP8Fe(III) into MP Fe(II), **3** being a better reductant than **1** and **2.** Accordingly, the substitution of one of the hydrogen atoms of the isopropyl group in **3** by an electron withdrawing aryl group, like in **4** (R = Ph-CH_2-CH-CH_3) and **5** (R = pCl-Ph-CH_2-CH-CH_3), led to a decrease of the initial rate of the reaction.

By contrast, the proportion of MP8-Fe(II)-N(O)R complex formed once the equilibrium was reached, was very similar whatever the N-monosubstituted hydroxylamines RNHOH used and ranged between 57 and 73% (Table 1). This indicated that the size and hydrophobicity of the R substituent of the nitrogen atom had little effect on the binding of the RNO ligand on the iron of MP8. It could be due to a lack of control of the binding of the nitrosoalkane on the iron atom which should occur at the opposite of the octapeptide arm on the non-hindered face of the heme. This behavior is totally opposite to that of hemoproteins which have a small active site, such as hemoglobin, myoglobin and catalase (8), and which are totally unable to form Fe(II)-nitrosoamphetamine complexes but can only accommodate small RNO ligands such as CH_3NO and C_2H_5NO. On the contrary, PGHS (10) and rat liver cytochromes (18), which possess a wide hydrophobic active site, are able to bind bulky hydrophobic ligands such as nitrosoamphetamine but are unable to form nitroso complexes from the highly hydrophilic CH_3NHOH and C_2H_5NHOH. Finally only nitric oxide synthase (NOS) was found to be able, like MP8, to bind a wide range of RNO ligands including the smaller one

CH_3NO as well as the large hydrophobic p-Cl-Ph-CH_2-CH(NO)-CH_3 (19).

As a conclusion, the formation of MP8-Fe(II)-nitrosoalkane or -arene complexes, either by oxidation of N-monosubstituted hydroxylamines or by reduction of nitroalkanes in the presence of sodium dithionite, constitutes a new reaction of microperoxidase 8. This is also the first example of fully characterized iron(II)-metabolite complexes of MP8. Such complexes constitute good models for those formed not only *in vitro* but also *in vivo* during the oxidative metabolism of drugs containing an amine function such as amphetamine or macrolids (12) and which lead to an inhibition of the catalytic functions of cytochromes P450. In addition the aforementioned results validate the use of this mini-enzyme as a convenient model for hemoproteins of interest in toxicology and pharmacology such as cytochromes P450 and peroxidases.

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