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COMPARISON OF THE HEMOGLOBIN REACTIONS WITH METHYL- AND PHENYL-HYDRAZINE:INTERMEDIATE FORMATION OF A HEMOGLOBIN Fe(II)-METHYLDIAZENE COMPLEX.

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Methylhydrazine reacts with hemoglobin and myoglobin in their Fe(III) or Fe(II) states, in the presence of limited amounts of oxygen, with quantitative formation of methyldiazene-Fe(II) complexes of these hemoproteins.These complexes are equally formed by direct reaction of Hb- or Mb-Fe(II) with methyldiazene itself. They are oxidized by oxygen or ferricyanide to stable complexes exhibiting visible spectra very similar to those previously described for complexes formed by aerobic reactions of phenylhydrazine with Hb or Mb.The reactions of Hb with CH_3NHNH_2 and $C_6H_5NHNH_2$ exhibit two major differences:(i) no intermediate phenyldiazene-Fe(II) complex could be detected from $C_6H_5NHNH_2$,(ii) the final Hb-Fe(III)-phenylhydrazine-derived metabolite complex is more stable than its methylhydrazine counterpart; moreover, an acidic heme extraction on the former leads to important amounts of N-phenyl-protoporphyrin IX whereas iron-protoporphyrin IX is mainly recovered from an identical treatment of the latter.

INTRODUCTION

Hydrazine derivatives are very effective inducers of hemoglobin(Hb) precipitation in the form of Heinz bodies within the red cells and ensuing hemolytic anemia(1-3).As yet, most studies have been concerned with arylhydrazines,and it has been shown that reactive intermediates such as phenyldiazene and phenyl radicals are formed upon oxidation of phenylhydrazine($C_6H_5NHNH_2$)by oxyhemoglobin(HbO_2)(4-6).However,the detailed mechanism of the reaction between hemoglobin and arylhydrazines is not yet completely clear despite the following results:(i) a hemoglobin-Fe(III)-phenylhydrazine-derived metabolite complex is quantitatively formed upon reaction of oxyhemoglobin and $C_6H_5NHNH_2$(2,6,7) (ii) very recently, N-phenyl-protoporphyrin IX(8,9) and β-mesophenyl-biliverdin IX(9) were isolated as final products of the reaction between HbO_2 and $C_6H_5NHNH_2$, after acidic treatment.The present report concerns the reactions of methylhydrazine CH_3NHNH_2 with hemoglobin and myoglobin and compares them to the correspon-

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ding reactions of phenylhydrazine.It provides evidence for the intermediate formation of iron(II)-methyldiazene complexes in the reactions of CH_3NHNH_2,which are the first examples of hemoprotein- or iron-porphyrin-Fe(II)-alkyldiazene complexes, and shows that these compounds are further oxidized into ferric complexes having spectral characteristics similar to those of the previously described ferric hemoglobin-$C_6H_5NHNH_2$-derived metabolite complexes(2).

MATERIALS AND METHODS

Materials: Methylhydrazine was used as supplied from Aldrich. Phenylhydrazine from Prolabo was distilled over NaOH pellets. Methyldiazene was prepared as described previously(10).Myoglobin type I equine skeletal muscle and hemoglobin type I from bovine blood were purchased from Sigma; they are almost completely in the ferric form.Deoxy-Fe(II)-Mb or -Hb were prepared by reduction of the commercial samples with nearly stoechiometric amounts of sodium dithionite under argon and purified by chromatography on a Sephadex G-10 column.Spectrophotometric measurements were performed on an Aminco DW2 spectrophotometer.

Reaction of methyldiazene with MbFe(II):Methyldiazene prepared as described previously(10) was bubbled through a 10^{-5}M solution of MbFe(II) in phosphate buffer pH 7.4(0.1M) during ca. 5mn, in strictly anaerobic conditions.The visible spectrum of the solution was recorded immediately and found almost superimposable to that of complex $\underline{A}_{Mb}$ (Fig.1)

Typical procedure for the isolation of complexes B and B': 1g of CH_3NHNH_2 is progressively added to an aerobic 50 ml solution of 1g of MbFe(III).After 3h, the hemoglobin complex is purified and separated from the excess of hydrazine by chromatography on a Sephadex G-10 column(0.1M phosphate buffer as eluent).A further lyophilization allowed to remove the remaining traces of hydrazine.

Acidic extraction of the heme of isolated complexes $\underline{B}_{Hb}$ and $\underline{B'}_{Hb}$: 20 mg of isolated complex $\underline{B}_{Hb}$ is treated by 30 ml of CH_3OH containing 1.5 ml of H_2SO_4 36M overnight at 4°C.The pigments are then extracted by a previously described procedure(8) and studied by visible spectroscopy and thin-layer chromatography on silicagel (CH_2Cl_2:acetone,80:20).The major pigment was found in this case nearly identical to that derived from iron(III)-protoporphyrin IX or HbFe(III) themselves treated in the same conditions; it is iron-protoporphyrin IX-dimethyl ester because of esterification of the COOH groups during the CH_3OH-H_2SO_4 extraction.The same reaction performed on $\underline{B'}_{Hb}$ gives a pigment which was found identical to N-phenyl-protoporphyrin IX-dimethyl ester(8), either as a free base or its Zn^{2+} complex.

RESULTS

Reaction of hemoglobin and myoglobin with CH_3NHNH_2 :

In anaerobic conditions, CH_3NHNH_2 does not bind to the iron of MbFe(II) and only reacts with MbFe(III) by reducing it to MbFe(II).When MbFe(III), or MbFe(II), is made to react with excess CH_3NHNH_2 in the presence of limited amounts of O_2(10 moles per mole of Mb) a new complex $\underline{A}_{Mb}$ exhibiting characteristic peaks at

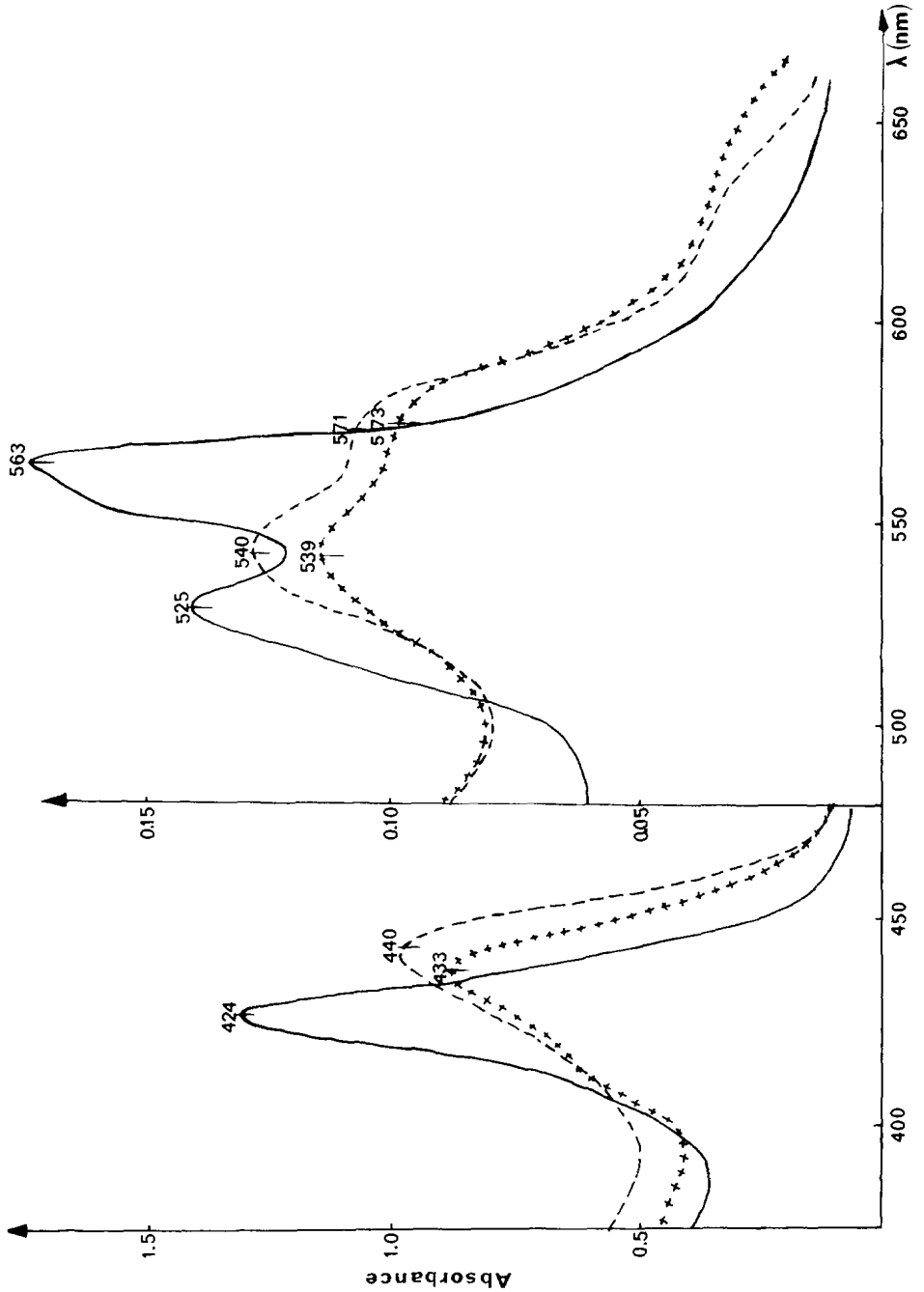


Figure 1: Electronic spectra of the complexes formed by reaction of Mb with CH_3NHNH_2 or $C_6H_5NHNH_2$, (——) complex $\underline{A}_{Mb}$ from reaction of 0.01 mM MbFe(III) with 0.1 mM CH_3NHNH_2 in phosphate buffer(0.1M) pH 7.4, in the presence of 0.1 mM O_2; (- - - -) complex $\underline{B}_{Mb}$ obtained upon further addition of 0.015 mM $Fe(CN)_6^{3-}$ (++++) complex $\underline{B'}_{Mb}$ from 0.01 mM MbFe(III) + 0.1 mM $C_6H_5NHNH_2$ in aerobic phosphate buffer(0.1M) pH 7.4.

Table 1

UV-visible spectral characteristics of the complexes formed by reaction of Mb and Hb with CH_3NHNH_2(A,B) or $C_6H_5NHNH_2$(B')(phosphate buffer pH 7.4,0.1 M)

	λ max(nm)			
A_{Mb}	423	525	555(sh)	563
A_{Hb}	429	528	555	
B_{Mb}	440	540	571(sh)	635(sh)
B_{Hb}	437	537	567(sh)	636(sh)
B'_{Mb}	433	539	573(sh)	641(sh)
B'_{Hb}	430	538	571(sh)	640(sh)

423, 525, 555 and 563 nm is almost quantitatively formed(Fig.1). Once formed, complex A_{Mb} is unchanged upon addition of a few equivalents of sodium dithionite but reacts immediately and quantitatively with CO to give MbFe(II)CO.Complex A_{Mb} is equally formed by reaction of MbFe(II) with $CH_3N{=}NH$ in anaerobic conditions. The visible spectrum of complex A_{Mb} is very similar to that previously described for the myoglobin complex formed by reaction of diimide NH=NH with MbFe(II), for which a MbFe(II)-(NH=NH) structure has been proposed(11).This spectral similarity, together with the formation of complex A_{Mb} by direct interaction of MbFe(II) with $CH_3N{=}NH$ and its fast transformation into MbFe(II)CO upon CO addition, indicates that complex A_{Mb} derives from the binding of $CH_3N{=}NH$ to the iron of MbFe(II).

Upon addition of one equivalent of ferricyanide to complex A_{Mb}, it is quantitatively oxidized to complex B_{Mb}, the visible spectrum(λ= 440, 544 and 570 nm, Fig.1)of which is very similar to that found for the complex B'_{Mb} formed by reaction of oxymyoglobin with $C_6H_5NHNH_2$(Table 1).Exposure of complex A_{Mb} to dioxygen also leads to complex B_{Mb} within 15 mn, and it is noteworthy that reaction of oxymyoglobin with CH_3NHNH_2 in usual aerobic conditions leads quantitatively to complex B_{Mb}.

Very similar reactions were observed with hemoglobin, the corresponding A_{Hb} and B_{Hb} complexes exhibiting the spectral characteristics indicated in Table 1.It is noteworthy that, as Mb or Hb complexes, compounds B_{Mb} and B_{Hb} are characterized by Soret peaks unusually shifted to the red.

Properties of complexes B_{Mb} and B_{Hb}; comparison with B'_{Mb} and B'_{Hb}:

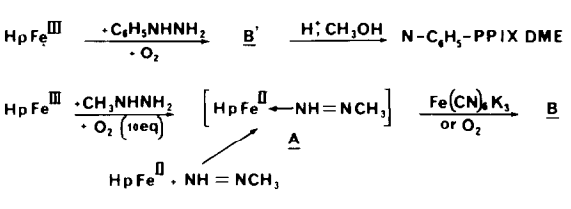
Complexes B_{Mb} and B_{Hb} exhibit visible spectra very similar to those described for the Mb- and Hb-"ferrihemochromes"(complexes

$\underline{B}'_{Mb}$ and $\underline{B}'_{Hb}$)formed by reaction of Mb and Hb with $C_6H_5NHNH_2$(Table 1) and previously interpreted as Mb or Hb-Fe(III) complexes with a $C_6H_5N{=}NH$ or $C_6H_5N{=}N^-$ ligand of the iron(2,7).Complexes $\underline{B}_{Mb}$ and $\underline{B}_{Hb}$ can be purified by column chromatography and are stable for hours in the presence of moderate excess of sodium dithionite.In the presence of a large excess of sodium dithionite, complexes $\underline{B}_{Mb}$ and $\underline{B}_{Hb}$ are slowly transformed into Mb- or Hb-Fe(II)(90% after 48h).When kept at 20°C in aerobic phosphate buffer pH 7.4(0.1M), they are slowly transformed into Mb- or Hb-Fe(III) that give Mb- or Hb-Fe(II)CO quantitatively upon dithionite reduction in the presence of CO.Complexes $\underline{B}'_{Mb}$ and $\underline{B}'_{Hb}$ are more stable in that sense they do not revert to Mb- or Hb-Fe(II)(or Fe(III))in identical conditions.

Extraction of the heme of isolated complex $\underline{B}'_{Hb}$ in the conditions described by Ortiz De Montellano et al.(8)(treatment by H_2SO_4 in CH_3OH and $CHCl_3$ extraction)leads to N-phenyl-protoporphyrin IX-dimethyl ester with a yield of about 40%.This is in agreement with the results previously reported for heme extraction on mixtures coming from reaction of Hb with $C_6H_5NHNH_2$ in excess(8,9).On the contrary, we were unable to detect the corresponding green N-methyl-protoporphyrin IX-dimethyl ester when isolated complex $\underline{B}_{Hb}$ was treated in the same conditions, iron-protoporphyrin IX-dimethyl ester being the main pigment recovered in this case.

DISCUSSION

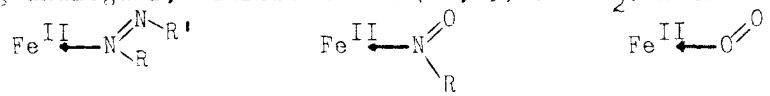
The aforementioned results give a detailed mechanism for the reaction of Mb- or Hb-Fe(II) or -Fe(III) with CH_3NHNH_2 in aerobic conditions(Scheme 1).The first intermediate(complex $\underline{A}_{Mb}$ or $\underline{A}_{Hb}$) derives from the oxidation of CH_3NHNH_2 by $HbFe(II)O_2$ or HbFe(III) leading to $CH_3N{=}NH$ and HbFe(II), and the binding of $CH_3N{=}NH$ to HbFe(II).Complexes $\underline{A}_{Mb}$ and $\underline{A}_{Hb}$, which are equally obtained by direct binding of $CH_3N{=}NH$ to the iron of HbFe(II) in anaerobic



Scheme 1

(Hp = Hb or Mb, PPIX DME = protoporphyrin-IX-dimethyl ester)

conditions, are the first reported alkyldiazene complexes of a ferrous hemoprotein. The existence of their $(CH_3N{=}NH)$-Fe(II) bond is further substantiated by a recent isolation and characterisation of a porphyrin-iron(II)-methyldiazene complex; Fe(tetraphenylporphyrin = TPP)$(CH_3N{=}NH)_2$, upon reaction of Fe(II)(TPP) with $CH_3N{=}NH$ (P. Battioni, J.P. Mahy, G. Gillet, D. Mansuy, to be published). The alkyldiazene ligands are isoelectronic to their oxygen-containing analogues, nitrosoalkanes (12,13) and O_2. Their affinity



for Fe(II)-porphyrins is however much lower than that of nitrosoalkanes, since contrary to the latter ligands they are easily displaced by CO and even by O_2 (when O_2 is bubbled through a 10^{-5}M solution of pure complex $\underline{A}_{Hb}$, it is rapidly transformed into HbFe(II)O_2). When CH_3NHNH_2 reacts with HbFe(III) or HbFe(II)O_2 in usual aerobic conditions, complex $\underline{A}_{Hb}$ is only formed as an intermediate, its concentration depending upon the relative starting concentrations of CH_3NHNH_2, Hb and O_2, since its diazene ligand may be exchanged with O_2 and since it is further oxidized by O_2 into complex $\underline{B}_{Hb}$.

In the case of the corresponding reactions with $C_6H_5NHNH_2$, a final complex $\underline{B}'_{Hb}$ (or $\underline{B}'_{Mb}$), exhibiting visible spectra very similar to those of $\underline{B}_{Hb}$ is also formed, but, in similar conditions to those mentioned above, no equivalent of complex $\underline{A}_{Hb}$ was ever observed. Accordingly, no iron(II)-$C_6H_5N{=}NH_2$ bond is formed upon anaerobic reaction of $C_6H_5N{=}NH$ and Hb- or Mb-Fe(II) (6,14). This could be related either to a greater steric hindrance or a lower binding affinity of $C_6H_5N{=}NH$ compared to $CH_3N{=}NH$. In that respect, it is noteworthy that nitrosoalkanes exhibit a much higher affinity for ferrous hemoproteins and porphyrins than nitrosoarenes (15). Another major difference between the reactions of CH_3NHNH_2 and $C_6H_5NHNH_2$ is the fate of isolated complexes $\underline{B}_{Hb}$ and $\underline{B}'_{Hb}$ upon acidic extraction of the heme. N-phenyl-protoporphyrin IX is formed in high yields from the latter, as found previously (8,9) whereas N-methyl-protoporphyrin IX could not be detected from the former, iron-protoporphyrin IX being mainly recovered in this case.

The precise structure of complexes of type $\underline{B}$ is not yet presently clear. Taking into account that complex $\underline{B}$ is formed by oxidation of $\underline{A}$ by $Fe(CN)_6K_3$ or O_2, a possible structure for it could involve the binding of either the diazene itself, $CH_3N{=}NH$, or its anion $CH_3N{=}N^-$ to Hb- (or Mb-) Fe(III). However, it is known that

oxygen-containing analogues of alkyldiazenes, nitrosoalkanes and O_2, do not bind to hemoproteins in their ferric state, making the Fe(III)-(NH=NR) structure unlikely.

Further work is in progress to determine the structure of complexes B and B' and to understand the different behaviour of B and B' upon heme extraction by acidic treatment.

REFERENCES

1. Beaven,G.H. and White,J.C.(1954) Nature 173, 389-391.
2. Itano,H.A.,Hirota,K.and Vedvick,T.S.(1977) Proc.Nat.Acad.Sci. U.S. 74, 2556-2560 and references cited there in.
3. Peisach,J.,Blumberg,W.E. and Rachmilewitz,E.A.(1975) Biochim. Biophys.Acta 393, 404-418.
4. Misra,H.P. and Fridovich,I.(1976) Biochemistry 15, 681-687.
5. Hill,H.A.O. and Thornalley,P.J.(1981) F.E.B.S. Letters 125, 235-238.
6. Itano,H.A. and Mannen,S.(1976)Biochim.Biophys.Acta 421, 87-96.
7. Itano,H.A. and Robinson,E.A.(1961)J.Am.Chem.Soc. 83, 3339-3340.
8. Ortiz De Montellano,P.R. and Kunze,K.L.(1981)J.Am.Chem.Soc. 103, 6534-6535.
9. Saito,S. and Itano,H.A.(1981)Proc.Nat.Acad.Sci.U.S. 78, 5508-5512.
10. Ackerman,M.N.,Ellenson,J.L. and Robinson,D.H.(1968)J.Am.Chem. Soc. 90, 7173.
11. Hanstein,W.G.,Lett,J.B.,Mc Kenna,C.E. and Traylor,T.G.(1967) Proc.Nat.Acad.Sci.U.S. 58, 1314-1316.
12. Mansuy,D.,Chottard,J.C. and Chottard,G.(1977) Eur.J.Biochem. 76, 617-623.
13. Mansuy,D.,Battioni,P.,Chottard,J.C. and Lange,M.(1977) J.Am. Chem.Soc. 99, 6441-6443.
14. Huang,P.K.C. and Kosower,E.M.(1968) Biochim.Biophys.Acta 165, 483-489.
15. Mansuy,D.,Beaune,P.,Cresteil,T.,Bacot,C.,Chottard,J.C. and Gans,P.(1978)Eur.J.Biochem. 86, 573-579.