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Very general formation of tetrahydropterin cation radicals during reaction of iron porphyrins with tetrahydropterins: model for the corresponding NO-synthase reaction

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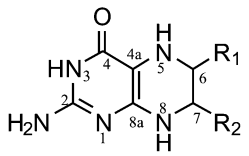
Electron transfer from tetrahydropterins to iron porphyrins, with formation of intermediate tetrahydropterin cation radicals, is a very general reaction that was shown to occur not only with tetrahydrobiopterin, as originally found in NO-synthases, but also with another important biological cofactor, tetrahydrofolate, and various iron porphyrins, either in their ferric state, or in the $Fe^{II}O_2$ state, as in the first model of the corresponding NO-synthase reaction described in this paper.

Tetrahydropterins, such as tetrahydrobiopterin (H_4B) or tetrahydrofolate (H_4F) (Scheme 1), are important cofactors involved in many biological processes.[1] In nitric oxide synthase (NO-synthase), H_4B is an essential cofactor for the monooxygenation of L-arginine to L-citrulline and NO.[2] It has been recently shown that, during the NO-synthase-dependent monooxygenation, H_4B transfers one electron to the heme, a second cofactor which is present in the active site in close proximity to H_4B. The $H_4B^{+\cdot}$ cation radical derived from this reaction has been detected by freeze–quench EPR methods.[3] This is so far the only evidence for the intermediate formation of $H_4B^{+\cdot}$ in a biological process. Moreover, in a more general manner, no data are presently available on electron transfer between tetrahydropterins and iron porphyrins. We have recently studied the reactions between various natural and synthetic tetrahydropterins and many iron porphyrins (Scheme 1) in order to mimic the electron transfer observed in NO-synthase and to ascertain whether this reaction is general or restricted to NO-synthase because of a special proximity and positioning of H_4B and the heme within the NO-synthase active site.

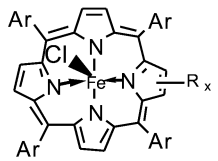
Addition of one equivalent of H_4B to a solution of Fe^{III}[*meso*-tetra(pentafluorophenyl)-β-octabromo-porphyrin](Cl),[4] $Fe^{III}(TF_5PBr_8P)Cl$ (Scheme 1), in CH_3CN : H_2O (9 : 1), under anaerobic conditions, led to the immediate formation of $Fe^{II}(TF_5PBr_8P)$, as shown by visible and EPR spectroscopy. Fast mixing (about 1 s) of these two reactants in an EPR Bruker AquaX cell at 20 °C led to signals centered at g = 2.003 (Fig. 1) very similar to those previously reported for $H_4B^{+\cdot}$ obtained by chemical oxidation of H_4B.[5] Simulation of this spectrum on the basis of literature data[3c,3d,5] gave a_{N5}, a_{H5}, a_{N8} and a_{H6} of 8.3, 9.7, 2.0 and 10.5 G respectively (for numbering of H_4B atoms, see Scheme 1). These values are similar to those previously reported for chemically generated $H_4B^{+\cdot}$ [5] and comparable to those found for $H_4B^{+\cdot}$ detected in NO-synthase,[3c,3d] allowing for the different environments of $H_4B^{+\cdot}$ in the chemical and enzymatic experiments.

Reaction of $Fe^{III}(TF_5PBr_8P)Cl$ either with H_4F in DMF : H_2O 9 : 1, or with $diMeH_4P$ under conditions identical to those used with H_4B, also resulted in fast reduction of the Fe^{III} porphyrin with intermediate formation of a species exhibiting EPR characteristics very similar to those of $H_4B^{+\cdot}$.

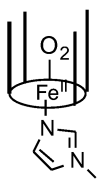
Similar reactions were performed between $diMeH_4P$ and seven other Fe^{III} porphyrins, $Fe(TDCPN_xP)Cl$, bearing from 0 to 7 β-nitro substituents and exhibiting redox potentials (*vs.* saturated calomel electrode) ranging from −225 to +560 mV for the Fe^{III}/Fe^{II} couple (in CH_2Cl_2).[6] All reactions were done at 20 °C in CH_3CN : H_2O (9 : 1) under strictly anaerobic conditions, and were followed by UV–vis, ^1H NMR and EPR spectroscopy. Complete reduction of the starting Fe^{III} porphyrin occurred in less than 10 s after addition of one equiv. of $diMeH_4P$ to $Fe^{III}(TDCPN_xP)Cl$ with $x > 2$. In fact, addition of increasing amounts of $diMeH_4P$ to $Fe(TDCPN_5P)Cl$ showed that complete formation of $Fe^{II}(TDCPN_5P)$ already occurred after addition of 0.5 equiv. of $diMeH_4P$, as expected if one considers that $diMeH_4P$ is a two-electron reducing agent. Complete reduction of Fe(TDCPP)Cl, $Fe(TDCPN_1P)Cl$ and $Fe(TDCPN_2P)Cl$ required the addition of 5–10 equiv. of $diMeH_4P$. EPR studies of all these reactions after fast mixing of the reactants in an EPR AquaX cell at room temperature always showed the intermediate formation of signals at g = 2.003 which are characteristic of $diMeH_4P^{+\cdot}$. Moreover, EPR studies of those reactions performed in the presence of various classical radical traps, such as α-(4-pyridyl-1-oxide)-*N*-*tert*-butyl nitrone (POBN), always showed the appearance of the signals expected[7] for the addition of a carbon-centered free radical derived from $diMeH_4P^{+\cdot}$ to the spin trap. For instance,



diMeH$_4$P $R_1=R_2=CH_3$
H$_4$B $R_1=CHOHCHOHCH_3$, $R_2=H$
H$_4$F $R_1=CH_2NH$–C$_6$H$_4$–$CONHCH(COOH)CH_2CH_2COOH$ $R_2=H$



Fe^{III}(TF$_5$PBr$_8$P)Cl Ar=C_6F_5 ; R_x=Br_8
Fe^{III}(TDCPN$_x$P)Cl Ar=2,6-diClPh ; R_x=$(NO_2)_x$



Fe^{II}(T$_{piv}$PP)(O$_2$)(NMeIm)

Scheme 1

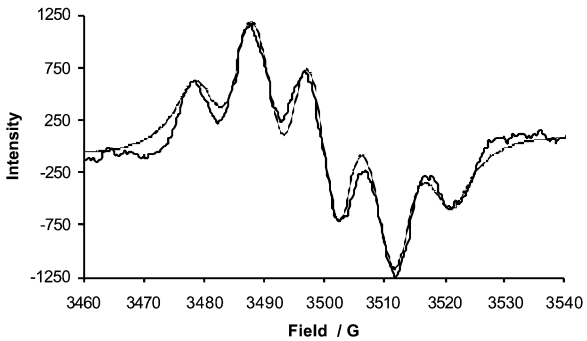


Fig. 1 EPR spectrum observed during reaction of H_4B with $Fe(TF_5PBr_8P)Cl$. (—) experimental spectrum obtained 10 s after fast mixing of 2 mM $Fe(TF_5PBr_8P)Cl$ and 2 mM H_4B in CH_3CN : H_2O (9 : 1) at 20 °C;(**---**) simulated spectrum with g = 2.003 and a_N = 8.3 and 2.0 and a_H = 9.7 and 10.5 G.

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reaction of $Fe(TF_5PBr_8P)Cl$ with $diMeH_4P$ in the presence of POBN (1 : 1 : 100 molar ratio) produced a six-line spectrum centered at $g = 2$, with $a_N = 14.5$ G and $a^{\beta}_{H} = 1.9$ G, corresponding to the trapping by POBN of a tertiary carbon-centered radical derived from $diMeH_4P^{+\cdot}$. A similar EPR spectrum has been recently observed during oxidation of H_4B by peroxynitrite in the presence of POBN.[8]

The above data showed that electron transfer from tetrahydropterins to iron (III) porphyrins, with intermediate formation of the corresponding tetrahydropterin cation radical, is a very general reaction [eqn. (1)].

$$diMeH_4P + (P)Fe^{III} \rightarrow (P)Fe^{II} + diMeH_4P^{+\cdot} \quad (1)$$

However, this electron transfer was not detected in the case of Fe^{III} porphyrins of very low redox potentials, such as Fe^{III} microperoxidase 11[9] (~ -375 mV *vs.* SCE) (data not shown), and does not occur between H_4B and the Fe^{III} heme (~ -500 mV *vs.* SCE) in NO-synthase.[2] Indeed, the intermediate formation of $H_4B^{+\cdot}$ in the NO-synthase reaction has been shown to result from an electron transfer from H_4B to the NO-synthase heme $Fe^{II}O_2$ complex.[3] In order to mimic that reaction, we have studied the reaction between $diMeH_4P$ and one of the few stable (porphyrin)$Fe^{II}O_2$ complexes reported in the literature, the $Fe^{II}(T_{piv}P\text{-}P)(O_2)(N\text{–}MeIm)$ complex (Scheme 1) obtained by exposure of the Fe^{II}("picket-fence" porphyrin) to O_2 in the presence of *N*-methylimidazole.[10]

Fast mixing of solutions of $Fe^{II}(T_{piv}PP)(O_2)(NMeIm)$ and $diMeH_4P$ (2 equiv.) in acetone : H_2O 9 : 1 at 20 °C in an EPR AquaX cell led to the formation of a species having EPR characteristics identical to those of $diMeH_4P^{+\cdot}$. The intensity of the $diMeH_4P^{+\cdot}$ signal reached its maximum value in 10 s and then decreased with a half life of about 120 s. UV–vis studies of the same reaction at 20 °C showed the disappearance of the bands of the starting complex and the appearance of the bands of the $Fe^{III}(T_{piv}PP)(OH)$ complex. EPR studies at 100 K of a similar reaction performed with addition of excess $diMeH_4P$, for 3 min at 180 K, led to signals at $g = 2.33$, 2.19 and 1.92. These *g*-values are highly similar to those previously reported for the Fe^{III}(TMP)(imidazole)(OOH) complex and for the hemoglobin–, P450cam– and heme oxygenase–$Fe^{III}OOH$ complexes (Table 1); they are considered to be characteristic of hexacoordinated porphyrin $Fe^{III}OOH$ complexes[15] and are clearly different from the *g*-values observed for the corresponding $Fe^{III}OO^{-}$ complexes.[15] Further warming of the solution from 180 K to 293 K led to the disappearance of these signals at temperature higher than 210 K. These data indicate that $diMeH_4P$ rapidly transfers an electron to the "picket-fence" porphyrin $Fe^{II}O_2$ complex with intermediate formation of $diMeH_4P^{+\cdot}$ and of a transient $Fe^{III}OO^{-}$ species which should be rapidly protonated at 180 K in the acetone–H_2O medium with formation of the corresponding $Fe^{III}OOH$ intermediate observed by EPR spectroscopy [eqn. (2)]. Such a protonation of the transient ("picket-fence" porphyrin)$Fe^{III}OO^{-}$ species at 180 K is in complete agreement with results reported on intermediate $HbFe^{III}OO^{-}$ generated by cryoreduction of $HbFe^{II}O_2$ at 77 K, which leads to $HbFe^{III}OOH$ upon warming at 180 K[12]. Reaction of eqn. (2) mimics well the electron transfer from H_4B to heme $Fe^{II}O_2$ in NO-synthase (NOS), that is supposed to occur with transient formation of $NOSFe^{III}OOH$[15] [eqn. (3)].

$$(P)Fe^{II}O_2 + diMeH_4P \xrightarrow{H^+} (P)Fe^{III}OOH + diMeH_4P^{+\cdot} \quad (2)$$

$$NOSFe^{II}O_2 + H_4B \xrightarrow{H^+} [NOSFe^{III}OOH] + H_4B^{+\cdot} \quad (3)$$

Our data show that the transfer of an electron from tetrahydropterins to iron porphyrins, with intermediate formation of tetrahydropterin cation radicals, is a very general reaction. It was shown to occur not only with H_4B, as described for the first time in the case of NO-synthase,[3] but also with $diMeH_4P$ or another important biological cofactor, H_4F, and various iron porphyrins, either in their ferric state, or in the $Fe^{II}O_2$ state, as in the first model of the NO-synthase reaction of that type described in this paper. These data suggest that such reactions should be found in enzymes using both a tetrahydropterin and a metallic centre in their active site; in that regard, it has been recently proposed that some bacterial NO-synthase could be associated with H_4F.[16]

Table 1 Comparison of the EPR data of the intermediate complex formed upon reaction of $Fe(T_{piv}PP)(O_2)(NMeIm)$ and $diMeH_4P$ with those previously reported for hexacoordinated porphyrin $Fe^{III}OOH$ complexes

Complex[a]	*g*-values			Ref.
$Fe(T_{piv}PP)(O_2)(NMeIm)$ + $diMePH_4$ at 180K	2.33	2.19	1.92	This work
Fe^{III}(TMP)(Im)(OOH)	2.32	2.19	1.94	11
β-Hb $Fe^{III}OOH$	2.31	2.18	1.94	12
P450cam $Fe^{III}OOH$	2.29	2.17	1.96	13
HOx $Fe^{III}OOH$	2.37	2.19	1.92	14

[a] TMP, β-Hb, P450cam and HO*x* are used for *meso*-tetramesitylporphyrin, the β chain of hemoglobin, cytochrome P450 101 and heme oxygenase, respectively.

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