Reduction of ferric haemoproteins by tetrahydropterins: a kinetic study

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We previously showed that one-electron transfer from tetrahydropterins to iron porphyrins is a very general reaction, with formation of an intermediate cation radical similar to the one detected in NO synthase. As a model reaction, the rates of reduction of eight haemoproteins by diMePH₄ (6,7-dimethyltetrahydropterin) have been studied and correlated with their one-electron reduction potentials, E_m (Fe^{III}/Fe^{II}). On the basis of kinetic data analyses, a bimolecular collisional mechanism is proposed for the electron transfer from diMePH₄ to ferrihaemoproteins. Haemoproteins with reduction potentials below –160 mV were shown not to be reduced by diMePH₄ to the corresponding ferrohaemoproteins. For haemoproteins with reduction potentials more positive than –160 mV, such as chloroperoxidase, cytochrome *b*₅, methaemoglobin and cytochrome *c*, there was a good correlation

between the second-order reduction rate constant and the redox potential, E_m (Fe^{III}/Fe^{II}):

$$\log [\text{rate (M}^{-1} \cdot \text{s}^{-1})] = -0.49 + (0.014 \times E_m)$$

The rate of reduction of cytochrome *c* by BH₄ [(6*R*)-5,6,7,8-tetrahydrobiopterin] was determined to be similar to that of the reduction of cytochrome *c* by diMePH₄. These results confirm the role of tetrahydropterins as one-electron donors to Fe^{III} porphyrins.

Key words: antioxidant, cytochrome *c*, haemoprotein, microperoxidase, NO synthase, tetrahydropterin.

INTRODUCTION

BH₄ [(6*R*)-5,6,7,8-tetrahydrobiopterin] is a cofactor required by several enzymes. Among tetrahydropterins, BH₄, and its analogues diMePH₄ (6,7-dimethyltetrahydropterin) and 6-methyltetrahydropterin, were equally effective in the hydroxylation reaction catalysed by non-haem iron-dependent aromatic amino acid hydroxylases, as described in recent reviews [1–3].

BH₄ is also an essential cofactor for the haem enzymes NOS (nitric oxide synthases) [4,5]. Increasing evidence suggests that BH₄ and synthetic analogues, such as diMePH₄, are redox-active in supporting the catalysis of oxidation of L-arginine into NO [6,7]. BH₄ rapidly transfers one electron to the NOS Fe^{II} (ferro)–O₂ intermediate, ensuring formation of the active oxygen species involved in arginine hydroxylation [8–10]. Rapid freeze–quench EPR experiments have provided useful information regarding the involvement of a BH₄ cation radical (BH^{•+}) in the arginine hydroxylation process [10–12].

Recent studies have shown that one-electron transfer from BH₄ to Fe^{III} (ferri) porphyrins is a very general reaction, with formation of an intermediate cation radical similar to the one detected in NOS [13]. This electron transfer occurred not only with BH₄, the natural cofactor, but also with the synthetic analogue, diMePH₄ with intermediate formation of a species exhibiting EPR characteristics very similar to those of BH^{•+}. Interestingly, these two compounds share common electrochemical properties [14–16].

To provide new data on the role of BH₄ and other tetrahydropterins as one-electron donors, we have studied the possible reactions of various Fe^{III} haemoproteins with diMePH₄, the synthetic and more readily available cofactor. The redox potential of these haemoproteins (for the Fe^{III}/Fe^{II} couple) varies from –400 to +260 mV. This panel includes haemoproteins with a haem active site similar to NOS and having a cysteinate-liganded haem group,

with the sixth iron ligand position that is either vacant or occupied by a water ligand, such as CYP (cytochrome P450) and CPO (chloroperoxidase). It also includes haemoproteins exhibiting a five-co-ordinated iron atom with a tyrosine axial ligand, such as catalase, or a histidine axial ligand, such as haemoglobin, HRP (horseradish peroxidase) and MP11 (microperoxidase 11), as well as haemoproteins that have a six-co-ordinate low-spin ferric haem such as cyt *b*₅ (cytochrome *b*₅) and cyt *c*.

Our data describe the spectral changes observed upon reaction of these haemoproteins with diMePH₄ and the kinetic features of the corresponding reactions. They show that all the studied Fe^{III} haemoproteins having a redox potential higher than –160 mV are easily reduced to their ferrous (Fe^{II}) state under the conditions used. The reductions follow simple second-order reaction kinetics and their rates are correlated with the one-electron reduction potential of the haemoproteins. These results confirm the role of tetrahydropterins as one-electron donors to Fe^{III} porphyrins.

MATERIALS AND METHODS

Proteins, chemicals and reagents

CPO from the marine filamentous fungus *Caldariomyces fumago*, catalase from bovine liver, cyt *c* from horse heart, haemoglobin from bovine blood, HRP and MP11 were purchased from Sigma and used as received. CYP2C5 (CYP 2C5/3LVdH) was a gift from Dr E. F. Johnson's laboratory (Department of Molecular and Experimental Medicine, Scripps Research Institute, La Jolla, CA, U.S.A.) [17]. The recombinant form of the trypsin-cleaved bovine liver microsomal cyt *b*₅ was a gift from Dr M. A. Sari of this institution and purified as described in [18]. diMePH₄ and BH₄ dihydrochloride were obtained from Sigma. All other chemicals used were of the highest grade available and were prepared daily

Abbreviations used: Asc, ascorbate; CYP, cytochrome P450; CYP2C5, CYP 2C5/3LVdH; CPO, chloroperoxidase; cyt, cytochrome; BH₄, (6*R*)-5,6,7,8-tetrahydrobiopterin; diMePH₄, 6,7-dimethyltetrahydropterin; Fe^{III}, ferri state; Fe^{II}, ferro state; HRP, horseradish peroxidase; methHb, methaemoglobin; MP11, microperoxidase 11; NOS, nitric oxide synthase.

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Table 1 Oxidation–reduction potentials (E_m) of various haemoproteins and their second-order rate constants (k_+) of reduction by diMePH₄

Reactions took place under anaerobic conditions and were monitored by the absorbance increase of the ferro-haemoprotein at the indicated wavelength (λ_{obs}).

Fe ^{III} haem protein	E_m for Fe ^{III} /Fe ^{II} (mV)	Reference	λ_{obs} (nm)	ε_{red} (mM ⁻¹ · cm ⁻¹)	$\Delta\varepsilon_{\text{ox-red}}$ (mM ⁻¹ · cm ⁻¹)	Reference	k_+ (M ⁻¹ · s ⁻¹)
CPO	-150	[31]	421	67	37.5	[19]	5×10^{-3}
Cyt <i>b</i> ₅	0	[32]	423	178	106	[21]	0.20
metHb	150	[28]	430	133	75	[23]	9.8
Cyt <i>c</i>	260	[29]	416	129	40	[22]	4.4×10^3
MP11*	-160	[36]	413	80	39	[20]	9.2

* Reaction in the presence of CO with λ_{obs} for Fe^{II}–CO species.

in distilled deionized water. All experiments were performed at 20 °C in 100 mM phosphate buffer, pH 7.

Haemoprotein solutions

Concentrations of CPO were determined by A_{403} using a ε value of 91.2 mM⁻¹ · cm⁻¹ [19]. MP11 was used at low concentration and the ε value of the monomer form was taken to be 148 mM⁻¹ · cm⁻¹ at 397 nm [20]. The cyt *b*₅ concentration was determined by spectrophotometry using a $\varepsilon_{412.5}$ value of 117 mM⁻¹ · cm⁻¹ [21]. The concentration of the cyt *c* solutions was determined by measuring A_{409} using a ε value of 106 mM⁻¹ · cm⁻¹ [22].

Preparation of metHb (methaemoglobin) solutions

metHb was freshly prepared before each experiment. Concentrated Hb · Fe^{II} · O₂ was oxidized by a fivefold excess of K₃Fe(CN)₆ and run through a Sephadex G-25 column to eliminate the oxidizing agent. The metHb concentration, always expressed per haem group, was determined using the peak at 405 nm with a ε value of 179 mM⁻¹ · cm⁻¹ [23].

Preparation of anaerobic or CO-saturated solutions for kinetics

Buffer solutions in glass tonometers were made anaerobic by at least six cycles of pumping under vacuum and flushing with oxygen-free argon gas. A concentrated aliquot of protein or reductant was added to the anaerobic solution with gas-tight syringes and the mixture was left to incubate at 4 °C under slight argon flushing. This solution was then transferred to a storage syringe of the flow system under a pressure of argon gas. For CO solutions, CO gas replaced argon gas.

Spectral measurements

Absorbance spectra, repetitive scans and kinetic absorbance measurements were collected on a Kontron UVICON 942 spectrophotometer interfaced with a computer to facilitate the collection, manipulation and analysis of data with the appropriate software. Therefore a series of spectra of oxidized and reduced haemoproteins recorded from 700 to 350 nm allowed one to calculate at each wavelength the individual absorbance coefficients ε_{ox} , ε_{red} and $\Delta\varepsilon_{\text{red-ox}}$ of bound haem (where _{ox} is oxidized and _{red} is reduced). The kinetic traces were obtained by following the time course of the reaction at a wavelength specific for the Fe^{II}-haemoprotein.

Rapid kinetic measurements

To circumvent the problems which had so far prevented analysis of the reaction by classical spectrophotometry, a sequential stopped-flow apparatus, SFM-3, from Bio-Logic Instruments, was used.

Anaerobic haemoprotein and diMePH₄ solutions were mixed together, resulting in a final haemoprotein concentration between 0.5 and 10 μ M and diMePH₄ concentrations ranging from 0.2 to 4 mM. The kinetic traces were recorded, saved and analysed using the BIO-KINE software application. The observed rate constants were obtained from the average of two or three traces.

Analysis of data

Formation of Fe^{II}-haemoprotein was monitored by the absorbance increase in the Soret band as indicated in Table 1. Experimental traces were monophasic and fitted to an equation for one exponential term using a non-linear regression program based on a least-squares criterion. At least three determinations of pseudo-first-order rate constants, k (s⁻¹), were performed for each diMePH₄ concentration. The second-order rate constant, k_+ (M⁻¹ · s⁻¹), of the haem iron reduction by diMePH₄ and the correlation coefficient (R^2) were derived from a plot of k (s⁻¹) versus [diMePH₄], using the Kaleidagraph V3.08 program.

RESULTS

Reactions of diMePH₄ with catalase, CYP2C5 and HRP

Incubation of anaerobic solutions of catalase, CYP2C5 or HRP with an excess (100–500 equiv.) of diMePH₄ for 1 h failed to produce any change in the UV–visible spectra of the starting Fe^{III}-haemoproteins. Thus there was no reduction with diMePH₄ of the Fe^{III} state of haemoproteins of very low oxidation–reduction midpoint potentials, i.e. $E_m = -460$ mV, -330 mV and -280 mV respectively [24–26] (all E_m values are given versus normal hydrogen electrode) under the conditions used. This result is consistent with the absence of reduction by BH₄ of the Fe^{III} haem of low redox potential present within the neuronal NOS active site (-250 mV) [27].

Reactions of diMePH₄ with metHb and ferricyt *c*

In contrast, a fast and total reduction of Fe^{III} haem to its Fe^{II} state was observed upon reaction of diMePH₄ with metHb and cyt *c*, both exhibiting positive redox potentials of $+150$ and $+260$ mV respectively [28,29]. The kinetics of the reduction of the Fe^{III} haem by diMePH₄ performed under anaerobic conditions were monitored under pseudo-first-order conditions (50–500 equiv. of diMePH₄) and monitored at the level of specific Soret band of each haemoprotein (Table 1).

A typical time course for the changes in cyt *c* absorbance is shown in Figure 1. Analyses of the traces to a mono-exponential process allowed to determine the pseudo-first-order rate constants, k (s⁻¹), which were then plotted against diMePH₄ concentration. The slope of the linear plot thus obtained (inset to Figure 1)

Figure 1 Reduction of cyt *c* by diMePH₄

The reaction trace observed at 416 nm on mixing 0.9 μM cyt *c* with 0.4 mM diMePH₄ in the stopped-flow apparatus is shown. The broken line shows a fit of the trace to a monoexponential process, $k = 2.0 \text{ s}^{-1}$ ($R^2 = 0.99$). The inset shows a second-order plot for the dependence of the reduction rate on diMePH₄ concentration: $k_+ = 5.2 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$ ($R^2 = 0.99$).

Figure 2 Time course of methHb reduction

A typical stopped-flow absorbance tracing recorded at 430 nm obtained at 4.2 μM and 1.4 mM final concentrations of methHb and diMePH₄ respectively is shown. Also shown is a biphasic fitting of the data to the time variation of the absorbance given by $a + b + c \cdot e^{-kt}$ (broken line; $a = 0.00046$, $b = 0.348$, $c = 0.072$, $k = 0.030$) ($R^2 = 0.99$). The magnitude of the absorbance change remained constant whatever the diMePH₄ concentration ($\Delta A = 0.080 \pm 0.010$, $n = 7$). The inset shows the effect of diMePH₄ on the pseudo-first-order rate constant of the fast initial reduction phase of methHb. The fit leads to $k_+ = 9.8 \text{ M}^{-1} \cdot \text{s}^{-1}$ ($R^2 = 0.98$).

yielded the second-order rate constant for the reduction of cyt *c* by diMePH₄. The mean value $\pm$ S.D. for four independent experiments corresponds to $k_+ = (4.4 \pm 0.8) \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$.

When the reaction of methHb with diMePH₄ in excess was performed, a more complicated picture was found (Figure 2). Biphasic kinetics were observed with an initial mono-exponential formation of Fe^{II} Hb, followed by a slow linear reduction phase. The total absorbance change observed on a longer time scale corresponded to the complete reduction of the whole haemoprotein. As the concentration of diMePH₄ was increased, the rate constant of the rapid initial phase increased (inset to Figure 2, $k_+ = 9.5 \text{ M}^{-1} \cdot \text{s}^{-1}$) and the magnitude of the absorbance change remained constant and corresponded to the rapid reduction of one subunit out of four in methHb. The observation of biphasic kinetics

Figure 3 Typical stopped-flow kinetics of MP11 reduction

Time course of the change in absorbance at 413 nm for the reduction of MP11 (11 μM) by diMePH₄ (1.4 mM) in the presence of CO. The broken line gives the fit of the trace to a monoexponential process; $k = 0.019 \text{ s}^{-1}$ ($R^2 = 0.99$). The inset shows a second-order plot for the dependence of the reduction rate on diMePH₄ concentration; $k_+ = 9.2 \text{ M}^{-1} \cdot \text{s}^{-1}$ ($R^2 = 0.95$).

was in agreement with the previously reported kinetic data on the reduction of metHb by ferrocylt *b*₅ [30], which indicated that the second and subsequent subunits may react at a rate different from that of the first one.

Reactions of diMePH₄ with CPO, cyt *b*₅ and MP11

For haemoproteins exhibiting a redox potential in the -150 to $+50$ mV range, an excess of diMePH₄ was required to detect the formation of the Fe^{II} state in CPO (-150 mV) [31] and cyt *b*₅ (0 mV) [32]. A slow and partial reduction was obtained with CPO and cyt *b*₅, with second-order rates of $2 \times 10^{-3} \text{ M}^{-1} \cdot \text{s}^{-1}$ and $0.2 \text{ M}^{-1} \cdot \text{s}^{-1}$ respectively.

Preliminary experiments showed that no reduction was observed after addition of an excess (50–500 equiv.) of diMePH₄ to Fe^{III}-MP11. Advantage was then taken of the relatively rapid reaction of Fe^{II}-haemoproteins with CO. Experiments were performed in the presence of 1 mM CO. Under these conditions, the binding reaction of CO to the Fe^{II}-MP11, i.e. $k_+ = 2.5 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ [33], is much faster than the reduction of Fe^{III}-MP11 by diMePH₄. As illustrated in Figure 3, the appearance of the haem Fe^{II}-CO complex was monitored as a means of measuring the electron-transfer rate between diMePH₄ and the Fe^{III} haem. The reduction was still slow, but total, for Fe^{III}-MP11, with a rate constant $k_+ = 10 \text{ M}^{-1} \cdot \text{s}^{-1}$.

Dependence of the rate constants of haemoprotein reduction by diMePH₄ on their reduction potential

The values of k_+ for the haemoproteins studied are listed in Table 1. The values covered more than six orders of magnitude, ranging from $2 \times 10^{-3} \text{ M}^{-1} \cdot \text{s}^{-1}$ for CPO to $5 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$ for cyt *c*. As shown in Figure 4, $\log(k_+)$ was linearly correlated with the reduction potential of the haemoproteins in a manner that is reminiscent of the Marcus theory of electron transfer [34,35]. Deviation from the correlation was observed for the rate of MP11 reduction by diMePH₄ in the presence of CO, which did not follow the reduction potential determined to be -160 mV for the Fe^{III}/Fe^{II} couple in MP11 [36]. As a consequence of this correlation, the rate of $10 \text{ M}^{-1} \cdot \text{s}^{-1}$ would be consistent with a redox potential value of $+100$ mV for the the Fe^{III}/Fe^{II}-CO couple in MP11.

Figure 4 Relationship between haemoprotein redox potentials and reduction rate by diMePH₄

A plot of $\log(k_+)$ versus E_m is shown. A linear correlation was observed for the reduction of several haemoproteins by diMePH₄ under anaerobic conditions [$\log k_+ = -0.49 + (0.0140 \times E_m)$] ($R^2 = 0.96$).

Figure 5 Reduction of cyt *c* by BH₄

The reaction trace observed at 416 nm on mixing 1.2 μ M cyt *c* with 0.36 mM BH₄ in the stopped-flow apparatus is shown. The broken line shows a fit of the trace to a mono-exponential process; $k = 0.29 \text{ s}^{-1}$ ($R^2 = 0.99$). The inset shows a second-order plot for the dependence of the reduction rate on BH₄ concentration; $k_+ = 1.8 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$ ($R^2 = 0.96$).

Reactions of BH₄ with ferricyt *c*

To further document the reduction of haemoproteins by tetrahydropterins, the reaction between cyt *c* and BH₄ was examined and typically illustrated in Figure 5. In the presence of an excess (100–1000 equiv.) of BH₄, a total reduction of ferricyt *c* to its Fe^{II} state was observed. Analyses of the traces to a mono-exponential process allowed us to determine the pseudo-first-order rate constants, k (s⁻¹), which were then plotted against BH₄ concentration. The mean value $\pm$ S.D. for three independent experiments yielded the second-order rate constant for the reduction of cyt *c* by BH₄ of $k_+ = (2.0 \pm 0.3) \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$, close to the value of $(4.4 \pm 0.8) \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$ determined for the reduction of cyt *c* by diMePH₄.

DISCUSSION

The present study reports, for the first time, experimental evidence for the anaerobic reduction of a group of structurally

diverse haemoproteins by tetrahydropterins from the Fe^{III} to the corresponding Fe^{II} state. These data essentially confirm that the electron transfer from tetrahydropterins to Fe^{III}-porphyrins recently described [13] is a very general reaction.

A fast and total reduction process was observed with Fe^{III}-haemoproteins exhibiting positive redox potentials $> 100 \text{ mV}$, such as metHb and cyt *c*, whereas a slow and partial reduction was observed for Fe^{III}-haemoproteins with redox potentials in the range -150 mV to 0 mV , such as CPO and cyt *b*₅. Under similar experimental conditions, in the presence of an excess (100–500 equiv.) of diMePH₄, no reduction of Fe^{III} haemoproteins with redox potentials $\leq -160 \text{ mV}$ was detected, i.e. for catalase, CYP2C5, HRP and MP11. These data indicate that the one-electron reduction potentials of haemoproteins are important parameters defining their reaction with tetrahydropterins. This kind of behaviour is consistent with previous observations that the rate constant for electron transfer will depend on the thermodynamic driving force for the reaction when analysed within the framework of the simple Marcus theory [34,35].

Indeed, a key thermodynamic property to consider is the reduction potential change (ΔE) from which predictions can be made. Polarographic experiments provide evidence that the oxidation of tetrahydropterins occurs via two overlapping one-electron steps [14–16]. The redox potentials of diMePH₄ and BH₄ were found to be rather similar and evaluated to be approx. $+150 \text{ mV}$ [14]. Thus the lack of spontaneous electron transfer within the active site of NOS between BH₄ and Fe^{III}-haem, which would have led to BH₄⁺ and Fe^{II}-haem, is consistent with the negative $\Delta E = (-250 - 150) = -400 \text{ mV}$. In contrast, the NOS-Fe^{II}-O₂ intermediate would have a sufficiently high redox potential to be reduced by BH₄, as discussed in [5].

The reduction of cyt *c* by diMePH₄ is thermodynamically feasible with a positive driving force ($\Delta E = 260 - 150 \text{ mV}$). Interestingly, the reduction rate of metHb was shown to be two orders of magnitude lower, as accounted for by a smaller $\Delta E (= 0)$. Several lines of evidence have emphasized that a negative redox potential change does not make a reaction impossible. Indeed, the cyt *b*₅ reaction with diMePH₄ corresponds to a partial and slow reduction, which nicely agrees with $\Delta E = -150 \text{ mV}$.

In line with our results, when the redox potentials of Fe^{III}-MP11 and diMePH₄ are considered, the large negative redox potential change, $\Delta E = -160 - 150 = -310 \text{ mV}$, indicates that the reduction is thermodynamically unfavourable. However, the complete reduction of Fe^{III}-MP11 was observed in the presence of CO, thus suggesting that the redox equilibrium was shifted as a result of the disappearance of the product Fe^{II}-MP11 by the further reaction of binding of CO, a strong ligand of the Fe^{II} haem, thereby pulling the reaction towards the formation of Fe^{II}·CO·MP11. Previous studies showed that the binding of *N*-acetylmethionine and imidazole to the second axial position for haem iron in microperoxidases, usually occupied by a water molecule, underlined a 70–120 mV upshift in the redox potential of the haem [37,38]. Therefore it is reasonable to conclude that the rate constant determined for the reduction of Fe^{III}-MP11 by diMePH₄ in the presence of CO would be very likely to be consistent with a redox potential for the Fe^{III}-MP11/Fe^{II}·CO·MP11 couple shifted to $E_m = +100 \text{ mV}$.

Comparative studies of tetrahydropterins emphasized that the cofactor binding affinity was dependent on the pterin side-chain structure, i.e. an aliphatic side chain attached to the pterin ring structure at the sixth position in BH₄, the natural cofactor, instead of the presence of methyl groups at the sixth and seventh positions in diMePH₄, a synthetic analogue, both in NOS and amino acid hydroxylases [1,5]. In contrast, BH₄ and diMePH₄ were shown to equally support electron transfer and catalysis in

these enzymes [5]. In the present study, the reduction of cyt *c* by diMePH₄ was only twofold faster than the reduction of cyt *c* by BH₄. Thus diMePH₄ can substitute for BH₄, not only in NOS and amino acid hydroxylase reactions, but also in the reduction of ferric haemoproteins.

Upon release from mitochondria, Fe^{III}-cyt *c*, which exhibits a peroxidase-like activity, participates in processes of cell injury and signalling [39]. The second-order rate constant for the reduction of cyt *c* by BH₄ was shown to be two orders of magnitude larger than that for the reduction by Asc (ascorbate) (15 M⁻¹ · s⁻¹) [40]. In addition, the consideration of the reduction potentials of these species [$E_m(\text{Asc}^{\bullet+}/\text{AscH}) = +330 \text{ mV}$ [41] and $E_m = +150 \text{ mV}$ for BH₄^{•+}/BH₄] indicates that cyt *c* may well be more easily reduced by BH₄ than by Asc. However, the ability of BH₄ to act as a cellular antioxidant will depend on the relative concentrations of the pterin to other low-molecular-mass antioxidants such as Asc and glutathione. The biological relevance of the reduction of cyt *c* by BH₄ remains to be determined.

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