

Generation of carbon monoxide and iron from heme proteins in the presence of 7,8-dihydroneopterin

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Abstract

7,8-Dihydroneopterin and neopterin are secreted by human and primate macrophages after activation by interferon- γ in a ratio of 2:1. 7,8-Dihydroneopterin is known to suppress radical-mediated processes, but it is also able in the presence of iron ions to generate superoxide radical anion and hydroxyl radicals from molecular oxygen. Effects of 7,8-dihydroneopterin were investigated on (met)myoglobin and (met)hemoglobin. Addition of 7,8-dihydroneopterin to heme proteins in air-saturated solution resulted in dose-dependent cleavage of the porphyrin moiety. The liberation of non-heme iron and carbon monoxide originating from the cleaved porphyrin was quantified. Both were generated at equimolar concentrations with a linear correlation coefficient of 0.9. Addition of ferrous iron significantly accelerated the pteridine-mediated cleaving of the porphyrin. However, the total yield of porphyrin cleaved was controlled by the pterin rather than by the ferrous ion concentration. 7,8-Dihydroneopterin is assumed to reduce the heme iron in intact protein molecules, thereby preparing the conditions for binding of oxygen and carbon monoxide as ligands. Beyond that, it is concluded that hydroxyl radicals might be generated via reduction of molecular oxygen to superoxide anion in the autoxidation process and dismutation to hydrogen peroxide and subsequent Fenton reaction. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

Despite the ubiquitous occurrence of pteridine derivatives in all living cells, their biological functions are not yet fully understood. Tetrahydrobiopterin, as an exception, has a clearly defined function as a cofactor in various enzyme systems [1–3]. Considerable clinical interest has been focussed upon neopterin, a highly sensitive indicator of cell-mediated immune activation in many diseases. Neopterin and its reduced form, 7,8-dihydroneopterin, are produced by human macrophages in a ratio 1:2 after activation by interferon- γ [4]. Despite numerous studies showing that neopterin concentrations in body fluids are a valuable indicator for activation of cell-mediated immunity, the biological role of neopterin and 7,8-dihydroneopterin remains unclear.

Pteridine derivatives may play an important role in radical-induced processes. According to their chemical structures, pteridine derivatives act either as scavengers or enhancers of radical-mediated phenomena [5–7]: aromatic pteridines were reported either to enhance, to scavenge or to show no effect at all on radical-mediated processes, while reduced pterins are able to rapidly (i.e., within minutes) scavenge free radicals [8]. Investigations on the effects of neopterin and 7,8-dihydroneopterin on the oxidation of low-density lipoproteins induced by copper(II) ions or 2,2'-azobis-(2-amidino propane) dihydrochloride showed a dramatic increase of the lag time of copper(II)-induced LDL oxidation by 7,8-dihydroneopterin, while neopterin and pterin did not exhibit any effect on this specific system [9]. Furthermore, micromolar concentrations of 7,8-dihydroneopterin can inhibit red blood cell hemolysis induced by 2,2'-azobis(amidino propane) dihydrochloride, hydrogen peroxide or hypochlorite [10]. 7,8-Dihydroneopterin is able to prevent oxidative damage in U937 cells [11].

On the other hand, highly reduced pterin structures such as tetrahydrobiopterin undergo autoxidation under air-satu-

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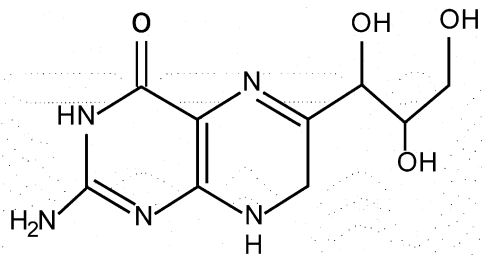


Fig. 1. Chemical structure of 7,8-dihydroneopterin.

rated conditions, thereby generating superoxide radicals. The stability of 7,8-dihydropterins on air is somewhat higher, but their half-life remains also shorter than 1 h [12].

Only recently a slow (i.e., within hours) formation of oxygen radicals in air-saturated solutions of 7,8-dihydroneopterin was reported [13].

Generally, the promotion of hydroxyl-radical formation by different 7,8-dihydropterins depends on their oxidizability [14].

The chemical structure of 7,8-dihydroneopterin is shown in Fig. 1.

The most important oxygen carriers in higher animals and humans, hemoglobin and myoglobin, were chosen to get more information about the role of 7,8-dihydroneopterin in an experimental system generating free radicals or activated species. Heme proteins constitute an important class of biomolecules which are involved in a variety of fundamental functions such as oxygen carriage and electron transfer. They can react with oxygen, hydroperoxides and other oxygen donors. Oxyhemoglobin and oxymyoglobin are known to undergo a slow but spontaneous autoxidation reaction with release of superoxide anion, forming met-hemoglobin and metmyoglobin [15,16]. Other heme proteins, like cytochromes *P450*, are even faster autoxidized [17] and we have recently observed that, for example, cytochrome *P450 cam* from *Pseudomonas putida* is degraded in the presence of aromatic pteridines indicated by a loss of the Soret absorption band (unpublished). Earlier experiments with rat liver microsomes [18] showed that the conversion of aminoantipyrine via the heme protein cytochrome *P450* from liver microsomes was diminished in the presence of aromatic pterins, presumably because of *P450* degradation. Comparison of the difference spectra induced by pterin derivatives with spectra from the reduced and CO-bound form of cytochrome *P450* indicated that carbon monoxide is formed and obviously bound to still intact heme protein molecules. The carbon monoxide must stem from cleaving the porphyrin [19].

The degradation products formed from heme protein degradation are hemin, biliverdin, carbon monoxide, free iron, protein peptides and amino acids [20]. To understand the possible role of pterin in degradation of heme proteins, we studied hemoglobin and myoglobin as representative examples in more detail. In this study, we investigated the effect of 7,8-dihydroneopterin upon heme protein degrada-

tion by measuring the liberation of carbon monoxide and non-heme iron.

2. Materials and methods

2.1. Reagents

7,8-Dihydroneopterin was obtained from Dr. Schirck's laboratory (Jona, Switzerland). Human hemoglobin (H 7379), myoglobin from horse heart (M1882) and catalase (C 9322) were purchased from Sigma (Vienna, Austria). Non-heme iron concentration in the commercial preparations was determined by the bleomycin assay [21] to be below 0.5 nmol per assay. Tris and sodium dithionite were obtained from Merck (Darmstadt, Germany). All solutions were prepared with nanopure water (Millipore, Bedford, MA, USA) under air.

2.2. Spectral analysis

Spectral analysis was performed on a Shimadzu dual wavelength spectrophotometer UV 2401 PC (Shimadzu Europe, Duisburg, Germany).

Heme concentrations were determined at 408 nm, using the extinction coefficient $\epsilon = 1.88 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ [22].

Incubation assays: 20 mg protein per milliliter was dissolved in Tris-buffer, 50 mM, pH 7.4, as a stock solution. 7,8-Dihydroneopterin was dissolved in the Tris-buffer and added to give concentrations of 40, 80 and 160 μM . Ferrous sulfate was added at increasing concentrations (0, 40, 80, 160 μM) with a constant concentration of 7,8-dihydroneopterin (80 μM).

Ten microliters of the protein stock solution was added to a cuvette with Tris-buffer to yield a final volume of 2.0 ml. Spectra were recorded 0, 15, 30, 45, 60, 120, 240 min after 7,8-dihydroneopterin addition and calculated as carbon monoxide-difference spectrum. Concentrations of carbon monoxide were determined by detecting ferrous carbon monoxide complex of myoglobin and hemoglobin as described [23]. Carboxy-hemoglobin and carboxy-myoglobin solutions as controls for the integrity of the remaining protein molecules after 24 h of incubation were prepared as follows: before recording the spectra, a few crystals of dithionite were added and CO was bubbled through the solution for 10 s.

Estimation of non-heme iron was performed with 100 μl from the same protein stock solution in the same time scale with the bleomycin assay [21].

The added ferrous ions were recovered as controls and subtracted from total non-heme iron.

2.3. Computations

The effects of the concentrations of 7,8-dihydroneopterin and ferrous ions on the kinetics of the release of carbon monoxide and non-heme iron were evaluated by fitting to

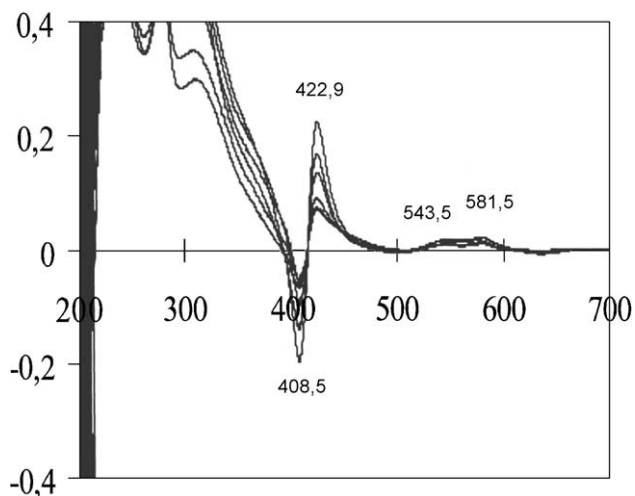


Fig. 2. CO-difference spectra of myoglobin incubated with 160 μM 7,8-dihydroneopterin recorded over 4 h.

the data by an exponential function of the form:

$$a(t) = a_{\text{max}}(1 - e^{-kt}).$$

$a(t)$ is the time-dependent amount of the products yielded per assay (2 ml), a_{max} is the final amount of these products, and k is the rate constant. Fitting of the parameters a_{max} and k was performed using the program “Scientist” (Micro-Math, Salt Lake City, UT, USA).

The comparison of liberated carbon monoxide and non-heme iron incubated with the same concentrations of 7,8-dihydroneopterin and ferrous ions was performed by linear regression and correlation analysis.

3. Results

3.1. Spectral scans

Regarding the spectroscopical scans of the heme protein suspensions, the Soret band was identified at 408 nm. This Soret band decreased after addition of 7,8-dihydroneopterin and even more in the presence of ferrous ions. In parallel, a

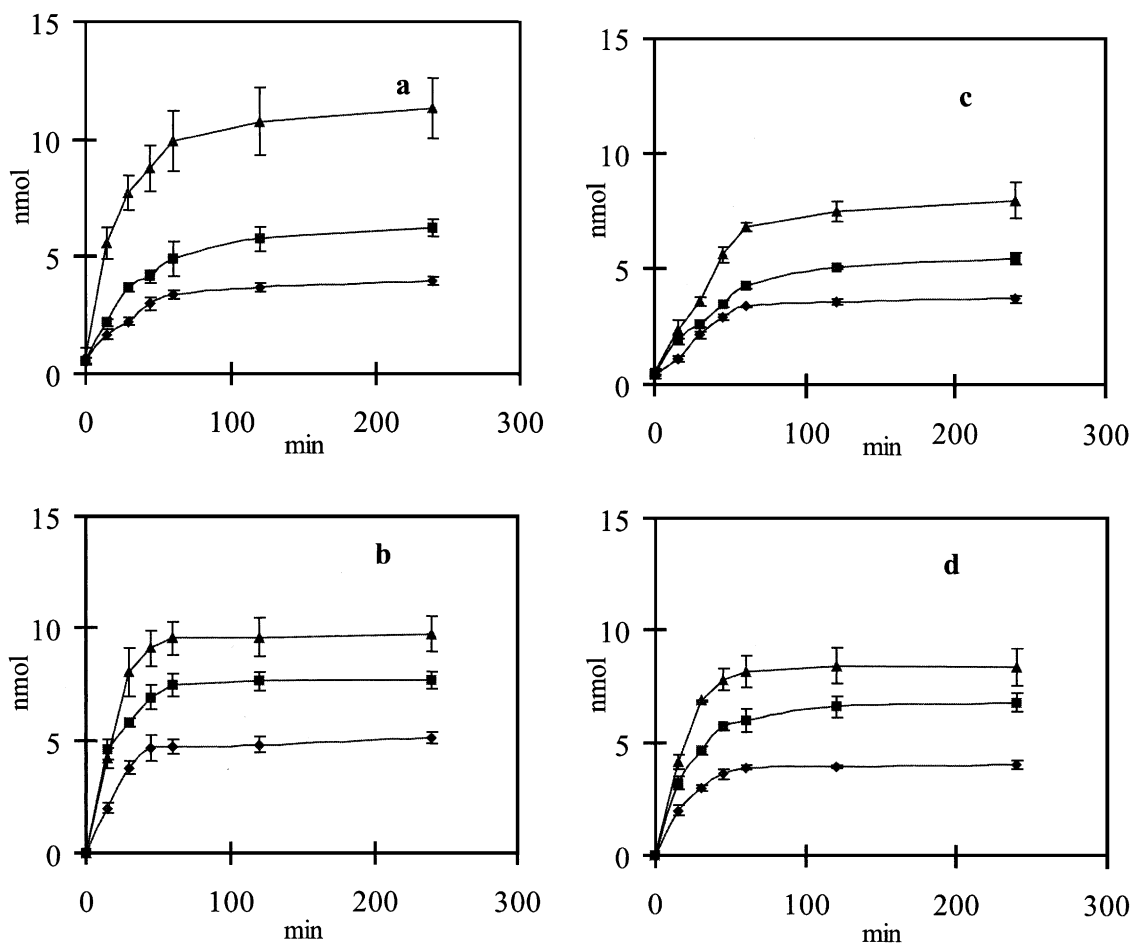


Fig. 3. Kinetic effects of 7,8-dihydroneopterin (rhombus 40 μM , square 80 μM and triangle 160 μM) on the generation of non-heme iron (a: from myoglobin, c: from hemoglobin) and carbon monoxide (b: from myoglobin, d: from hemoglobin).

Table 1

Myoglobin and hemoglobin were incubated with increasing concentrations of 7,8-dihydroneopterin. Liberation of carbon monoxide and non-heme iron from porphyrin of the heme proteins were estimated. Exponential curves were fitted to the data shown in Fig. 3 and their maximum amounts per assay (a_{\max}) and time constants (k) were calculated

7,8-Dihydroneopterin (μM)	40	80	160
Kinetic effects of 7,8-dihydroneopterin on Fe- and CO-liberation from myoglobin			
a_{\max} Fe (nmol)	3.98 ± 0.16	6.33 ± 0.23	11.33 ± 0.35
k (nmol/min)	0.030 ± 0.004	0.030 ± 0.002	0.038 ± 0.004
a_{\max} CO (nmol)	5.08 ± 0.13	7.67 ± 0.12	9.92 ± 0.03
k (nmol/min)	0.042 ± 0.004	0.045 ± 0.004	0.047 ± 0.005
Kinetic effects of 7,8-dihydroneopterin on Fe- and CO-liberation from hemoglobin			
a_{\max} Fe (nmol)	3.90 ± 0.15	5.70 ± 0.17	8.09 ± 0.26
k (nmol/min)	0.028 ± 0.003	0.034 ± 0.002	0.024 ± 0.002
a_{\max} CO (nmol)	4.13 ± 0.05	6.51 ± 0.14	8.67 ± 0.15
k (nmol/min)	0.044 ± 0.020	0.040 ± 0.003	0.047 ± 0.003

shoulder at 423 nm appeared. This shoulder could be identified as the carbon monoxide-bound form of the heme protein. This was verified by reducing a heme protein control sample with dithionite and gassing with carbon monoxide. Simultaneously, the absorbance at 370 nm (porphyrin absorption), and at 280 nm (aromatic amino acids) increased [22]. The spectral change of the cleaved porphyrin and of the aromatic amino acids was not quantified because of the absorption of 7,8-dihydroneopterin between 200 and 350 nm.

Fig. 2 shows the carbon monoxide-difference spectrum for metmyoglobin incubated with 160 μM 7,8-dihydroneopterin over 4 h.

3.2. Effects of 7,8-dihydroneopterin on the cleavage of heme proteins

Fig. 3 shows the dose-dependent liberation of iron and carbon monoxide from the porphyrin in hemoglobin and myoglobin under the influence of three concentrations (40, 80 and 160 μM) of 7,8-dihydroneopterin. The amounts of the liberated products were about 20% higher from myoglobin than from hemoglobin. A significant increase of porphyrin cleaving activity by 7,8-dihydroneopterin was observed during the first 60 min.

To confirm the hypothesis that 7,8-dihydroneopterin may support redox reactions via superoxide radical anion and hydrogen peroxide, the heme proteins were incubated with additional 1000 U catalase per assay (500 U/ml). The decrease of the Soret band and subsequently the increase of the CO-bound form were extremely inhibited to less than 1 nmol during an observing period of 4 h.

The effects of 7,8-dihydroneopterin kinetics on the generation of the porphyrin products carbon monoxide and non-heme iron were evaluated from the data in Fig. 3. From these fits, time constants and maximum concentrations were

calculated. 7,8-Dihydroneopterin in the assay causes the maximal formation of the porphyrin products within approximately 1 h (Table 1). The kinetic rate constant k is not significantly influenced by the addition of increasing pterin concentrations.

3.3. Addition of ferrous ions

In the presence of 7,8-dihydroneopterin, ferrous ions (FeSO_4) significantly increases the generation of carbon monoxide and non-heme iron in a dose-dependent way. Added ferrous ions without the pteridine in the assay showed a weak effect (less than 2 nmol per assay after 4 h) on the liberation of porphyrin products. In Fig. 4, the effect of increasing concentrations of ferrous ions in the presence of a moderate dihydroneopterin concentration (80 μM) on the liberation of CO from myoglobin is compared.

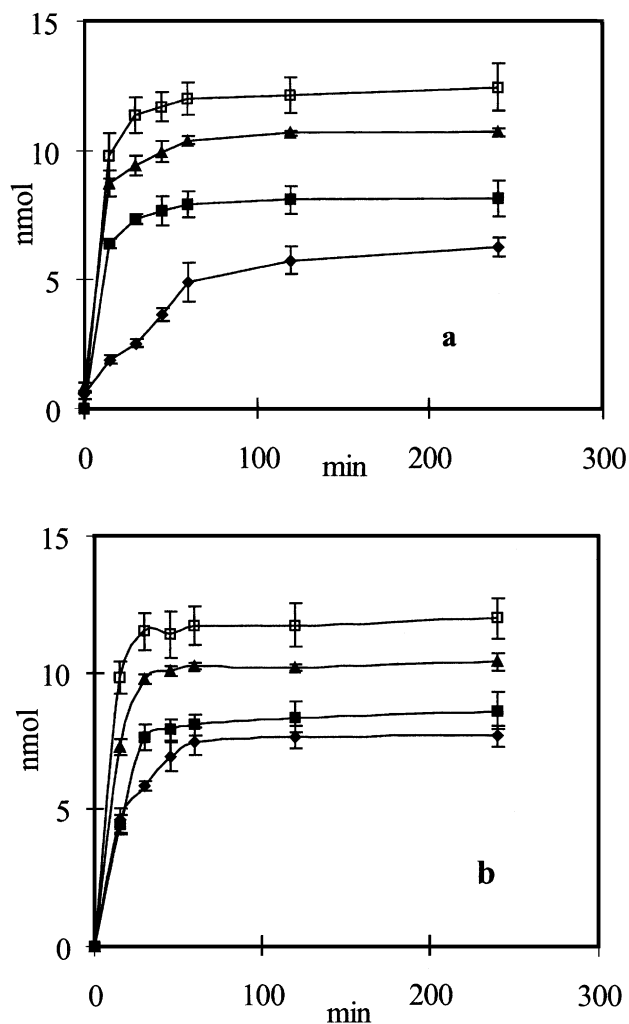


Fig. 4. Kinetic effects of FeSO_4 on the liberation of non-heme iron (a) and carbon monoxide (b) from myoglobin in the presence of a constant concentration of 7,8-dihydroneopterin (80 μM). The concentrations were: filled rhombus: 0/80, filled square: 40/80, filled triangle: 80/80, open square: 160/80, μM pteridine/ FeSO_4 .

Table 2

Myoglobin and hemoglobin were incubated with different concentrations of ferrous ions with 80 μM 7,8-dihydroneopterin. Liberation of carbon monoxide and non-heme iron from porphyrin of the heme proteins were estimated. Exponential curves were fitted to the data shown in Fig. 4 and their maximum amounts per assay (a_{max}) and time constants (k) were calculated

Fe^{2+} (μM)	0	40	80	160
7,8-Dihydroneopterin (μM)	80	80	80	80
Kinetic effects of 7,8-dihydroneopterin and ferrous ions on Fe- and CO-liberation from myoglobin				
a_{max} Fe (nmol)	6.33 ± 0.23	8.01 ± 0.09	10.43 ± 0.02	12.16 ± 0.13
k (nmol/min)	0.030 ± 0.002	0.100 ± 0.008	0.108 ± 0.017	0.104 ± 0.008
a_{max} CO (nmol)	7.67 ± 0.12	8.56 ± 0.18	10.36 ± 0.07	11.84 ± 0.12
k (nmol/min)	0.045 ± 0.004	0.082 ± 0.003	0.121 ± 0.003	0.116 ± 0.009
Kinetic effects of 7,8-dihydroneopterin and ferrous ions on Fe- and CO-liberation from hemoglobin				
a_{max} Fe (nmol)	5.70 ± 0.17	8.62 ± 0.12	10.71 ± 0.06	11.60 ± 0.03
k (nmol/min)	0.034 ± 0.002	0.096 ± 0.005	0.109 ± 0.006	0.112 ± 0.002
a_{max} CO (nmol)	6.51 ± 0.14	9.45 ± 0.17	11.28 ± 0.06	13.51 ± 0.16
k (nmol/min)	0.040 ± 0.003	0.103 ± 0.004	0.126 ± 0.020	0.115 ± 0.011

In contrast to 7,8-dihydroneopterin, ferrous ion addition also shows a distinct effect on the kinetic rate constant to the reaction (Table 2).

3.4. Correlation between the liberated non-heme iron and carbon monoxide

In Fig. 5, the liberation of CO versus non-heme iron from myoglobin is compared by linear regression analysis: a good correlation exists between the formation rate of both cleavage products. The linear correlation coefficient is 0.89. Importantly, the liberation of both products is essentially equimolar, indicated by a regression coefficient of 0.829 [95% confidence interval (CI) from 0.65 to 1.00]. The intercept is 1.95 [95% CI from 0.43 to 3.47].

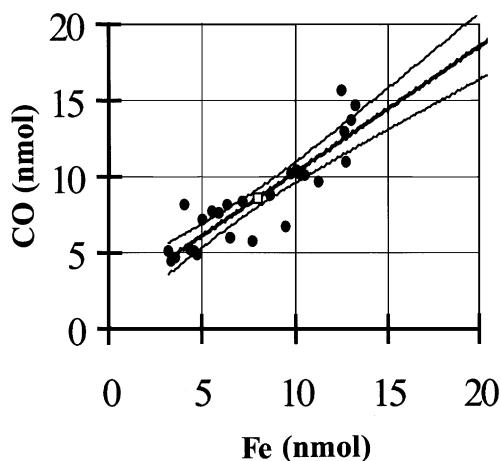


Fig. 5. Linear regression analysis of carbon monoxide versus iron liberation from myoglobin in the presence of 7,8-dihydroneopterin. The regression line is shown with the 95% confidence interval.

4. Discussion

7,8-Dihydroneopterin in lower concentrations is known to act as a scavenger of radical-mediated reactions on a short time scale (within minutes) [5–7]. This effect is attributed to a chemical reduction of the active species, and the pteridine compound is oxidized to xanthopterin [13]. High concentrations above 1 mM, however, were reported to enhance radical activity [24]. A recent study demonstrated that 7,8 dihydroneopterin in a concentration of only 50 μM is able to yield hydroxylation products of salicylic acid via generation of free hydroxyl radicals from oxygen in a simple chemical assay. This reaction is accelerated by the presence of iron ions [13,14]. Obviously, the effect of dihydroneopterin is comparable to the role of ascorbate as a reducing agent which reduces redox-active metals such as iron, thereby increasing the pro-oxidant chemistry of these metals [25].

We found that the presence of 7,8-dihydroneopterin leads to a reduction of Fe^{3+} to Fe^{2+} in the heme proteins which allows molecular oxygen to bind. At the same time, 7,8-dihydroneopterin in concentrations higher than 40 μM supports also the cleavage of the porphyrin ring in (met)myoglobin and (met)hemoglobin, reflected in the decrease of the Soret band amplitude. The porphyrin cleavage products carbon monoxide and non-heme iron appear in equivalent concentrations with good correlation. The formed carbon monoxide binds to the reduced heme protein and produces the specific spectral changes observed. Lower concentrations of 7,8-dihydroneopterin (5, 10, 20 μM , data not shown) failed to yield carbon monoxide and non-heme iron.

The rate of generating carbon monoxide and non-heme iron is significantly increased in the presence of added ferrous iron which suggests that similar reactive intermediates are relevant in the pteridine as well as ferrous iron-mediated degradation. Hydrogen peroxide formed from superoxide anion released by autoxidation of the oxy-heme protein might be such intermediate. Hydrogen peroxide may react with heme proteins in the oxy or the ferric state thus

forming the ferryl state [20]. The reaction of the ferryl heme protein with an additional hydrogen peroxide molecule leads to degradation of the porphyrin ring [26]. With proceeding reaction via the heme autoxidation process, however, the formed CO inhibits further degradation (product inhibition). In the case of added ferrous sulfate, the well-known Fenton reaction with hydrogen peroxide occurs yielding OH radicals which also destroy the heme protein.

An alternative or additional source of the required hydrogen peroxide may be the direct reaction of the pteridine with molecular oxygen to generate superoxide radical anion and hydroxyl radicals. In fact, *in vitro* experiments [13,14] have provided ample evidence that the radical enhancing effect of 7,8-dihydroneopterin is achieved by its capacity to reduce ferric to ferrous ions. Thus, 7,8-dihydroneopterin may be regarded as a potent electron-generating system, facilitating the iron-catalysed series of redox reactions leading from molecular oxygen via superoxide radical anion and hydrogen peroxide finally to hydroxyl radicals, one of the most aggressive free radical species. In other words, 7,8-dihydroneopterin may function as electron source in the sense of a co-substrate of the reactions.

As very low concentrations of the pterin in the assay cause only negligible effects on porphyrin degradation, 40 μM of 7,8-dihydroneopterin seems to be the threshold value, whereby the kinetic rate at higher concentrations of ferrous ions exceeds the low rate of 0.03 up to 0.100 nmol/min. Macrophages also generate superoxide upon activation in concentrations as high as 100–600 μM [15]. Neopterin and 7,8-dihydroneopterin are generated simultaneously with this oxidative burst of a macrophage, probably enhancing these radical-induced processes, even with higher kinetic rates in the presence of iron ions. The formation of methemoglobin and metmyoglobin happens rapidly and at a constant rate of 1–3% *in vivo* [27]. These ferric heme proteins serve as a source for degradation products as hemin, biliverdin, and iron [20].

Our findings could provide additional explanation for hitherto unexplained observations: high neopterin and 7,8-dihydroneopterin levels, observed in patients with anemia associated with chronic immune stimulation, correlate well with the degree of anemia [28,29]. One might speculate, that the activated macrophages in these patients produce free radicals and 7,8-dihydroneopterin over a longer period. In the presence of ferrous iron which is present in blood and cells, this combination of 7,8-dihydroneopterin and oxidative species according to our results might contribute to degradation of the porphyrin in heme proteins, and thus enhance the disturbances of iron homeostasis in such patients.

A problem with this hypothesis is the fact that the concentrations of 7,8-dihydroneopterin used in our experiments were much larger than those seen in body fluids. We can only speculate that the local 7,8-dihydroneopterin concentrations in the vicinity of stimulated macrophages may be much higher than the levels in the circulating blood.

Unfortunately, according to our knowledge, there are no data available on the actual concentration of 7,8-dihydroneopterin within inflammatory sites [11].

Therefore, many putative biological effects such as degradation of heme proteins caused by endogenously synthesised 7,8-dihydroneopterin still remain to be proven. In particular, further experiments involving superoxide dismutase and/or chelating agents seem to be promising.

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