

Influence of magnetic field on enzymatic ONOO^- production

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Abstract. Enzymatic oxidation of L-arginine catalyzed by inducible nitric oxide synthase gives nitric oxide as the main product and superoxide anion as a side reaction product. Recombination of these radicals gives a very reactive species – peroxynitrite, which is involved in many biochemical processes. In the current work it was shown that such a system can be a usable model system for investigating the influence of magnetic field on enzymatic peroxynitrite formation. Using a selective fluorescent probe for peroxynitrite - coumarin boronic acid and an adopted for the experimental purpose incubation mixture, magnetic field experiments have been done at 11.7T. The averaged magnetic field effect is equal to $2.8 \pm 0.9\%$.

1. Introduction

In our previous work [1] we have shown that the recombination of nitric oxide (NO) and superoxide anion ($\text{O}_2^{\bullet-}$) that forms peroxynitrite (ONOO^-) is magnetosensitive. The radical pairs were formed by decomposition of 3-morpholinocydonimine (SIN-1) in aqueous buffered solution at pH=7.6 (1).



As described in numerous studies (see, e.g., [2]) magnetic field can affect spin dynamic in radical pairs and thus the yield of the spin-selective recombination product. The measured magnetic field (MF) effect in the system of SIN-1 in the field of 18 T equals $5.5 \pm 1.6\%$ [1].

The purpose of current work is trying to detect this effect in a more complicated biochemical system, where the radical pairs are formed during enzymatic oxidation of L-arginine catalyzed by inducible nitric oxide synthase (iNOS) (2).



Nitric oxide synthases (NOS) are a family of enzymes catalyzing the production of NO from L-arginine (L-arg) [3] (3).



NO can be produced by nervous tissue (by neuronal NOS), by endothelium (endothelial NOS), and by macrophages (inducible NOS) [3]. For the purpose of this project inducible NOS was chosen, since iNOS is producing 100-1000 times more NO than other NOS isoforms. This allows NO



detection at lower NOS concentration. This is important as NOS is thermally unstable and quite expensive.

NOS isoforms also catalyze other leak and side reactions, such as superoxide production. For all these isoforms superoxide production has also been detected [4-6]. So, in the catalytic cycle of NOS diffusion radical pairs $\text{NO}/\text{O}_2^{\bullet-}$ are generated.

2. Preliminary experiments

2.1. Optimization of incubation mixture

The main experimental challenge was to measure the yield of peroxynitrite formed as a result of enzymatic oxidation of L-arg. For this purpose the composition of iNOS incubation mixture was optimized, since most of the components are potential targets for peroxynitrite (including the buffer – HEPES [7]). Step by step these components (such as tetrahydrobiopterin BH4, dithiothreitol DTT, NADPH, and HEPES) were excluded or varied checking iNOS activity. The activity of NOS was detected by measuring the rate of NO formation. This was done by the hemoglobin method [8], which is based on the reaction of NO with oxyhemoglobine to form the oxidized form of hemoglobine (methemoglobine). Oxyhemoglobine has a characteristic absorption band, so we can observe the decreasing optical density of oxygemoglobine at its characteristic maximum with time. The slope of this curve gives the rate of NO formation in the incubation mixture. It was estimated as 0.1-0.2 mkM/min. Checking the activity of iNOS at each step, it was decided to exclude from the incubation mixture BH4 and DTT, which is usually added to prevent oxidation of BH4. Also the concentration of NADPH was decreased and the concentration of Mg^{2+} was optimized in the new buffer (decreased) without a serious loss of NOS activity. HEPES buffer was changed to PBS. So, the new optimized incubation mixture for MF experiments on the NOS system was as follows: PBS (50 mM, pH=7.4), DTPA (50 mkM), MgCl_2 (4.5 mM), iNOS (1-3 U/mL), L-arginine (1 mM), NADPH (50 mkM).

2.2. Peroxynitrite detection

In our work [1] peroxynitrite was detected using its reaction with dihydrorhodamine-123 DHR-123 and measuring the specifically colored product spectrophotometrically. This is not possible in the new system, since peroxynitrite readily reacts with NADPH ($k=4 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$) [9] (rate constant for DHR-123 is $8.2 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$ [10]). Therefore, for peroxynitrite detection coumarin boronic acid (CBA), a more specific probe, was used. CBA reacts with peroxynitrite with rate constant $k=1 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ forming hydroxycoumarin COH [11] that has a characteristic fluorescence band ($\lambda_{\text{excit}}=322 \text{ nm}$, $\lambda_{\text{emit}}=455 \text{ nm}$, Figure 1).

In order to estimate the yield of peroxynitrite formed during the enzymatic reaction, COH signal was calibrated as follows. To a known amount of CBA (25 mkM) an excess amount of SIN-1 (150 and 500 mkM) was added. The produced yield of COH, equal to 25 mkM, gives the fluorescence intensity reaching 700 mV. During the enzymatic oxidation the COH signal was rising up to 150 mV. So, the estimated yield of peroxynitrite is about 5 mkM. Taking into account the fact that the error of fluorescence signal measurements is about 0.5%, the yield of peroxynitrite should be enough to see a MF effect about 2-3% that is expected in this system.

Using the CBA probe the effect of NOS concentration on the rate of COH formation was investigated. It was found that NOS concentration affects the initial rate of peroxynitrite formation and also the running time of peroxynitrite formation. Beyond a certain NOS concentration a plateau was reached. This fact can be explained by autoinhibition effect. So, for better experimental conditions, the concentration of NOS of 1-3 U/mL was chosen. This concentration is enough to produce peroxynitrite yield with good fluorescent signal of COH (not being at the plateau). The running time at this concentration is about 1.5 hours.

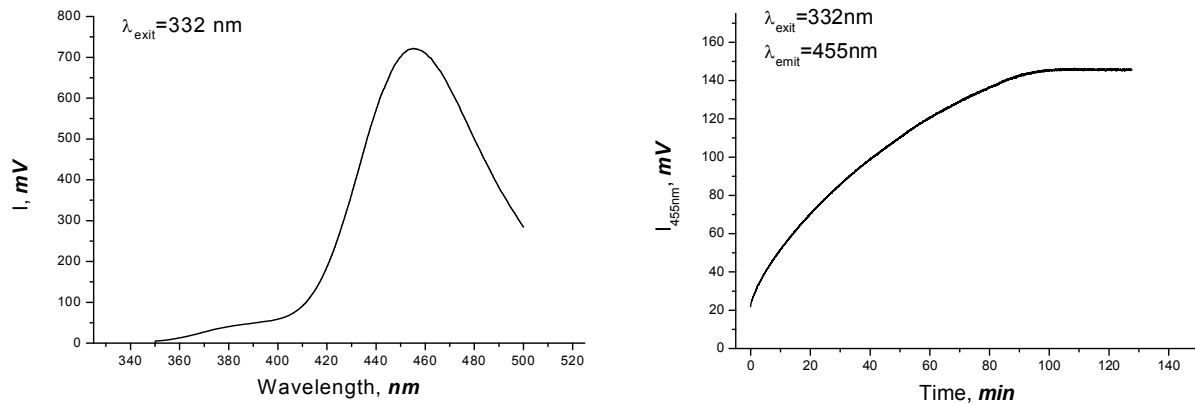


Figure 1. Left panel: fluorescence spectrum of COH, the product of CBA oxidation ($\lambda_{\text{excit}}=332 \text{ nm}$, $\lambda_{\text{emit}}=455 \text{ nm}$). Right panel: kinetics of COH formation in NOS incubation mixture during CBA oxidation by peroxynitrite (PBS (50 mM, pH=7.4), DTPA (50 mM), MgCl_2 (4.5 mM), iNOS (1.5 U/mL), L-arginine (1 mM), NADPH (50 mM), CBA (25 mM). $T=T_{\text{room}}$.

3. Materials and methods

Reagents: Standard phosphate buffer solutions of pH 7.6 were prepared from deionized water, K_2HPO_4 , and KH_2PO_4 (99.0 %, Fluka). To eliminate traces of transition metal ions, diethylenetriamine pentaacetic acid (DTPA, 99.0 %, Fluka) was added at a concentration of 50 mM. Inducible NOS (Cayman Chemicals) was stored at -70°C . Stock 1 mM solutions of SIN-1 (3-morpholinomethylsydnimine hydrochloride, Invitrogen) in pH 7.6 buffer was also stored at -20°C after preparation. Oxyhemoglobine was prepared from Hemoglobine (Sigma) as described in ref. [8]. For quantifying oxyhemoglobine characteristic absorption maxima ($\epsilon_{577 \text{ nm}}=15.4 \text{ mM}^{-1}\text{cm}^{-1}$) was used [8]. CBA (Cayman Chemicals) 1 mM stock solution in DMSO was stored at -20°C .

The absorption spectra were taken on a Shimadzu UV-2401 UV/Vis spectrometer. The fluorescent measurements were carried out on a Hitachi MPF4 fluorimeter or Perkin Elmer Victor X3 2030 Multilabel Reader.

4. Magnetic field experiments

MF experiments were performed in the magnet of a high field MRI scanner (11.7 T) Bruker BioSpec 117/16. To control the temperature two identical thermostats were built. One was placed inside the magnet, and the other was left in the room for the control sample. Temperature stabilization was achieved by pumping water through the two thermostats connected in parallel. The thermostats were checked before the experiments, the temperature inside them was equal to within 0.2°C .

MF experiments were carried out using two groups of samples (for incubation in the magnet and for control) that were prepared from the same incubation mixture. Each group comprised 8 samples (16 altogether). Incubation time was 3 hours.

For the fluorescence measurements in MF experiments a multi-channel fluorimeter was used, which can measure all the samples (one by one at the fluorescence maximum) in a very short time. One run of such measurements takes only about 10 sec. For reliability, the samples were placed at different positions over the plate (a type of plate formed by individual strips) and then the results were averaged. The volume of each sample was 200 μL .

Magnetic field effect was calculated as $\varphi = \frac{\bar{I}_m - \bar{I}_c}{\bar{I}_c}$, where $\bar{I}_m$ and $\bar{I}_c$ are averaged over eight samples fluorescence intensities for samples incubated in magnetic field and controls, respectively. Ten MF experiments have been done in the field 11.7 T (Figure 2 (a)). The error of the measured

$$S_{\varphi} = \frac{S}{\sqrt{n}}$$

effect was calculated as the standard error of the mean $S_{\varphi} = \frac{S}{\sqrt{n}}$, where n is the number of experiments in the field 11.7 T. Several otherwise identical experiments in the bore of the magnet that was quenched have also been done. The results are shown in Figure 2 (b). Significance of the magnetic field effect was estimated by two-way ANOVA with factors “magnet” and “day of experiment”.

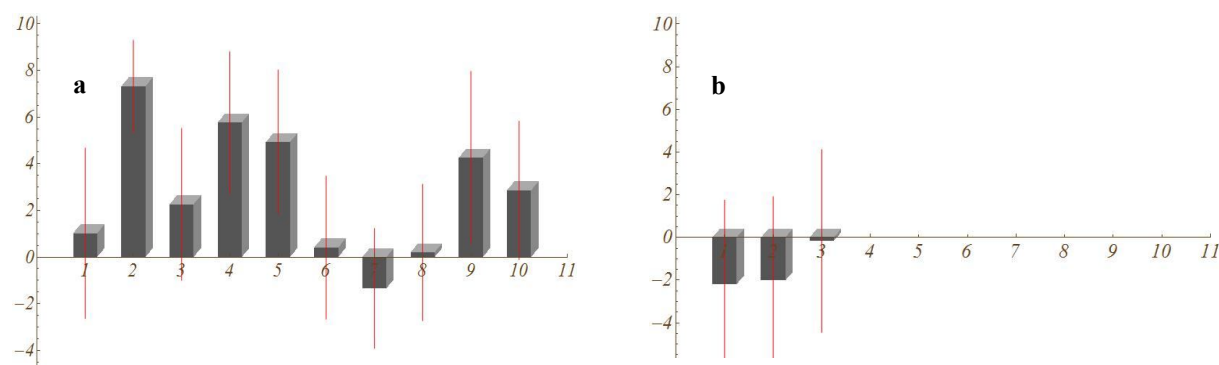


Figure 2. (a) Results of 10 MF experiments: (1.0±3.7)%, (7.3±2.0)%, (2.3±3.3)%, (5.8±3.0)%, (4.9±3.0)%, (0.4±3.1)%, (-1.3±2.6)%, (0.2±2.9)%, (4.3±3.7)%, (2.9±3.0)%. The averaged magnetic field effect is equal to (2.8±0.9)%. **(b)** Results of zero field experiments: (-2.2±4.0)%, (-2.0±3.9)%, (-0.2±4.3)%. The averaged effect is equal to (-1.5±0.6)%.

5. Results and Discussion

In continuation of the work [1] experiments to search for the magnetic field effect on the more complex biochemical system of enzymatic oxidation of L-arginine catalyzed by inducible NO-synthase were prepared and carried out. As a result of this reaction, as in the case of decomposition of 3-morpholinosydnonimine, radical pairs NO and $O_2^{\bullet -}$ are formed, promising to give a magnetically sensitive recombination to form peroxynitrite. The expected magnetic effect is 2-3% of the yield of peroxynitrite.

In order to make possible measurements of the yield of peroxynitrite, the composition of iNOS incubation mixture was optimized. Potential targets for peroxynitrite were excluded or varied without a serious loss of NOS activity. For detection of peroxynitrite a selective fluorescent probe - coumarin boronic acid (CBA) was used.

In the current work ten magnetic field experiments have been done at 11.7 T. The averaged magnetic field effect has been calculated, which is equal to 2.8±0.9% ($F_{1,142}=35.9$, $p<0.001$). Control experiments at zero field give the result -1.5±0.6% ($F_{1,42}=2.7$, $p>0.1$). This may indicate a probable magnetic field sensitive step in the enzymatic formation of peroxynitrite.

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