Ultrafast dynamics of heme distortion in the O₂-sensor of a thermophilic anaerobe bacterium

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Supplementary Fig. S1. Equilibrium absorption spectra of Ct H-NOX in various coordination states. (a) Thawed Ct H-NOX (Fe$^{2+}$ – O$_2$) and reduced (Fe$^{2+}$) species, together with the calculated difference. (b) NO-bounded (Fe$^{2+}$ – CO). (c) NO-bounded (Fe$^{2+}$ – NO). (d) Oxidized form of Ct H-NOX (Fe$^{3+}$) and NO-bounded (Fe$^{3+}$ – NO). See Experimental procedures for the preparation. Optical path length = 1 mm. In panel a, the blue portion of the spectrum represents the range probed by time-resolved absorption.
**Supplementary Table S1.** Peak positions in equilibrium absorption spectra of *Ct* H-NOX.

| Proteins | Coordination | Soret band (nm) | Q-band (nm) |
|----------|--------------|-----------------|-------------|
|          |              |                 |             |
| **Unliganded** |               |                 |             |
| sGC      | Fe(II) 5c-His| 431.5           | 555         |
| sGC      | Fe(III) 5c-His| 393          | 550         |
| *Ct* H-NOX | Fe(II) 5c-His| 431 – 432     | 558 – 570   |
| *Ct* H-NOX | Fe(III) 6c-His-(H₂O) | 409      | 548 – 589   |
| *Ct* H-NOX | Fe(III) 5c-His transient | 390 – 400 | –           |
|          |              |                 |             |
|          |              |                 |             |
| **Heme-NO complex** | |                 |             |
| sGC      | Fe(II) 5c-NO| 398            |             |
| *Ct* H-NOX | Fe(II) 6c-NO| 420            | 550 – 572   |
| *Ct* H-NOX | Fe(III) 6c-NO| 424.5       | 538 – 572   |
|          |              |                 |             |
|          |              |                 |             |
| **Heme-O₂ complex** | |                 |             |
| *Ct* H-NOX | Fe(II) 6c-O₂| 416            | 555 – 590   |
| Mb       | Fe(II) 6c-O₂| 420            |             |
Supplementary Fig. S2. Dynamics in ferrous O$_2$-liganded Ct H-NOX. (a) Raw transient difference absorption spectra (spectra at positive delay minus spectrum at negative delay) after the photodissociation of O$_2$ from ferrous heme at increasing time delay. The wavelengths at which kinetics were analyzed (panels c and d) are indicated. (b) Spectrum at 2.5 ps from which the contribution of dissociated O$_2$ (bleaching) was removed by adding the equilibrium absorption spectrum of O$_2^-$-liganded Ct H-NOX (red). (c and d) Kinetics at particular wavelengths (see panel a) fitted to a sum of two exponentials with time constants in two time ranges (Supplementary Table S2).
**Supplementary Table S2.** Time constants of the fitted raw kinetics for photodissociated O$_2$–Ct H-NOX.

| Wavelength (a) (nm) | $\tau_1$ ($A_1$) ps (%) | $\tau_2$ ($A_2$) ps (%) | $A_{\text{constant}}$ (%) |
|---------------------|--------------------------|--------------------------|---------------------------|
| 400                 | 5.2 (75)                 | 58 (18)                  | 7                         |
| 410                 | 4.7 (74)                 | 114 (3)                  | 23                        |
| 420                 | $\sim$1 (22)            | 54 (10)                  | 68                        |
| 430                 | 6.7 (24) $\sim$1 (3)    | 53 (7)                   | 66                        |
| 435                 | 5.9 (31) $\sim$1 (12)   | 60 (6)                   | 51                        |
| 440                 | 5.2 (50) $\sim$1 (29)   | 44 (5)                   | 16                        |

(a) These wavelengths do not correspond to the absorption maximum of the different species, but to wavelengths at which species can be probed efficiently in the transient spectra.
Supplementary result and discussion.

**Excited states relaxation of unliganded *Ct* H-NOX sensor.**

We recorded the heme excited states relaxation of ferrous unliganded *Ct* H-NOX (Supplementary Fig. S3). An instantaneous bleaching appears at 430.5 nm which decreases and is stabilized at 17 ps, centered at 428 nm, whereas the maximum of the induced absorption (449 nm) fastly shifts and decreases. Simultaneously, the isosbestic point shifts from 443 nm (at 1 ps) to 439 nm (at 7 ps), then shifts back to 445 nm after ~20 ps (Supplementary Fig. S3a), indicating the occurrence of at least two processes. We analyzed the time-wavelength data matrix using the Singular Value Decomposition method which yielded individual spectral components (Supplementary Fig. S3b). The associated kinetics (Supplementary Fig. S3c) comprise two fast exponential components (\( \tau_1 = 2 \pm 0.1 \) ps and \( \tau_2 = 5.1 \pm 0.2 \) ps). They are assigned to excited states decay and heme vibrational cooling, as similarly observed in other ferrous heme proteins excited either in the Q-bands\(^1,^2,^3\) or in the Soret band\(^4,^5\) and are well established as the "classical route" for heme electronic relaxation.

![Graph](image)

**Supplementary Fig. S3.** Excited states dynamics of ferrous unliganded *Ct* H-NOX. (a) Difference absorption spectra of the ferrous unliganded sensor at increasing time delay after photo-excitation at 564 nm. (b) Spectral components from Singular Value Decomposition of the time-wavelength data matrix. The relative absorbance is obtained by multiplying with the respective singular value. (c) SVD kinetic components with time constants of the fitted exponentials.

Importantly here, after the decrease of the induced absorption (440 – 460 nm) from excited states, the induced bleaching at 428 nm does not reach the original baseline. The electronic excited heme does not relax to the initial absorption of unliganded ferrous *Ct* H-NOX after 200 ps, even though the heme ground state is 5-coordinate (5-c) so that no ligand can be photodissociated. The spectral component SVD1 represents the formation of this final species which occurs simultaneously to the vibrational relaxation with time constant \( \tau_2 = 5.1 \)
ps. Since the absorption decrease is centered at 428 nm, the hypothesis of photo-oxidation should be considered for this process. However, no induced absorption band appears in the region 393 – 405 nm where the 5-c ferric heme absorbs, or at any other wavelengths, once the induced absorption vanished after 17 ps (Supplementary Fig. S3a) and photo-oxidation can be discarded as the mechanism.

The present result means that photo-excitation of the Ct H-NOX unliganded ferrous heme transiently changes its conformation but does not change its redox state, so that its absorption spectrum is close to that of the initial ferrous ground state, but with a lower absorption coefficient, which can originate from a change of orbitals overlap due to a change of heme distortion.

**Supplementary Fig. S4.** Spectral components from Singular Value Decomposition analysis of the time-wavelength data matrix of Ct H-NOX–O₂ measured to 5 ns.
Supplementary Table S3. Time constants of the O$_2$ geminate rebinding for various heme proteins.

| Protein       | Function                        | $\tau_{G1}$ ($A_1$) ps (%) | $\tau_{G2}$ ($A_2$) ps (%) | $A_{\text{constant}}$ (a) (%) | Reference |
|---------------|--------------------------------|-----------------------------|-----------------------------|------------------------|-----------|
| Ct H-NOX      | Sensor (putative O$_2$)         | 5.1 (36)                    | 100 (3)                     | 61                     | this work |
| L16A-Cyt c'   | NO transporter                 | 7.5 (27.5)                  | 120 (22) 2000 (45.3)        | 5.2                    | (7)       |
| Myoglobin     | O$_2$ transporter               | 6.3 (28.5)                  | 291 (6)                     | 65.5                   | (8)       |
| HbI           | H$_2$S transporter              | 6.0 (20.5)                  | 396 (18)                    | 61.5                   | (8)       |
| HbII-III      | H$_2$S transporter              | 5.8 (72)                    | 100 (5)                     | 23                     | (8)       |
| DosH          | O$_2$ Sensor                    | 5.3 (96)                    | –                           | 4                      | (9)       |
| FixL          | O$_2$ Sensor                    | 4.7 (90)                    | –                           | 10                     | (9)       |
| Af GcHK (globein-coupled histidine kinase) | O$_2$ Sensor | – | 2000 – 3000 (> 90) | < 10 | (10) |

(a) The constant term $A_C$ quantifies the amount of O$_2$ which exits the protein and does not rebind geminately.
(b) From the bacterium *Alcaligenes xylosoxydans* Leu16Ala mutant. The wild type protein does not bind O$_2$.
(c) From horse heart.
(d) From the mollusc *Lucina pectinata*.
(e) From the bacterium *Escherichia coli*.
(f) From the bacterium *Bradyrhizobium japonicum*.
(g) From the bacterium *Anaeromyxobacter* sp.
Supplementary Table S4. Time constants of the NO geminate rebinding for various heme proteins.

| Protein          | Function                      | $\tau_{G1}$ ($A_1$) ps (%) | $\tau_{G2}$ ($A_2$) ps (%) | $A_{\text{constant}}$ (a) (%) | Reference       |
|------------------|-------------------------------|----------------------------|----------------------------|--------------------------------|-----------------|
| Ct H-NOX         | Sensor (putative $O_2$)       | 6.8 (78)                   | 200 (4)                    | 18                             | this work       |
| Cyt c' (b)       | NO transporter                | 7.5 (99)                   | –                          | 1                              | (11)            |
| sGC (c)          | NO receptor                   | 7.5 (97)                   | –                          | 3                              | (1)             |
| Myoglobin (d)    | $O_2$ transporter             | 13 (40)                    | 148 (50)                   | 10                             | (8)             |
| Myoglobin (d)    | Ferric heme                   | 24 (14)                    | 208 (48)                   | 38                             | (8)             |
| HbI (e)          | $H_2S$ transporter            | 8.0 (36)                   | 90 (62)                    | 2                              | (8)             |
| HbII-III (e)     | $H_2S$ transporter            | 11 (83)                    | 61 (15)                    | 2                              | (8)             |
| DosH (f)         | $O_2$ Sensor                  | 5.0 (85)                   | 20 (15)                    | 0                              | (12)            |
| FixL (g)         | $O_2$ Sensor                  | 5.3 (62)                   | 20 (24)                    | 6                              | (9)             |
| E. Coli YddV    | $O_2$ Sensor                  | 90 (25)                    | 650 (45)                   | 30                             | (13)            |

(a) The constant term $A_C$ quantifies the amount of NO which exits the protein and does not rebind geminately.
(b) From *Alcaligenes xylosoxydans*.
(c) From bovine lung.
(d) From horse heart.
(e) From the mollusc *Lucina pectinata*.
(f) From the bacterium *Escherichia coli*.
(g) From the bacterium *Bradyrhizobium japonicum*.

Supplementary Table S5. Ratio of the singular values of SVD1 and SVD2 components associated with heme structural relaxation and ligands binding, respectively.

| Diatoms | SVD1 / SVD2   | $K_D$ (M)       |
|---------|---------------|-----------------|
| $O_2$   | 0.958 / 0.143 | $10^{-8}$       |
|         | $\gamma = 6.7$|                 |
| NO      | 0.324 / 0.162 | $2.3 \times 10^{-11}$ |
|         | $\gamma = 2$ |                 |
| CO      | $\gamma < 1$  | $1.6 \times 10^{-7}$ |

For $O_2$ we used the value of $k_{\text{on}}$ that we have measured here by time-resolved spectroscopy and the value of $k_{\text{off}}$ in Wu et al.\textsuperscript{14}
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