Electronic Supplementary Information

ESI 1. Materials

The immobilizing material was well-ordered kaolinite KGa-1b from The Clay Minerals Society (Source Clays Repository, University of Missouri, Columbia, MO), completely sodium exchanged as reported in reference (1) (Nak, hereafter). The M80A mutant of *S. cerevisiae* iso-1-cytochrome *c* was produced as extensively described elsewhere.[2] This variant carries an alanine residue replacing the axial heme iron methionine ligand.[2]

Buffer solutions at pH 3.5 were prepared from HAc glacial; sodium phosphate buffer solutions at pH 7 and I = 5 mM were obtained from Na_2HPO_4 and NaH_2PO_4; the stock of buffer solutions at pH 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5 and at pH 7, 7.5, 8, 8.5, 9 were obtained adding $HClO_4$ or NaOH respectively to the buffer solutions at pH 7. All chemicals were reagent grade: HAc glacial from Riedel-de Haën; pellets of NaOH 99%, NaCl and $HClO_4$ 65% from Carlo Erba; Na_2HPO_4 e NaH_2PO_4 from J. T. Baker; guaiacol (2-methoxyphenol) and H_2O_2 from Sigma.

ESI 2. Preparation of the solid catalyst Nak-M80A

M80A was adsorbed on Nak at pH 3.5. The starting M80A solution was prepared by dilution of the mother solution in the buffer at pH 3.5, then passed through a preconditioned exclusion column (Sephadex G-15). Its concentration was checked spectrophotometrically (UV-Vis JASCO mod. V-570) and adjusted to reach an absorbance value of 0.62 at the λ_{max} of the Soret band, following a standardized procedure.[3] The batches of suspensions were prepared mixing 2 mg of Nak with 1 ml of M80A solution followed by shaking at 250 rpm in an orbital incubator (Stuard Scientific Orbital Incubator SI50) for 2 hours at 25°C. After the solid-liquid separation, UV-Vis spectra of the clarified supernatants were recorded using a Jasco V-570 spectrophotometer in order to calculate the moles of adsorbed M80A. The solid phase was washed three times with the buffer. Washing caused no loss of M80A. The washed solid constitutes the solid catalysts Nak-M80A. The number of adsorbed protein moles per kilogram of kaolinite was calculated both for M80A and wild-type yeast cytochrome *c* (YCC) at pH 3.5. The obtained mean values were 0.74 ± 0.03 and 0.71 ± 0.02 mmoles/Kg for M80A and YCC, respectively.

ESI 3. Diffuse-Reflectance (DR) UV-Vis measurements.

DR UV-Vis spectra were recorded on a V-570 Jasco instrument equipped with an integrating sphere attachment (Jasco model ISN-470) in the 190-800 nm range using a bandwidth of 0.5 nm. Powder sample holder (model PSH-001) was used. Kaolinite wetted with the same buffer of the measurement was used as blank instead of $BaSO_4$.

This technique was employed to perform different batches of measurements:

- DR UV-Vis spectra of adsorbed M80A as a function of pH; the samples were the solid catalyst Nak-M80A wetted with drops of buffers at different pH values.
- DR UV-Vis spectra of adsorbed M80A as a function of time at 50° and 80°C; the samples were the solid catalyst Nak-M80A wetted with drops of buffer at pH 7. Measurements were performed using a proper thermostated cell.
- DR UV-Vis spectra of adsorbed M80A as a function of time at pH 7 in the presence of H_2O_2. The samples were the solid catalyst Nak-M80A wetted with a 12.2 mM H_2O_2 solution at pH 7 for different times. After a proper contact time, the solid catalyst was separated by filtration from the reaction products in solution, washed with water and subjected to DR UV-Vis measurements.
- DR UV-Vis spectra of adsorbed M80A as a function of time at pH 7 in the presence of H_2O_2 and guaiacol. The samples were the solid catalyst Nak-M80A wetted with a 12.2 mM H_2O_2 solution at pH 7 in the presence of guaiacol for different times. After a proper contact time, the solid catalyst was separated by filtration from the reaction products in solution, washed with water and subjected to DR UV-Vis measurements.
- DR UV-Vis spectra of adsorbed M80A at pH 7 after repeated catalytic cycles; the samples were the solid catalyst Nak-M80A already used in any catalytic cycles, washed three time with the buffer at pH 7.

All the measurements were performed at 25°C, unless otherwise specified.

ESI 4. DR UV-Vis spectra of M80A adsorbed on kaolinite as a function of pH

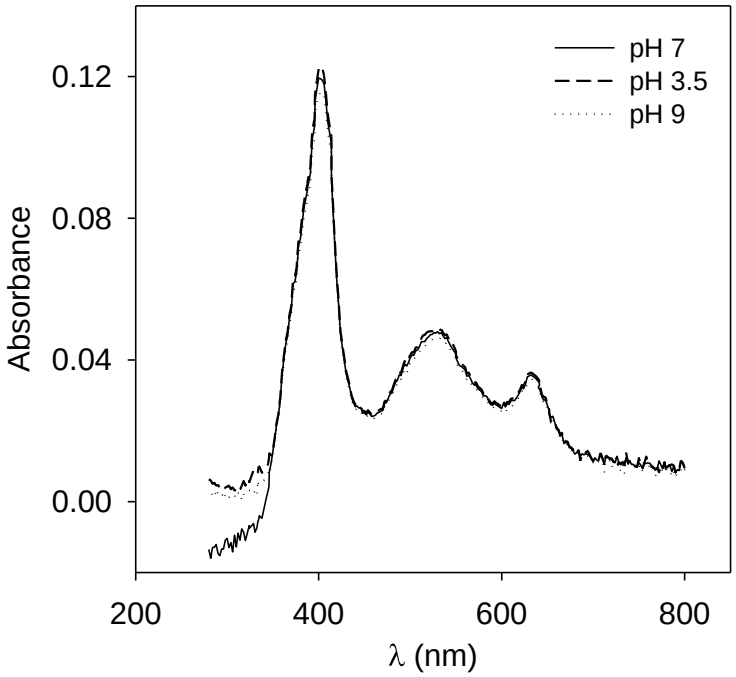


Fig. ESI 4. DR UV-Vis spectra of M80A adsorbed on kaolinite in buffer solutions at pH 3.5, 7 and 9.

ESI 5. UV-Vis measurements in solution.

UV-Vis spectra of M80A in solution were recorded on a V-570 Jasco instrument in the 190-800 nm range using a bandwidth of 0.5 nm.

Different batches of UV-Vis measurements in solution were performed as follows:

- UV-Vis spectra of M80A in solution as a function of time at 50° and 80°C; the samples were solutions of M80A in buffer at pH 7. Measurements were performed using a proper thermostated cell.
- UV-Vis spectra of M80A in solution as a function of time at pH 7 in the presence of H_2O_2; the samples were solutions of M80A and 12.2 mM H_2O_2 at pH 7.
- UV-Vis spectra of M80A in solution as a function of time at pH 7 in the presence of H_2O_2 and guaiacol; the samples were solutions of M80A and 12.2 mM H_2O_2 at pH 7 in the presence of guaiacol at different concentrations.

All the measurements were performed at 25°C, unless otherwise specified.

ESI 6. Time dependence of UV-Vis and DR UV-Vis spectra of M80A in solution and adsorbed on kaolinite at pH 7 in the presence of H_2O_2.

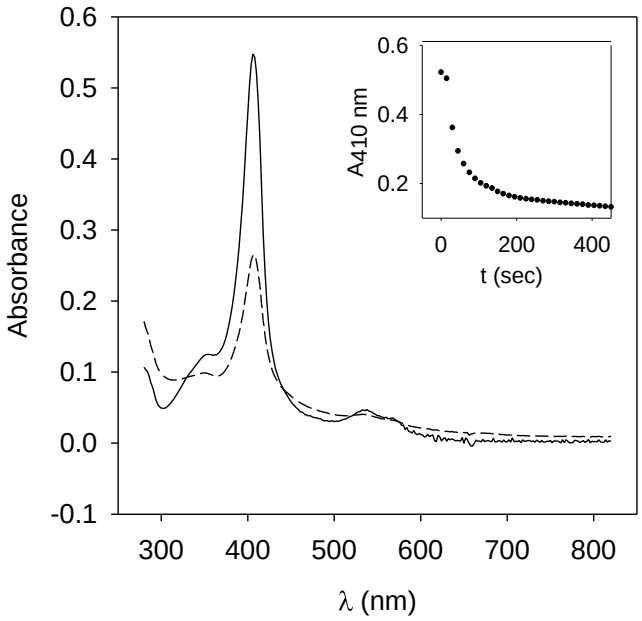


Fig. ESI 6a. UV-Vis spectra of M80A in solution at pH 7 in the presence of 12.2 mM H_2O_2 at t = 0 s (continuous line) and t = 60s (dotted line), $[M80A]_0$ = 4.5 μM. Inset: absorbance of the Soret band (λ = 410 nm) vs. time.

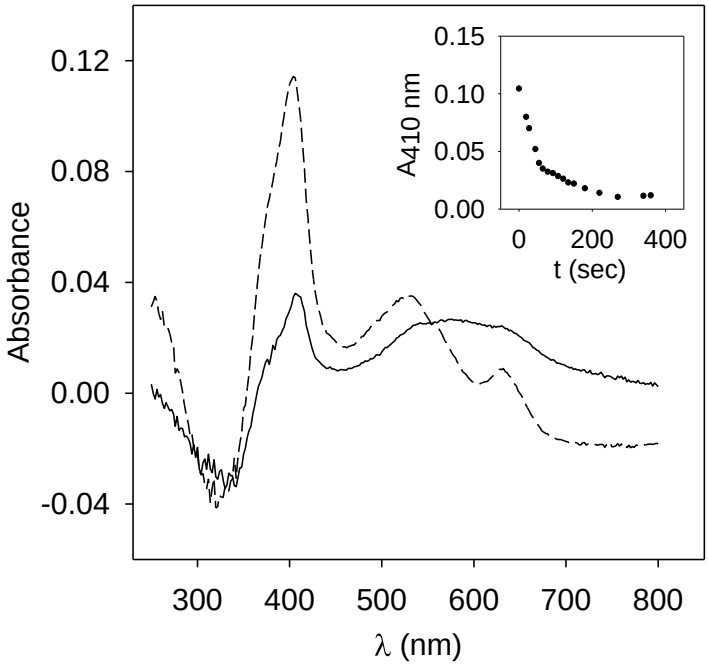


Fig. ESI 6b. DR UV-Vis spectra of M80A adsorbed on kaolinite in the presence of 12.2 mM H_2O_2 at t = 0 s (dotted line) and t = 60 s (continuous line), pH = 7. Inset: absorbance of the Soret band (410 nm) vs. time.

ESI 7. Time dependence of UV-Vis and DR UV-Vis spectra of M80A in solution and adsorbed on kaolinite at pH 7 in the presence of H_2O_2 and guaiacol.

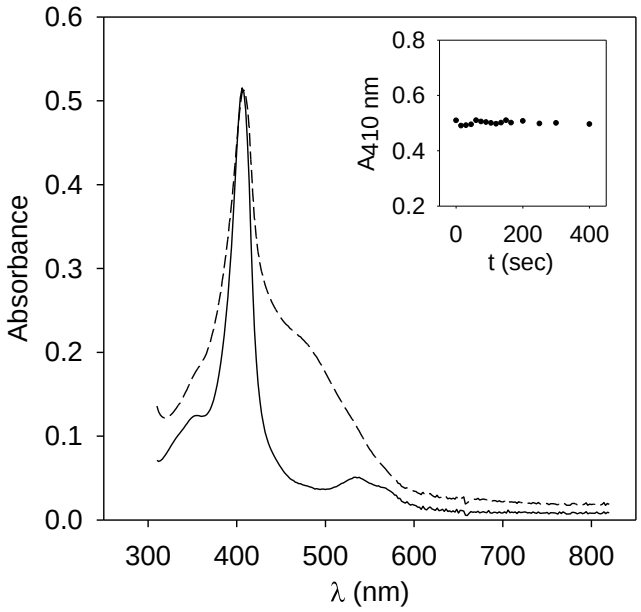


Fig. ESI 7a. UV-Vis spectra of M80A in solution at pH 7 in the presence of 12.2 mM H_2O_2 and 14 mM guaiacol at t = 0 s (continuous line) and t = 300 s (dotted line), $[M80A]_0$ = 4.2 μM. The shoulder at 480 nm is due to formation of tetraGc. Inset: absorbance of the Soret band (λ = 410 nm) vs. time.

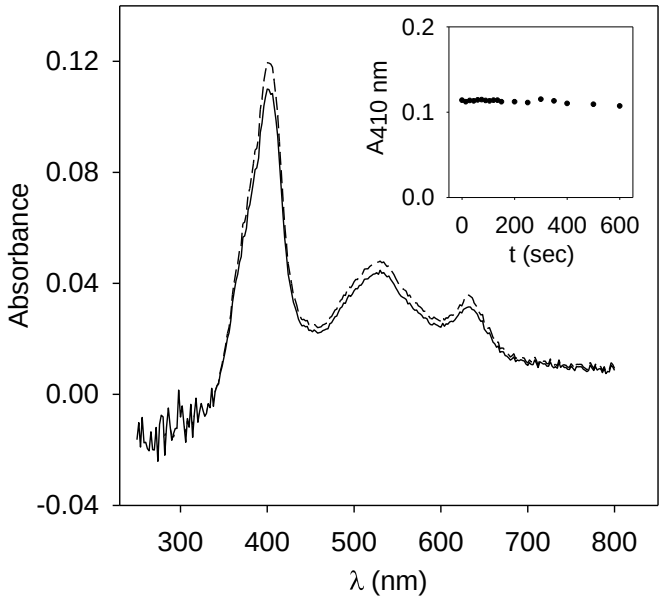


Fig. ESI 7b. DR UV-Vis spectra of the solid catalyst Nak-M80A after different contact times (t) with 12.2 mM H_2O_2 and 14 mM guaiacol: t = 0 s (dotted line) and t = 600 s (continuous line), pH = 7. Inset: absorbance of the Soret band (λ = 410 nm) *vs.* time. The contribution of tetraguaiacol to DR UV-Vis spectra is absent (at the difference of Fig. ESI 7a), since the solution phase has been removed before spectroscopic measurements.

ESI 8. Catalyst efficiency measurements of M80A adsorbed on kaolinite

The peroxidase activity of Nak-M80A was determined at 25°C through guaiacol (Gc) oxidation to tetraguaiacol (tetraGc) in the presence of H_2O_2, following the reaction[5]:

$$4Gc + 4H_2O_2 \rightarrow tetraGc + 8\,H_2O \qquad (1)$$

The initial reaction rate (V_0) is defined as the concentration of reagent Gc, in nanomolarity, which was consumed per second and per micromole of adsorbed cytc (μmol_{cyt}), namely:

$$V_0 = [Gc] /(\Delta t \cdot \mu mol_{cyt}) \qquad (2)$$

$$[V_0] = nM_{Gc} /(s \cdot \mu mol_{cyt}) \qquad (3)$$

V_0 of reaction 1 was measured as a function of H_2O_2 starting concentration at different pH values, given by the pH of the Gc solution. The experimental conditions were as follows: $[Gc]_0 = 1.46 \cdot 10^{-4}$ M at pH = 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9; $[H_2O_2]_0$ = 6, 9, 12, 15, 18, 21, 26, 30, 35, 40 mM; the time of contact between the solid catalyst Nak-M80A and the substrate H_2O_2 was 15 seconds; T=25°C. UV-Vis spectra were used to follow the formation of tetraGc (characteristic band at λ = 480 nm, ε_{480} =26600 M^{-1} cm^{-1}; the extinction coefficient was confidently considered constant within the pH range investigated, as previously reported in literature[6-8]). The detailed experimental procedure was reported in reference (3). From the absorbance of the product tetraGc at 480 nm ($A_{\lambda=480nm}$) and the measured time of contact between the adsorbed M80A and H_2O_2 (Δt), the initial reaction rate (V_0) was obtained from the experimental data as follows:

$$V_0 = 4 \cdot 10^9 \cdot \Delta A_{\lambda=480nm} / (\Delta t \cdot \varepsilon_{tetraGc,480} \cdot d \cdot \mu mol_{cyt}) \qquad (4)$$

where:

$\Delta A_{\lambda=480nm}$ is the difference between the $A_{\lambda=480nm}$ value recorded at t = Δt and at t = 0; $\varepsilon_{tetraGc,480}$ is the molar extinction coefficient of the tetraGc at λ = 480 nm ($\varepsilon_{tetraGc,480}$ = 26600 $M^{-1}cm^{-1}$); d is the optic pathway of the UV-Vis cell (1 cm); the coefficient $4 \cdot 10^9$ is needed to transform tetraGc molarity into Gc nanomolarity; μmol_{cyt} is the number of micromoles of adsorbed M80A. The average time of contact (15 seconds) is within the linear dependence of $A_{\lambda=480nm}$ *vs.* t.

ESI 9. Michaelis-Menten model applied to M80A adsorbed on kaolinite.

The plots of V_0 for reaction (1) at 25°C as a function of H_2O_2 starting concentration at pH values 2.5, 3.5, 4.5, 5.5, 6.0, 6.5, 7.5, 8.5 are reported in Fig. ESI 9a (pH = 2.5, 3.5, 4.5), in Fig. ESI 9b (pH = 5.5, 6.0, 6.5) and in Fig. ESI 9c (pH = 7.5, 8.5) together with the curve of the data fitting performed with *Sigma Plot* v. 11.0 (Dundas Software LTD, Germany). The experimental conditions were as follows: $[Gc]_0 = 1.46 \cdot 10^{-4}$ M; $[H_2O_2]_0$ = 6, 9, 12, 15, 18, 21, 26, 30, 35, 40 mM.

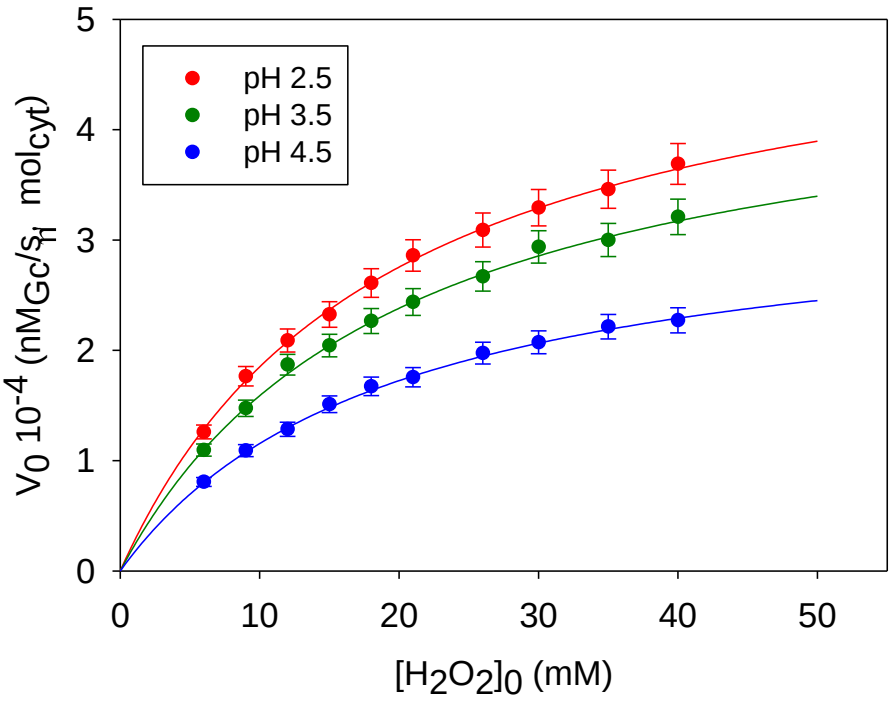


Fig. ESI 9a. V_0 of reaction 1 at 25°C as a function of H_2O_2 starting concentration; pH = 2.5, 3.5, 4.5; $[Gc]_0 = 1.46 \cdot 10^{-4}$ M; solid catalyst: Nak-M80A.

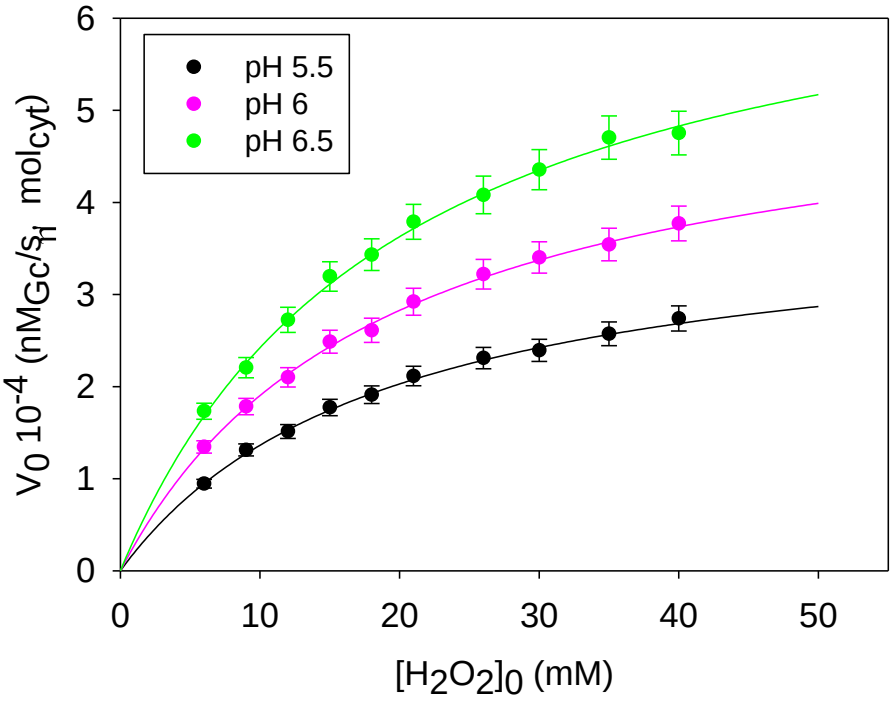


Fig. ESI 9b. V_0 of reaction 1 at 25°C as a function of H_2O_2 starting concentration; pH = 5.5, 6.0, 6.5; $[Gc]_0 = 1.46 \cdot 10^{-4}$ M; solid catalyst: Nak-M80A.

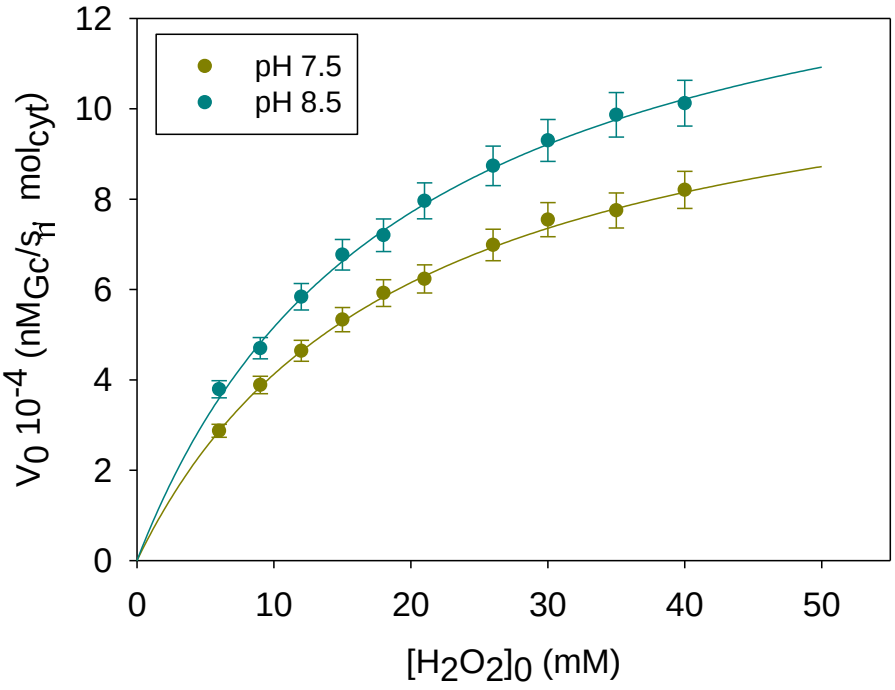


Fig. ESI 9c. V_0 of reaction 1 at 25°C as a function of H_2O_2 starting concentration; pH = 7.5, 8.5; $[Gc]_0 = 1.46 \cdot 10^{-4}$ M; solid catalyst: Nak-M80A.

ESI 10. Catalyst efficiency measurements of M80A in solution

The peroxidase activity of M80A in solution was determined at 25°C by measuring guaiacol (Gc) oxidation to tetraguaiacol (tetraGc) in the presence of H_2O_2, according to the reaction[5]:

$$4Gc + 4H_2O_2 \rightarrow tetraGc + 8\ H_2O \qquad (1)$$

The initial reaction rate (V_0) is defined as the concentration of reagent Gc, in nanomolarity, which was consumed per second and per micromolarity of cytc (μM_{cyt}), namely:

$$V_0 = [Gc] / (\Delta t \cdot \mu M_{cyt}) \qquad (2)$$

$$[V_0] = nM_{Gc} / (s \cdot \mu M_{cyt}) \qquad (3)$$

V_0 of reaction 1 was measured as a function of H_2O_2 starting concentration at different pH values, given by the pH of the Gc solution. The experimental conditions were as follows: [M80A] = 0.75 μM; $[Gc]_0 = 1.46 \cdot 10^{-4}$ M at pH = 3.5 and 7; $[H_2O_2]_0$ = 6, 9, 12, 15, 18, 21, 30, 40 mM; T=25°C. UV-Vis spectra were detected to follow the formation of tetraGc (characteristic band at λ = 480 nm, ε_{480} =26600 M^{-1} cm^{-1}; the extinction coefficient was confidently considered constant within the investigated pH range, as previously reported[6-8]). Under the above experimental conditions, absorbance of tetraguaiacol at 480 nm was found linear with time within 80 s, for this reason we selected a time of 15 s to calculate V_0.

From the absorbance of the product tetraGc at 480 nm ($A_{\lambda=480nm}$) and the measured time of contact between the adsorbed M80A and H_2O_2 (Δt), the initial reaction rate (V_0) was obtained from the experimental data as follows:

$$V_0 = 4 \cdot 10^9 \cdot \Delta A_{\lambda=480nm} / (\Delta t \cdot \varepsilon_{tetraGc,480} \cdot d \cdot \mu M_{cyt}) \qquad (4)$$

where:

$\Delta A_{\lambda=480nm}$ is the difference between the $A_{\lambda=480nm}$ value recorded at t = 15s and at t = 0; $\varepsilon_{tetraGc,480}$ is the molar extinction coefficient of the tetraGc at λ = 480 nm ($\varepsilon_{tetraGc,480}$ = 26600 $M^{-1}cm^{-1}$); d is the optic pathway of the UV-Vis cell (1 cm); the coefficient $4 \cdot 10^9$ is needed to transform tetraGc molarity into Gc nanomolarity; μM_{cyt} is the concentration of M80A in micromolarity.

ESI 11. Michaelis-Menten model applied to M80A in solution.

The plots of V_0 for reaction 1 at 25°C as a function of H_2O_2 starting concentration at pH values 3.5 and 7 are reported in Fig. ESI 12 together with the curve of the data fitting performed with *Sigma Plot* v. 11.0 (Dundas Software LTD, Germany).

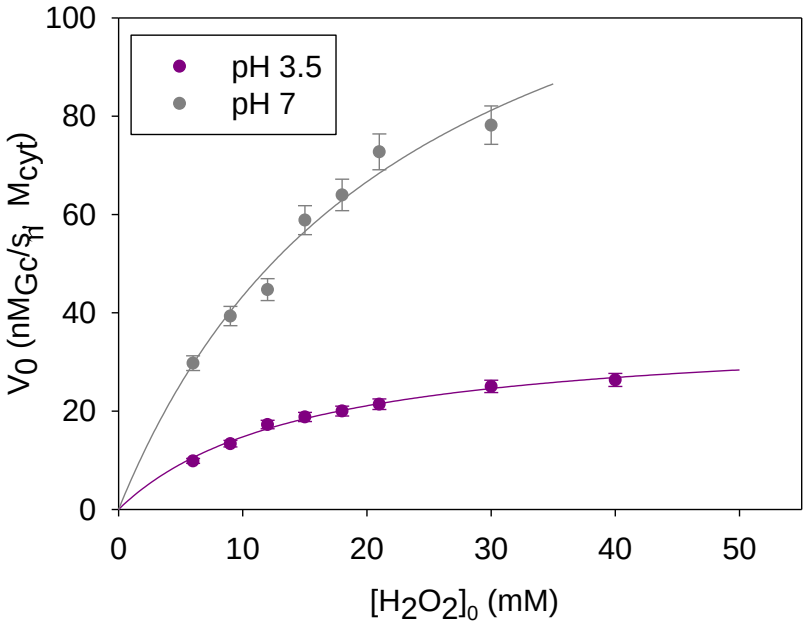


Fig. ESI 11. V_0 of reaction 1 at 25°C as a function of H_2O_2 starting concentration; pH =3.5, 7; $[Gc]_0$ = $1.46 \cdot 10^{-4}$ M; catalyst: M80A in solution, [M80A] = 0.75 µM.

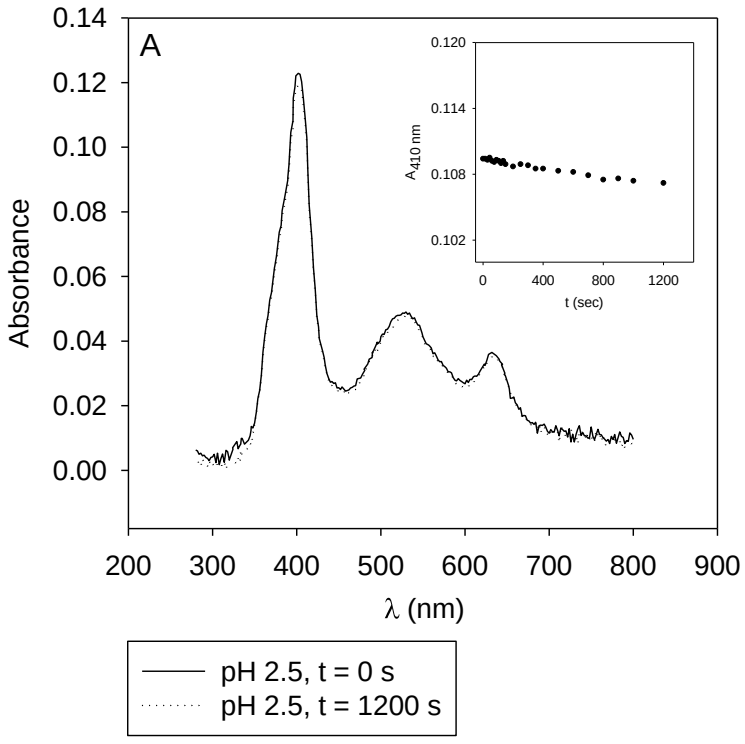
The values of K_M and V_{max} were calculated from the best fit of the data with *Sigma Plot*. To compare the data with those obtained in the adsorbed phases, we have performed the measurements

under the same experimental conditions used for the immobilized phase. In particular, for each sample we have employed the same number of protein moles acting in the adsorbed phase (mean value = 0.0015 μmoles), which have been solubilised in the same volume of guaiacol (V = 2 ml) at the same concentration used in adsorbed phase ($[Gc]_0 = 1.46*10^{-4}$M). Moreover, we have employed the same starting H_2O_2 concentrations ($[H_2O_2]_0$= 6, 9, 12, 15, 18, 21, 30, 40 mM). In such a way, we have been able to refer V_{max} not only to the M80A concentration but also to the number of protein moles in solution (see Table 1 in the text).

ESI 12. Solvent-accessible surface area (SASA) measurement

Measurement of solvent-accessible surface area (SASA) was performed with the VMD program[4] using a sphere of 1.4 Å radius as a probe. The PDB codes for the structure files used for SASA calculation for wild-type cytochrome *c* and M80A mutant were 1YCC.pdb and 1FHB.pdb, respectively.

ESI 13. DR UV-Vis spectra of adsorbed M80A at pH 2.5 and 9 as a function of time



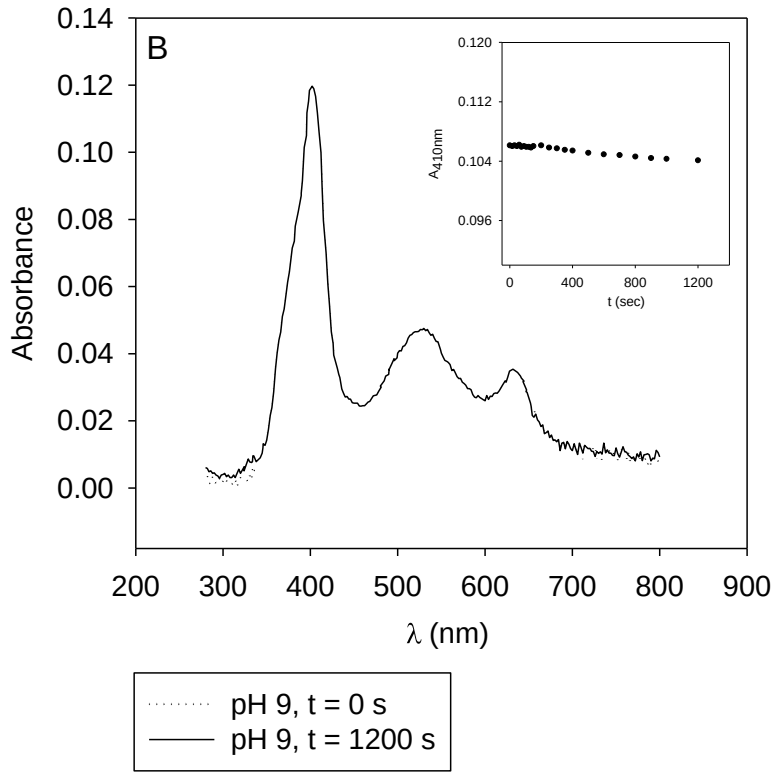


Fig. ESI 13. DR-UV-Vis spectra of M80A adsorbed on kaolinite, at t = 0 s and t = 1200 s of exposition time to solutions at pH 2.5 (A) and 9 (B). The inset shows the absorbance at λ = 410 nm *vs.* exposition time to the solution at pH 2.5 (A) and 9 (B).

ESI 14. Peroxidase activity of M80A adsorbed on kaolinite and in solution as a function of time at 50° and 80°C

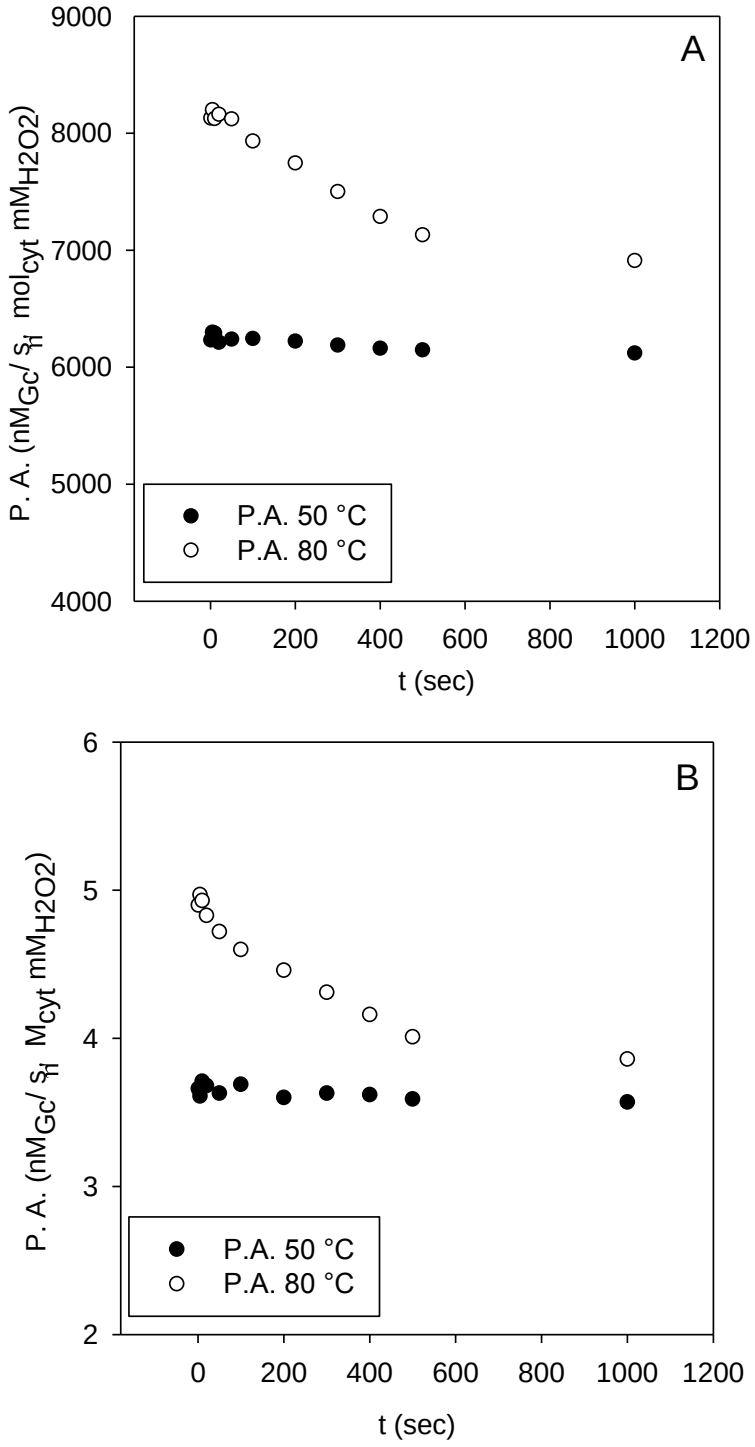


Fig. ESI 14. Peroxidase activity of M80A adsorbed on kaolinite (A) and in solution (B) *vs.* time at T = 50°C and T = 80°C , pH 7 and $[H_2O_2]_0$=12.2 mM. The scale of P.A. in A is referred to the number of adsorbed M80A micromoles, while in B it is referred to the concentration (micromolarity) of M80A in solution (see page 2, lines 44-49, ESI 8 and ESI 10).

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