Supramolecular mimetic peroxidase based on hemin and PAMAM dendrimers†

Maria Angela Castriciano,[a] Andrea Romeo,[a] Maria Camilla Baratto,[b] Rebecca Pogni[b] and Luigi Monsù Scolaro*[a]

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An acid–base interaction between hemin and PAMAM dendrimers affords supramolecular non-covalent peroxidase systems whose catalytic activity is enhanced after spontaneous electrostatic self-assembling onto a solid surface.

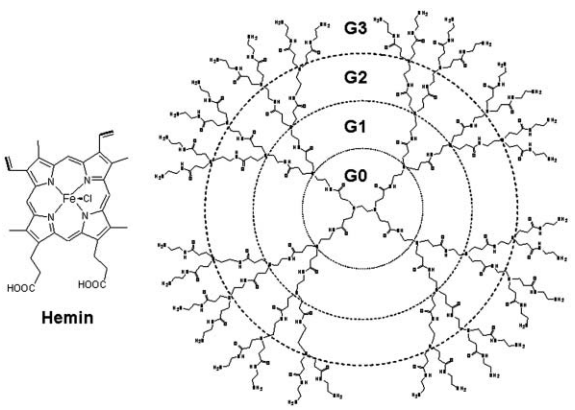
Heme peroxidases are a class of macromolecules able to catalyze oxidation of organic substrates by Compound I, an intermediate formed by reaction with hydrogen peroxide as electron acceptor.[1] Compound I is a heme π-cationic radical containing an oxyferryl heme (Fe(IV)=O),[2,3] which undergoes two consecutive one-electron transfer processes with two molecules of reducing substrate, and reconverts to the Fe(III) resting state passing through a second intermediate, Compound II.[2] This system has been the inspiration for synthetic analogues, and different Fe(III) and Mn(III) porphyrins have been proposed as models for the oxidative activity of different natural peroxidases.[4–7] Most of the synthetic heme-peroxidase analogues have been investigated in non-aqueous solvents.[8,9] On the contrary very few examples have been reported in aqueous media,[10,11] in which is necessary to cope with low solubility, large tendency to self-aggregate or to form μ-oxo dimers.[12]

Here we report a supramolecular approach to prepare an artificial peroxidase (Scheme 1) soluble in aqueous solution. This system can be easily obtained by interacting poly(amidoamine) PAMAM dendrimers (generation 2.0, 16 terminal amine groups, G2; or 3.0, 32 terminal amine groups, G3) with solid hemin in 1 : 1 ratio.

The resulting supramolecular species (HPG) is readily soluble in methanol and water, remaining stable for months. The structure is stabilized through electrostatic interactions among the anionic carboxylate groups of hemin and the charged protonated nitrogen atoms of PAMAM, similarly to other strictly related systems.[13]

Spectroscopic evidence in methanol suggests the formation of a six-coordinated low-spin heme species.[14–16] UV/Vis spectra display a strong B-band located at 408 nm ($\varepsilon = 8 \times 10^5$ M^{-1} cm^{-1} for G2 and $\varepsilon = 9.5 \times 10^5$ M^{-1} cm^{-1} for G3) accompanied by a weak Q-band at 538 nm (Fig. 1). The Beer law is held up to 15 μM, pointing to the absence of aggregation phenomena. The ^{1}H NMR spectra of HPG in deuterated methanol show a typical pattern of a six-coordinated low-spin species through an up-field shift of the methyl resonances with respect to the corresponding signals in the penta-coordinated high-spin hemin free base.[14,15] EPR experiments reveal the presence of low-spin Fe(III) at $g = 3.6$ and $g = 2.3$ (the third g value is too weak to be observed)[17] and a small contribution of non-heme orthorhombic species with $g = 4.3$. Generally for heme proteins g_x lies in the range 1.25–1.93, g_z ranges from 3.15 to 2.41, while g_y remains fairly constant at about 2.2–2.5.[17,18] Furthermore a significant number of heme proteins have strong g_{max} signals either in their native states or in the presence of specific modifying agents such as pyridines or aliphatic amines.[19,20] In this case iron could axially coordinate an amine group of the PAMAM and a solvent molecule which is easily removable in the presence of Fe(III) specific ligands.

Reduction of Fe(III) has been carried out by adding a large excess of ascorbic acid (molar ratio >100 : 1 with respect to the hemin adduct). The instantaneous formation of Fe(II) species in methanol solution leads to a hypsochromic shift of the B-band (λ_{max} = 398 nm, Fig. 1), together with a rapid decrease of the signals for low-spin Fe(III) at $g = 3.6$ and $g = 2.3$ in the EPR spectra. The Fe(II) species is not stable in air and it evolves rapidly to a high-spin Fe(III) species as confirmed by a bathochromic shift of the B-band (λ_{max} = 403 nm) accompanied by an hypochromic effect and the appearance of a high-spin contribution at $g = 5.9$ and $g = 2.0$ in the EPR spectrum. The formation of an oxyferryl heme complex is evident after the addition of a moderate excess of H_2O_2 (molar ratio 8 : 1). The UV/Vis spectra undergo a decrease in intensity and broadening of the B-band, pointing to the conversion



Scheme 1 Molecular structure of hemin and PAMAM dendrimers.

[a]*Dipartimento di Chimica Inorganica, Chimica Analitica e Chimica Fisica, and C.I.R.C.M.S.B., Università di Messina, Salita Sperone 31, 98166 Vill.S.Agata, Messina, Italy. E-mail: lmonsu@ unime.it; Fax: 39 090 393756; Tel: 39 090 6765711*
[b]*Dipartimento di Chimica Università di Siena Via A. De Gasperi, I-53100, Siena, Italy. E-mail: pogni@unisi.it; Fax: 39 0577 234239; Tel: +39 0577 234258*

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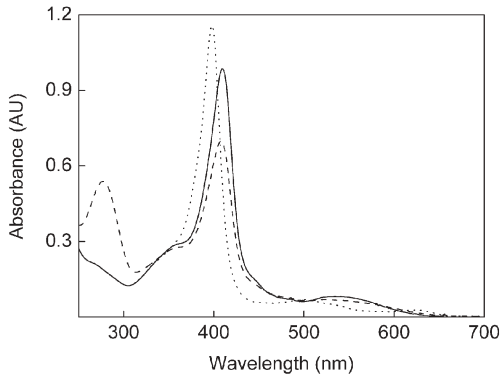

Fig. 1 UV-Vis spectra of HPG3 in methanol solution (solid line), in the presence of ascorbic acid (dotted line) and in presence of H_2O_2 (dashed line). ([HPG3] = 1 μM, [ascorbic acid] = 100 μM, [H_2O_2] = 8 μM, T = 298 K).

of the native system to Compound 0, in which hydrogen peroxide is coordinated in the sixth axial site.[21] The formation of an oxyferryl heme complex coupled to a porphyrin π-cation radical (Compound I) has been also evidenced in aqueous solutions by UV/Vis and EPR spectroscopy. The electronic spectra of HPG in water solution exhibit an hypsochromic shift and a splitting of the B-band with respect to methanol solution. The corresponding EPR spectra show the contemporary presence of high-spin Fe(III) with g = 6.0 and g = 2.0 and low-spin species with g = 3.6 and g = 2.2, in addition to the orthorhombic signal at g = 4.3 (Fig. 2(a)). The iron spin state is substantially coincident with the case of the system dissolved in methanol, even if a contribution of the high-spin species is evident. The EPR spectrum and its g-value after the addition of H_2O_2 to the system in aqueous solution, resemble Compound I formation. Fig. 2(b) shows the appearance of a new radical-like signal at g = 2.00, indicative of the involvement of the hemin iron in the radical formation with a reduction of the high-spin contribution and the disappearance of the low-spin species. EPR measurements were performed at different temperatures and microwave power, showing the typical behavior of Compound I as a porphyrinyl radical. The asymmetry in the lineshape of the EPR spectrum recorded at T = 4 K and saturation measurements show the presence of a radical in ferromagnetic exchange interaction with the oxyferryl heme iron.[22–24]

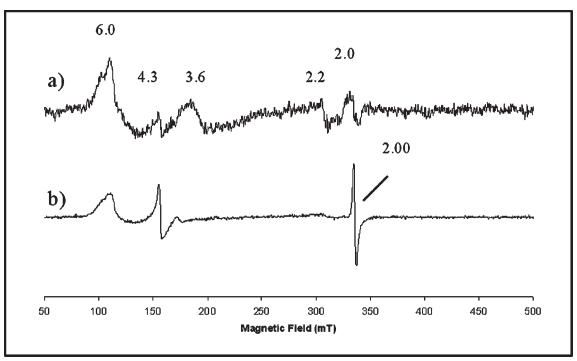

Fig. 2 20 K EPR spectra of HPG2 in water solution: (a) in the native state, (b) 10 s after the addition of H_2O_2. Experimental conditions: ν = 9.39 GHz, modulation amplitude = 1 mT, 2 mW microwave power, 100 kHz modulation frequency.

Table 1 Kinetic parameters for the catalytic oxidation of ABTS by H_2O_2 in aqueous solutions at 298 K

Catalyst	k_{cat}/s^{-1}	K_m/mM	$(k_{cat}/K_m)/s^{-1}\ M^{-1}$
HPG2[a]	0.39 ± 0.02	5.54 ± 0.54	0.7 × 10^2
HPG3[a]	0.16 ± 0.02	2.68 ± 0.50	0.6 × 10^2
HPG2[c]	1.92 ± 0.01	0.50 ± 0.02	3.84 × 10^3
HRP[b]	45.5	0.64	7.11 × 10^4

[a] 0.1 M Phosphate buffer pH 7. [b] Data from ref. 32 [c] Thin film.

The catalytic activity of HPG complexes has been tested in aqueous solutions in the oxidation of the standard substrate diammonium 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS).[16,25] The formation of the $ABTS^{+\bullet}$ radical has been detected through both UV/Vis[26] and EPR spectroscopy. In particular, the occurrence of a new intense radical signal at g = 2.0036 is evident in presence of ABTS and H_2O_2 in the EPR spectra of the supramolecular system, suggesting its involvement as a catalytic agent in the oxidation process. In order to verify possible catalyst degradation by an excess of H_2O_2, kinetic measurements have been performed as a function of oxidant concentration. The values of initial rates *vs.* [H_2O_2] exhibit a saturation profile which levels off for concentrations higher than 4 mM. Consequently, we decided to keep [H_2O_2] = 2 mM, and the kinetics for oxidation of ABTS have been performed reporting the initial rates as a function of substrate concentration. The obtained profiles exhibit typical Michaelis–Menten saturation behavior, from which the values of k_{cat}, Michaelis constant (K_m) and k_{cat}/K_m can be determined. These parameters are collected in Table 1, and compared with the values of native horseradish peroxidase (HRP). The value of k_{cat} (turnover s^{-1}) and the ratio k_{cat}/K_m (indicative of the catalytic activity at very low substrate concentration) are consistent with values reported for other heme analogue systems obtained by a covalent approach.[16]

Interestingly, the supramolecular HPG systems exhibit a high propensity to electrostatically adsorb onto glass substrates. When a methanolic solution of HPG is left in contact with a glass or silica surface for 24 h, a red colored film is formed (Fig. 3). This layer is no longer soluble in methanol or water and is stable for months. UV/Vis absorption spectra of the films are very similar to those of the parent HPG adduct in methanol solution, suggesting a closely similar coordination environment for the central metal ion. The HPG films retain their catalytic ability in the oxidation of ABTS

Fig. 3 Thin film of HPG2 adsorbed on a wall of a fluorescence cuvette. Kinetic runs can be spectrophotometrically monitored through the clean area of the front window.

with H_2O_2. The initial rates as a function of substrate concentration for this heterogeneous process show Michaelis–Menten profiles similar to the homogeneous solution.[27,28] Even in this case, the initial rates *vs.* $[H_2O_2]$ exhibit a saturation profile excluding extensive degradation of the catalyst due to high concentration of the oxidant agent.[29]

The HPG2 complex possesses a more efficient catalytic activity in the heterogeneous phase in comparison with the homogenous phase (Table 1). This effect can be probably ascribed to the different molecular conformations of PAMAM dendrimers in different solvents. Theoretical and experimental results on low-generation PAMAMs indicated a compact structure for these molecules in aqueous solutions.[30] On the other hand, in methanol the dendrimers should undergo a structural cross-over from an open (fractal-like) structure to a closed shell structure only after generation four.[31] In aqueous solution, hemin could be located in an inner pocket of a closed spatial conformation of the dendrimer, rendering the hemin iron center less accessible to the reagent molecules. In methanol, the HPG adducts could have a more open conformation and this could be retained or even enhanced by adsorption on glass or silica charged surfaces.

The supramolecular approach affords a simple way to access a non-covalent peroxidase mimetic system. The PAMAM dendrimers provide a charged scaffold which prevents hemin from the formation of μ-oxo dimers or aggregation. At the same time, the positively charged amino-terminal groups of PAMAM lead to electrostatic interactions with the negatively charged carboxylate on hemin stabilizing the resulting adduct. Furthermore, these groups allow a facile electrostatic self-assembling on charged surfaces giving adsorbed films which exhibit an enhanced catalytic activity. We expect that such an approach could be the basis for developing new and more efficient mimetic peroxidase systems effective both in homogeneous and heterogeneous environments.

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