Artificial peroxidase-like hemoproteins based on antibodies constructed from a specifically designed ortho-carboxy substituted tetraarylporphyrin hapten and exhibiting a high affinity for iron-porphyrins

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Received 10 July 1996

Abstract **In order to get catalytic antibodies modelling peroxidases BALB/c mice have been immunized with iron(III)-α,α,α,β-*mesotetrakis*-orthocarboxyphenyl-porphyrin (Fe-(ToCPP))-KLH conjugates. Monoclonal antibodies have been produced by the hybridoma technology. Three antibodies, 2 IgG_1 and 1 IgG_{2a}, were found to bind both Fe(ToCPP) and the free base $ToCPPH_2$ with similar binding constants. None of those antibodies was found to bind tetraphenylporphyrin. Those results suggest that the recognition of Fe(ToCPP) by the antibodies was mainly due to the binding of the carboxylate groups to some amino acid residues of the protein. True K_d values of 2.9×10^{-9} M and 5.5×10^{-9} M have been determined for the two IgG_1-Fe(ToCPP) complexes. Those values are the best ones ever reported for iron-porphyrin-antibody complexes. UV-vis. studies have shown that the two IgG_1-Fe(ToCPP) complexes were high-spin hexacoordinate iron(III) complexes, with no amino acid residue binding the iron, whereas the IgG_{2a}-Fe(ToCPP) complex was a low-spin hexacoordinate iron(III) complex with two strong ligands binding the iron atom. Both IgG_1-Fe(ToCPP) complexes were found to catalyze the oxidation of 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS) 5-fold more efficiently than Fe(ToCPP) alone whereas the binding of IgG_{2a} to this iron-porphyrin had no effect on its catalytic activity. k_{cat} values of 100 min^{-1} and 63 min^{-1} and k_{cat}/K_m values of 105 M^{-1} s^{-1} and 119 M^{-1} s^{-1} have been found respectively for the two IgG_1-Fe(ToCPP) complexes.**

Key words: Catalytic antibody; Peroxidase; Artificial hemoprotein; Porphyrin

1. Introduction

In the last few years, many monoclonal antibodies elicited against carefully designed transition state analogs have been reported to catalyze a wide range of chemical reactions [1–3]. Only a few catalytic antibodies modelling enzymes which require a cofactor such as flavins [4], metal ions [5–8] or metal complexes [9–19] have been described. Those antibodies were designed either to bring into close proximity the cofactor and the substrate or to bind tightly the cofactor to enhance its reactivity. A target of choice for antibody-cofactor catalysis was the modelling of heme proteins such as peroxidases which catalyze the oxidation of various substrates [20] by hydrogen peroxide and alkyl hydroperoxides. For that purpose, antibodies have been elicited against various free base [10,14,19], *N*-substituted- [11,12,18] and Sn- [13,15] or Pd-porphyrins [16,17]. However, only three of the obtained antibodies have shown a significant peroxidase activity [12,18,19]. We report here that antibodies elicited against a more sophisticated hapten, the iron(III)-*meso*-α,α,α,β-*tetrakis*-orthocarboxyphenyl-porphyrin (Fe(ToCPP), **1**) bind **1** with the highest affinity reported so far for iron-porphyrins. A detailed spectroscopic and kinetic study of the resulting IgG-**1** complexes shows that they are either high-spin or low-spin Fe(III) complexes and only the high spin complexes exhibit a peroxidase activity 5-fold higher than **1** alone and stable until complete conversion of the reducing cosubstrate.

2. Materials and methods

2.1. Synthesis of meso-α,α,α,β-tetrakis-orthocarboxyphenyl-porphyrin 2 and its iron(III) complexes 1

Condensation of ortho-carbomethoxybenzaldehyde with pyrrole according to an already described procedure [21] gave a mixture of 4 atropoisomers of *meso-tetrakis*-ortho-carbomethoxyphenyl-porphyrins [22]. The α,α,α,β atropoisomer was isolated by column chromatography on silica gel (eluent: CH_2Cl_2/Et_2O: 80/20) and identified by its UV-visible and ^1H NMR spectra which were identical to those reported in the literature [22]. **2** was then obtained after hydrolysis of the methyl esters at 40°C in 50% H_2SO_4 (v/v). ^1H NMR spectrum in CD_3OD: δ 8.57 (d, 8H); 8.32 (d, 4H); 8.15 (m, 4H); 7.99 (bs, 8H); −2.65 (d, 2H). **1** was obtained by reaction at room temperature with excess ferrous acetate generated in situ in glacial acetic acid. UV-visible spectrum in DMSO: λ_{max} (nm), ε (mM^{-1}): 422 (81), 534 (11), 693 (3.1).

2.2. Preparation of monoclonal antibodies

1 was activated by *N*-hydroxy-succinimide and covalently attached to KLH (keyhole limpet hemocyanin) and to BSA (bovine serum albumin) in PBS pH 7.5. The conjugates were then purified by column chromatography on Biogel P10. Hapten-carrier protein ratios determined spectrophotometrically were in the range of 15/1 to 20/1. Two 5 week old, female BALB/c mice were immunized with the hapten-KLH conjugate, and the mouse showing the best immune response 12 days after the third immunization was killed. Its splenocytes were fused with PAI myeloma cells according to Köhler and Milstein [23]. The resulting hybridomas were screened by ELISA for binding to the hapten-BSA conjugate using peroxidase-linked goat anti-mouse antibodies [24]. Positive hybridomas were cloned twice and produced in large amounts. Antibodies were then purified from hybridoma supernatants on a column of protein A and their homogeneity and purity were checked by SDS gel electrophoresis.

2.3. Determination of the binding constants

The binding constants were measured by competitive ELISA as follows: mixtures of a given antibody (at a concentration equal to its titer) and increasing amounts of **1** or **2** (10^{-9}–10^{-6} M) were incubated overnight at 4°C and then poured in microtiter wells previously coated with **1**-BSA in PBS, pH 6.0 (100 ng/well) and saturated with casein (3%). After incubation for 2 h at RT, anti-porphyrin antibodies were revealed with goat-antimouse antibodies labelled with peroxidase, using ABTS as a substrate. The binding constants were then determined as the concentration of porphyrin inhibiting 50% of the binding of the antibody to the immobilized antigen.

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PII S0014-5793(96)01006-X

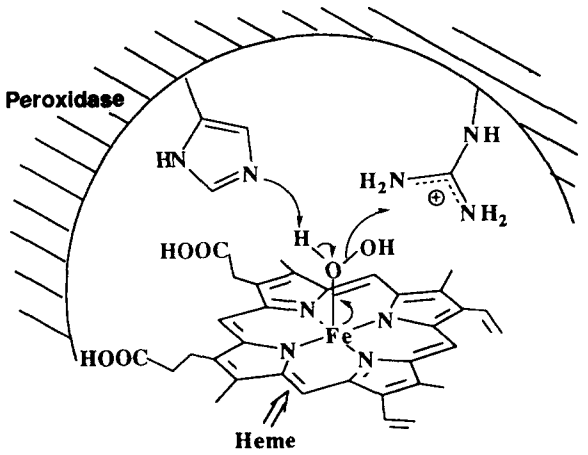

Fig. 1. Mechanism of the heterolytic cleavage of the O–O bond of hydroperoxides catalyzed by peroxidases.

True K_d values were determined from Scatchard plots as described by Friguet et al. [25].

2.4. UV-visible spectroscopic studies

The UV-visible characteristics of the porphyrin **1** and **2** and of the IgG-**1** complexes were determined in 0.1 M PBS, pH 6.5, using an UVIKON 860 spectrophotometer.

2.5. Assay of peroxidase activity

In a typical experiment, the oxidation of 0.2 mM **3** by 1 mM H_2O_2 was performed at 20°C in 0.1 M acetate buffer, pH 5, without catalyst or in the presence of either 0.2 μM **1** alone or 0.2 μM **1** and 0.5 μM 13G10 protein as catalysts. The absorbance at 414 nm was monitored by UVIKON 860 UV-visible recording spectrometer and the rates of oxidation were measured using an ε value of 36000 M^{-1} cm^{-1} [12].

In order to establish Lineweaver-Burk plots, the concentration of H_2O_2 ranged from 0.2 mM and 5 mM and 0.2 μM **1** was used either alone or in the presence of 0.5 μM IgG_1 protein (13G10 or 14H7) or 1 μM IgG_{2a} protein (6F12) as a catalyst.

3. Results and discussion

3.1. Strategy

The active site of peroxidases is relatively narrow and only allows the oxidant to interact with the iron, whereas the reducing cosubstrate only interacts with the meso edge of the heme [26]. The key step of the mechanism is the heterolytic cleavage of the O-O bond of H_2O_2 or ROOH assisted by two amino acid residues [20], a histidine and an arginine (Fig. 1), which leads to an highly reactive iron(V)=O species. This complex is further reduced to iron(III) by two one electron transfer reactions occurring through the porphyrin ring. The choice of **1** as a hapten was thus made to generate in antibodies not only a binding site for an iron-porphyrin but also, opposite to the ortho-carboxylate substituents of the phenyl rings, amino acids such as histidine or arginine which would assist the heterolytic cleavage of the O–O bond of H_2O_2.

3.2. Production of monoclonal antibodies and dissociation constants

BALB/c mice were immunized with the **1**-KLH conjugate and antibodies were produced using the hybridoma technology. Three monoclonal antibodies were found to bind **1**, two were IgG_1, 13G10 and 14H7, and one was IgG_{2a}, 6F12. After purification on protein A, apparent binding constants of **1** and its free base $ToCPPH_2$ (**2**) to the antibodies were measured by a competitive ELISA procedure (Table 1). 6F12 bound **1** with a binding constant of 1.2×10^{-6} M whereas 13G10 and 14H7 both bound **1** with a binding constant of about 2×10^{-8} M. Affinities of the three antibodies for the metallated and non-metallated porphyrins, **1** and **2**, were similar as shown by the binding constants (Table 1). None of the three antibodies bound *meso*-tetraphenylporphyrin. Those results suggest that the carboxylate groups were essential for the recognition of the hapten by the antibodies. True equilibrium K_d values were determined for the 13G10- and 14H7-**1** complexes and found to be 2.9×10^{-9} M and 5.5×10^{-9} M respectively (Table 1). Those values are the best ones ever reported for the binding of an iron-porphyrin to an antibody [10–19] and are in the range of the K_d values generally observed for natural heme-protein complexes (10^{-12}–10^{-8} M) [16].

*3.3. UV-visible characteristics of the IgG-**1** complexes*

The binding of **1** to the three aforementioned IgGs was studied by UV-visible spectroscopy. The spectrum of **1** showed bands at 396, 532 and 680 nm (Fig. 2) characteristic of a high-spin hexacoordinate iron(III) species [27], probably resulting from the coordination of two water molecules to the iron atom. Only minor changes were induced by the insertion of **1** into 13G10 or 14H7: a 5 nm shift and a slightly greater absorbance of the Soret band (Fig. 2) consistent with the binding of **1** in a hydrophobic pocket with no change of the iron(III) spin state. On the contrary, the binding of **1** to 6F12 led to dramatic changes in its UV-visible spectrum. An increase in absorbance and a 25 nm shift of the Soret band towards higher wavelengths were observed together with the appearance of new bands at 534 and 566 nm (Fig. 2). Such a spectrum, characteristic of a low spin hexacoordinate iron(III) species such as Fe^{III}(porphyrin)(imidazole)$_2$ [28] could be due to the coordination to the iron atom of two strong ligands such as histidine residues of the antibody. The UV-visible titration of 13G10 by **1** showed that two molecules of **1** were bound per molecule of IgG. This was confirmed by the quenching of protein tryptophan fluorescence which was op-

Table 1
Binding parameters of the antibodies and kinetic constants for the oxidation of ABTS by H_2O_2 catalyzed by **1**-antibody complexes

Antibody	Binding constants (M)		K_d (M)	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
	Fe(ToCPP)	$ToCPPH_2$	Fe(ToCPP)			
13G10	1.3×10^{-8}	1.0×10^{-8}	2.9×10^{-9}	16	100	105
14H7	2.7×10^{-8}	1.6×10^{-8}	5.5×10^{-9}	9	63	119
6F12	1.2×10^{-6}	3.5×10^{-7}	nm	nm	nm	nm
Fe(ToCPP)				42	51	20

nm: not measured.

timal for the addition of stoichiometric amounts of **1** to 13G10.

3.4. Peroxidase activity of the IgG-1 complexes

The oxidation of 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) **3** by H_2O_2 was used to assay the peroxidase activity of the **1**-IgG complexes. Whereas the reaction was negligible in the absence of catalyst (Fig. 3a), it was catalyzed both by **1** and 13G10-**1** (Fig. 3b,c). Moreover, it proceeded about 5 times faster with 13G10-**1** than with **1** as catalyst (Fig. 3b,c). With **1** alone, the reaction rate slowed down after 15 min and the reaction stopped after only 40% of the starting ABTS was oxidized (~30 min). On the other hand, in the presence of 13G10-**1**, the reaction rate remained constant until all the ABTS was converted. This showed the protecting effect of the antibody towards the oxidative degradation of the porphyrin ring by H_2O_2. The reaction really did occur inside the binding site of the antibody since: (i) 13G10 alone did not catalyze the reaction, (ii) addition of 0.5 μM non-related antibody protein to 0.2 μM **1** did not enhance its catalytic activity, (iii) preincubation of 13G10 with a stoichiometric amount of **2** inhibited the oxidation of ABTS and, (iv) the rate of oxidation of **3** by H_2O_2, when plotted versus the **1**/13G10 protein ratio ρ, increased linearly up to a ρ value of 2 and then levelled off (Fig. 3, inset). Additionally, the specific catalytic activity of the 13G10-**1** complex remained constant after several rounds of purification of the IgG protein on a column of protein A, which showed that the catalytic activity was not due to the presence, in the antibody preparations, of trace amounts of a contaminating peroxidase like protein devoid of its Fe-porphyrin cofactor.

A pH dependence study showed that the optimal rate of oxidation of **3** was observed within a pH range of 4–5. Further experiments, performed at pH 5, showed that the catalysis by the 13G10-**1** complex was optimal for concentrations of **3** between 0.1 and 1 mM. Such a wide range for acceptable reducing cosubstrate concentrations suggested

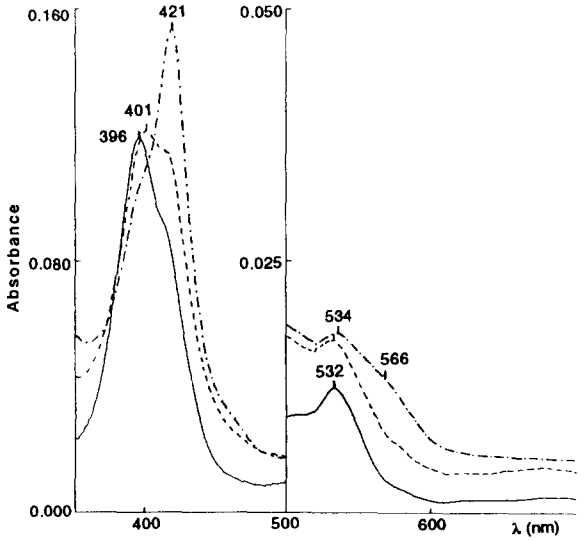

Fig. 2. Visible spectra of free **1** (—), 13G10-**1** (—) and 6F12-**1** (-·-·-) complexes in 0.1 M PBS, pH 6.5. In all cases the concentration of **1** was 2 μM. The antibody protein concentrations were 5 μM for 13G10 and 10 μM for 6F12.

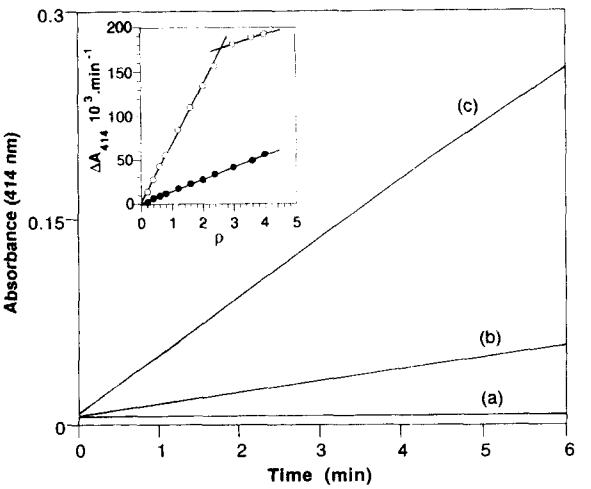

Fig. 3. Peroxidation of 0.2 mM ABTS by 1 mM H_2O_2; (a) uncatalyzed, (b) in the presence of 0.2 μM **1**, (c) in the presence of 0.2 μM **1** and 0.5 μM 13G10 protein. Inset: variations of the rate of ABTS peroxidation: ● as a function of the concentration of **1** between 0.1 and 2 μM; ○ as a function of the ρ ratio **1**/13G10 protein. The concentration of 13G10 protein was 0.5 μM and that of **1** varied between 0.1 and 2 μM.

that ABTS probably did not bind specifically to the antibody. H_2O_2 concentration dependence was therefore investigated for **1** and the three IgG-**1** complexes at pH 5 and using a concentration of **3** of 0.2 mM.

The 6F12-**1** complex did not increase the rate of oxidation of **3** by H_2O_2, presumably because the iron atom was tightly bound to two strong axial ligands. On the contrary, 13G10- and 14H7-**1** complexes as well as **1** alone led to peroxidation reactions displaying saturation kinetics with respect to H_2O_2. The enzymatic kinetic parameters determined by Lineweaver-Burk plots are listed in Table 1. A higher K_m value was observed for **1** (42 mM) than for both 13G10- and 14H7-**1** complexes (16 mM and 9 mM respectively), which showed that H_2O_2 had a better affinity for the **1**-antibody complexes than for **1** alone. Both 13G10- and 14H7-**1** complexes exhibited higher k_{cat} values (100 min^{-1} and 63 min^{-1} resp.) and k_{cat}/K_m values (105 M^{-1} s^{-1} and 119 M^{-1} s^{-1} resp., Table 1) than **1** alone (k_{cat} = 51 min^{-1} and k_{cat}/K_m = 20 M^{-1} s^{-1}). Such values are comparable to those already reported for the peroxidase reaction catalyzed by iron(III)-porphyrin antibody complexes [18,19].

4. Conclusion

Our results show that the immunization of mice with **1**-KLH conjugates leads to the production of antibodies having a peroxidase activity with no destruction of the catalyst by H_2O_2 even after complete conversion of the reducing cosubstrate. Preliminary results using a series of differently substituted porphyrins show that the recognition probably occurs via the binding of the *o*-carboxylate substituents of the phenyl rings, suggesting that our attempt to generate amino acid residues opposite to the carboxylates was successful. It is thus reasonable to think that those residues could be involved in the catalysis of the heterolytic cleavage of the O–O bond of H_2O_2. Further work is in progress to improve the catalytic efficiency of such artificial peroxidases.

Acknowledgements: We are indebted to Bernadette Desfosses for her important technical assistance and to the European Network of Antibody Catalysis for a postdoctoral fellowship award (Rebeca Quilez).

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