FEBS Letters 355 (1994) 314–316

FEBS 14834

Monoclonal antibodies against metalloporphyrins. Specificity of interaction with structurally different metalloporphyrins

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Received 18 October 1994

Abstract Monoclonal antibodies against Pd-coproporphyrin I have been obtained. The antibody specificity for free as well as for conjugated Pd-coproporphyrin I is characterized. Affinity constants are estimated for 3 monoclonal antibodies effectively interacting with free Pd-coproporphyrin I. A comparative study on the binding of monoclonal antibodies with analogues and derivatives of Pd-coproporphyrin I has revealed that the antigen is mainly located inside the antibody paratope. The protein adjoins complementary to the metalloporphyrin in such a manner that antibodies obtained discern only isomer I, and to some degree, isomer III of coproporphyrin.

Key words: Monoclonal antibody; Metalloporphyrin; Pd-coproporphyrin; Hemoprotein; Abzyme

1. Introduction

There are a number of proteins in Nature that bind hemin specifically and form molecules with different functional activity: hemoglobins, myoglobins, cytochromes and hemeperoxidases. As a rule, heme in these proteins is bound noncovalently with an association constant of 10^{-12}–10^{-8} M [1,2]. Heme is involved directly in the active site formation. Another example of such high-specific interaction is the reaction between porphyrins and antibodies. Antibodies against metalloporphyrins have recently received considerable attention as new reagents for phosphorescent immunoassay [3,4] and as new enzyme-like catalysts [5,6]. The first experience in abzyme catalysis of the porphyrin metallation and oxidative reactions has been reported in [6,5]. Antibodies against natural porphyrins are the most interesting ones because they clarifies the general rules in the formation of heme-binding domains of hemoproteins. The present paper is devoted to the description of hybridomas producing monoclonal antibodies against coproporphyrin I, 6 hybridomas being characterized by specificity of their interaction with natural and synthetic coproporphyrin analogues.

2. Experimental

2.1. Materials

Coproporphyrin I (CPI), coproporphyrin III (CPIII), Fe-coproporphyrin I (FeCPI), Pd-coproporphyrin I (Pd-CPI), Pd-coproporphyrin III (Pd-CPIII), Cu-coproporphyrin I (CuCPI),Zn-coproporphyrin I (ZnCPI), cyclopentenecoproporphyrin I (CPIc), Pd-cyclopentencoproporphyrin I (Pd-CPIc) were obtained from Innovation Biotechnologies Ltd. (Leninsky pr., 33, Moscow, 117071 Russian Federation). Protoporphyrin IX (PPIX) was from Calbiochem, hematoporphyrin IX (HPIX) from Koch-Light Laboratories. 1-Cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate, diammonium salt of 2,2′-azinobis(3-ethylbenzo-thiazoline-6-sulfonic acid) (ABTS), Trizma base, Tween 20 and Triton X-405 were purchased from Sigma. Sephadex G-50, Sepharose 6B were from Pharmacia. Bovine serum albumin (BSA), soybean trypsin inhibitor (STI), egg ovalbumin (OVA) were from Reachim (Russian Federation). Class and subclass specific goat anti-mouse antibodies were purchased from Serotec. HAT, HT, DMEM, fetal bovine serum, glutamine, Na-pyruvate were obtained from Flow. All other reagents were of fine grade quality.

2.2. Methods

2.2.1. Stock solutions of porphyrins. Stock solutions of porphyrins were prepared by dissolving a crystalline sample in a few drops of 0.1 M NaOH and subsequently diluted with deionized water to obtain slightly alkaline solution of porphyrin (or slightly acid solution for FeCPI).

2.2.2. Covalent binding of Pd-CPI to proteins. Conjugates were synthesized by the procedure described in [7] with minor modifications. A small amount of Pd-CPI was dissolved in 0.3 ml of 0.1 N KOH and then 0.3 ml water was added. The solution was neutralized with 0.1 N HCl to pH 6.0. A water solution of 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate was added immediately after preparation. Hydrochloric acid (0.01 N) was used to maintain pH 6.0 up to the end of the pH drift (for 15 min). The resultant solution of activated Pd-CPI was added to a protein solution in 0.1 N carbonate buffer (pH 9.0). The reaction mixture was incubated overnight in the dark at 4°C. Isolation of Pd-CPI/protein conjugates from the reaction mixture was performed on a column packed with two gels. The upper gel was Sephadex G-50 (7 cm) and the lower Sepharose 6B (25 cm). The column was equilibrated with 50 mM Tris buffer containing 150 mM NaCl, pH 7.4. The fraction of the second peak was collected. Absorption was measured at 280 nm. The Pd-CPI to protein ratio in the conjugate was determined in accordance with [4].

2.2.3. Monoclonal antibodies against Pd-coproporphyrin. Monoclonal antibodies against Pd-coproporphyrin I were obtained by the conventional procedure [8]. Mice BALB/c were immunized by the conjugate of Pd-CPI with BSA (porphyrin/protein ratio more than 10). Mouse immune lymphocytes were fused with myelome cells Sp2/O and pipetted into 480 wells of 96-wells microtiter plates. Growth of the hybridoma was observed in 260 wells and 30 of them had positive response in the ELISA. The Pd-CPI conjugated with STI was used as an antigen for adsorption on the microtiter plates). Only 23 hybridomas retained the ability to synthesize monoclonal antibodies and were cloned in semi-liquid agar.

2.2.4. Indirect enzyme immunoassay. Pd-CPI conjugated with STI (porphyrin/protein = 3) were adsorbed on the surface of polystyrene microtiter strip wells as follows. 100 ml of a protein solution (1 mg per ml in O.1 M carbonate buffer, pH 9.0) were added in the wells and incubated overnight at 4°C. To saturate adsorption centers of the wells 200 ml of 6% mannite solution in 0.01 M Na-phosphate buffer with 0.15 M NaCl, pH 7.4 (PBS), were added in each well. The plates were incubated for 2 h at 37°C and then washed six times with PBS, containing 0.05% Tween 20 (PBST). The plates were stored at 4°C. The antibodies in PBST buffer with egg albumin (5 mg/ml) were added to the microtiter wells coated with Pd-CPI-STI, incubated for 1 h and washed six times with PBST. Rabbit anti-mouse antibodies labeled with perox-

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SSDI 0014-5793(94)01221-0

idase were added and the strips were incubated for 1 h and washed in the same manner. The substrate mixture (4 mM H_2O_2, 1 mM ABTS in 0.05 M citrate buffer, pH 4.0) was added into the wells and in 15 min the enzyme activity was measured by changes in the absorbance at 410 nm using a Titertek Multiskan MCC/340.

2.2.5. Competitive immunoassay. The specificity of the interaction of antibodies with free and conjugated Pd-CPI was determined by comparing the ability of monoclonal antibodies to bind either to immobilized Pd-CPI-STI or to porphyrin in solution. The quantity of antibodies bound to immobilized Pd-CPI-STI was measured by indirect ELISA as described above.

2.2.6. I_{50} index. Index I_{50} is the concentration of porphyrin that inhibits the binding of monoclonal antibodies to free Pd-CPI by 50%. In competitive immunoassays, the concentration of antigen that provides half-maximal binding of antibodies correlates with the affinity constant [9,10]. The $I_{50porphyrin}/I_{50Pd\text{-}CPI}$ ratio reflects variations in the affinity constant caused by changes in porphyrin structure. Hence we have:

$$I_{50porphyrin}/I_{50Pd\text{-}CPI} = K/K^*,$$

where $I_{50Pd\text{-}CPI}$ and $I_{50porphyrin}$ are the concentrations of half-maximal inhibition, and K and K^* are the affinity constants for Pd-CPI and different porphyrins, respectively.

3. Results and discussion

Eighteen hybridomas belonged to IgM class of immunoglobulins and five to different subclasses of IgG. According to the results of competitive immunoassay, monoclonal antibodies can be divided into 3 groups (Table 1).

(1) Antibodies interacting only with conjugated Pd-CPI (C2.B5, G6.D3, F7.B3, B3.D5).

(2) Antibodies interacting only with conjugated protein or with the spacer taking part in the formation of a covalent bond between Pd-CPI and protein (C2.F3, D7.B4, D11.C2, B8.D9, D9.E6);

(3) Antibodies interacting with free Pd-CPI (D5.E3, D3.F5, B5.C6).

The affinity constants were determined by the solid-phase immunoassay method [1] for 3 monoclonal antibodies that interacted efficiently with free Pd-CPI. The constants calculated from a Scatchard plot are presented in Table 2. The specificity of interaction of the other monoclonal antibodies with different porphyrins was determined using the I_{50} index (Table 3). All the monoclonal antibodies obtained proved to be sensitive to the presence and nature of the metal ion in the porphyrin. The results presented in Table 3 show that metal-free coproporphyrin is bound to monoclonal antibodies 10–100 times less efficiently than Pd-coproporphyrin. The change of Pd-ion to Zn-ion results in 10-fold (or even more) deterioration of the binding depending on the clone of monoclonal antibodies. The influence of the side substituents of the porphyrin ring on the interaction with monoclonal antibodies D3.F5 was studied in detail by the I_{50} index (Table 3). Monoclonal antibodies D3.F5 bind to isomer III of coproporphyrin less efficiently than isomer I. This demonstrates that the change of the location of a single propionic acid residue within a single pyrrol ring results in small change in the binding efficiency. The appearance of the cyclopentane ring in coproporphyrin I does not influence the binding of Pd-CPc to antibodies and improves the interaction of free CPc to antibodies as compared with free CPI. At the same time antibodies D3.F5 do not virtually bind to natural isomers IX of porphyrin (hematoporphyrin IX). Antibodies D5.E3 are more sensitive to substituents in the porphyrin ring than antibodies D3.F5, while antibodies D9.E6 are less sensitive than antibody D3.F5 and antibodies B5.C6, C2.B5, G6.D3 are almost insensitive. If we compare these data with the affin-

Table 1
Specificity of monoclonal antibodies to free and conjugated Pd-coproporphyrin I

No.	Clone	Indirect ELISA	Competitive ELISA with Pd-CPI-SIT conjugate	Competitive ELISA with free Pd-CPI
1	C2.B5	+++	+++	+–
2	D5.E3	+++	+++	+++
3	D3.F5	+++	+++	+++
4	D11.C2	++	+++	–
5	G6.D3	+++	+++	+–
6	F7.B3	++	+	+–
7	D9.E6	++	+	+
8	B8.D9	+++	+++	–
9	B5.C6	+++	+++	++
10	B3.D5	++	+	+–
11	C2.F3	++	–	–
12	D7.B4	+	–	–

Indirect ELISA	Competitive ELISA
+++ = OD > 1.5	+++ = OD < 0.9
++ = OD = 0.9–1.5	++ = OD = 0.9–1.5
+ = OD < 0.9	+ = OD > 1.5

Table 2
The main features of monoclonal antibodies to Pd-coproporphyrin I

No.	Clone	Antibody	Ascitic fluid		Isoelectrofocusing		Binding constant (M)	
		Class	Titre	Quantity (mg/ml)	pI main band	Number of bands	free Pd-CP	Conjugate Pd-CP-SIT
1	D3.F5	IgG_1	500,000	10	7.2	4	4.76×10^{-10}	3.15×10^{-10}
2	D5.E3	IgG_1	200,000	10	6.5	6	4.99×10^{-9}	5.48×10^{-9}
3	D9.E6	IgG_{2b}	100,000	5	5.5	5	2.11×10^{-8}	1.45×10^{-8}
4	G6.D3	IgG_1	500,000	11	6.8	7	–	–
5	C2.B5	IgG_1	500,000	10	6.0	5	–	7.30×10^{-9}
6	C2.F3	IgG_{2a}	20,000	8	–	–	–	–
7	B8.D9	IgM	500,000	8	–	–	–	–
8	B3.D5	IgM	25,000	5	–	–	–	–
9	D11.C2	IgM	500,000	11	–	–	–	–
10	F7.B3	IgM	50,000	8	–	–	–	–
11	B5.C6	IgM	5,000	4	–	–	–	–
12	D7.B4	IgM	20,000	8	–	–	–	–

Table 3
Specificity of different antibodies to porphyrins: I_{50} index; ratio: $I_{50porphyrin}/I_{50Pd\text{-}CPI}$

		Antibodies											
		D3.F5		D5.E3		D9.E6		B5.C6		C2.B5		G6.D3	
No.	Porphyrin	I_{50}	ratio	I_{50}	ratio	I_{50}	ratio	I_{50}	ratio	I_{50}	ratio	I_{50}	ratio
1	Pd-CPI	1.73×10^{-8}	1.0	4.17×10^{-8}	1.0	3.00×10^{-8}	1.00	8.00×10^{-7}	1.00	7.25×10^{-5}	1.00	7.20×10^{-6}	1.00
2	Pd-CPIII	1.29×10^{-7}	7.46	–	–	–	–	–	–	–	–	–	–
3	Pd-CPIc	1.82×10^{-8}	1.05	–	–	–	–	–	–	–	–	–	–
4	CPI	3.70×10^{-7}	21.39	1.26×10^{-6}	30.22	1.71×10^{-7}	5.7	1.30×10^{-5}	16.23	4.17×10^{-5}	0.58	4.20×10^{-5}	5.83
5	CPIII	4.68×10^{-7}	27.05	2.34×10^{-5}	561.15	1.58×10^{-6}	52.67	1.40×10^{-6}	17.48	–	–	–	–
6	CPIc	5.49×10^{-8}	3.17	9.33×10^{-6}	223.7	9.05×10^{-7}	30.17	8.10×10^{-6}	10.11	7.33×10^{-5}	1.01	7.30×10^{-5}	10.14
7	HPIX	9.33×10^{-5}	5393.06	–	–	1.29×10^{-6}	43.00	3.90×10^{-6}	0.87	3.50×10^{-5}	0.48	3.50×10^{-5}	4.86
8	PPIX	–	–	–	–	–	–	1.50×10^{-6}	1.87	–	–	–	–
9	Zn-CPI	3.39×10^{-6}	195.95	4.17×10^{-5}	1000.0	7.54×10^{-7}	5.13	6.10×10^{-5}	76.16	1.80×10^{-4}	2.62	1.80×10^{-4}	25.0
10	Cu-CPI	1.66×10^{-8}	0.96	–	–	–	–	–	–	–	–	–	–
11	Fe-CPI	2.34×10^{-7}	14.05	–	–	–	–	–	–	–	–	–	–
12	Pd-CPI-SIT	3.47×10^{-9}	0.20	1.99×10^{-9}	0.048	5.14×10^{-9}	0.17	9.60×10^{-7}	1.20	5.47×10^{-8}	0.0008	2.15×10^{-9}	0.0003
13	Pd-CPI-BSA	1.95×10^{-9}	0.11	1.40×10^{-9}	0.034	4.57×10^{-9}	0.15	8.45×10^{-7}	1.05	4.10×10^{-8}	0.0006	1.40×10^{-9}	0.0002
14	Pd-CPI-OVA	2.29×10^{-9}	0.13	1.82×10^{-8}	0.4	46.38×10^{-9}	0.21	9.58×10^{-7}	1.20	5.23×10^{-8}	0.0007	1.93×10^{-9}	0.0003

ity constants and I_{50} indices for the same antibodies, it becomes clear, that there is a simple rule for antibodies against porphyrins: the higher is the affinity, the higher is the specificity.

On the basis of the data on the interaction of different derivatives of Pd-CPI with highly specific antibodies D3.F5 and D5.E3, it can be supposed that Pd-CPI is mainly located inside the antibody paratope. One of the propionic acid residue and one of the neighboring mezo-bridge are located most closely to the surface of antibody. If we compare natural hemoproteins with the antibodies obtained, some general features of the interaction of metalloporphyrins with proteins become clear. In both cases, the metalloporphyrin is located inside the protein and only its small part is on the surface. The protein complementary adjoins the metalloporphyrin and natural hemoproteins recognize only isomer IX of porphyrin [12], while the antibodies obtained only isomer I and III. It should be noted that all natural hemoproteins bind to porphyrin more tightly than the monoclonal antibodies obtained. This phenomenon can be explained by the restricted number of antibodies as compared the proteins evolutionary selected for efficient binding of heme.

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