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Specific Interaction of Coproporphyrin I with Antibodies in Water and Reverse Micelles: Dimerization of Antibodies Affects Antigen Binding Site

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The antigen-antibody interaction between coproporphyrin I and anti-coproporphyrin antibodies was studied by a fluorescence method in water and a reverse micellar system: *n*-octane/Aerosol OT. Coproporphyrin fluorescence was quenched, and coproporphyrin emission maximum was shifted to the long-wavelength region after binding to the antibodies or Fab′-fragments. The mechanism of this quenching is static, most probably, by a tryptophan residue (or maybe lysine or methionine). Apparent dynamic quenching, in this case, arises from protein backbone motion. A special kind of antibody Fab′-Fab′ dimerization was proposed.

KEY WORDS: Coproporphyrin; monoclonal antibodies; reverse micelles; Aerosol OT; fluorescence quenching.

INTRODUCTION

Monoclonal antibodies against porphyrins have recently attracted considerable attention as models for studying apoprotein-prosthetic group interactions and new enzyme-like catalysts [1]. Structural similarity of the location of metalloporphyrins in peroxidases and in complexes with antibodies was demonstrated [2]. The mechanism of ligand binding by monoclonal anti-coproporphyrin antibodies was studied by steady-state and time-resolved fluorescence spectroscopy on a picosecond laser system [3].

Recently, analytical chemists have paid increasing attention towards immunoassays based on labeled antibodies or antigens. Antibodies against Pd-coproporphyrin were effectively used in different immunochemical methods based on bridge structure (UniPhia, universal phosphorescent immunoassay) [4] and bispecific monoclonal antibodies [5].

In this paper we studied the binding of monoclonal antibodies (Ab) to coproporphyrin, isomer I (CP), in two homogeneous media, an aqueous solution and a well-known reverse micellar system consisting of Aerosol OT [AOT, sodium bis(2-ethylhexyl)sulfosuccinate] in *n*-octane. Luminescence study of hapten-antibody interaction presents a unique opportunity for understanding a more detailed mechanism of antibody-antigen complex formation.

EXPERIMENTAL

AOT was purchased from Serva. *n*-Octane was purchased from Reachim (Russia). It was purified by shaking with sulfuric acid (sulfuric acid/octane 1:6 v/v) and washing with water. This extraction with sulfuric acid and washing with water was repeated 3 times. Then *n*-octane was washed with an aqueous solution of sodium carbonate and one more time with water, and distilled before use. Other chemicals were of analytical grade.

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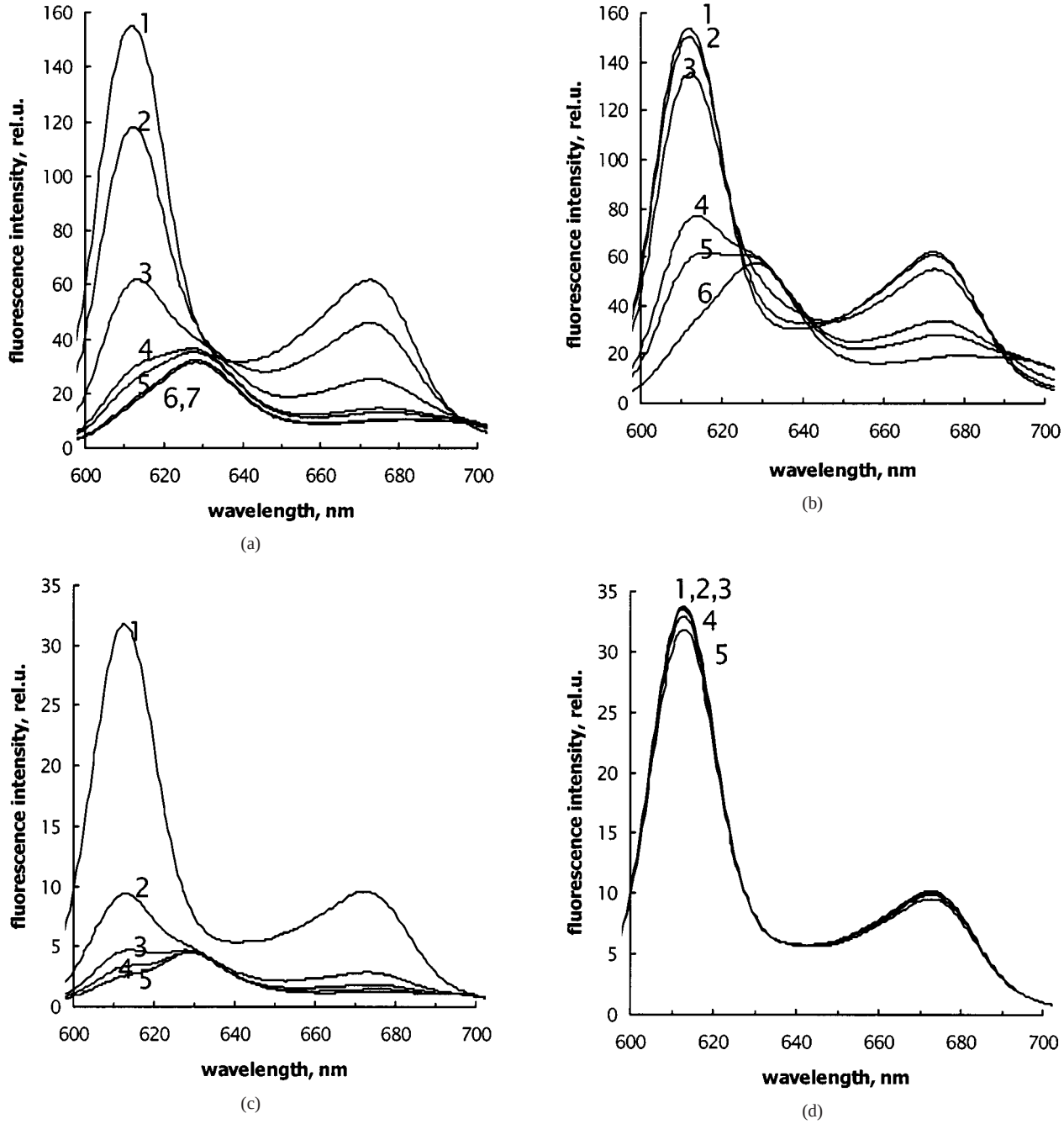


Fig. 1. CP fluorescence spectra in water in the absence of antibodies and in the presence of different clones of specific anti-CP antibodies (a, c), or Fab′ (b), or non-specific (anti-insulin) antibodies (d). (a) [CP] = 1 μM; [Ab] = 0 (1), 0.2 (2), 0.5 (3), 0.6 (4), 0.7 (5), 0.8 (6), and 1.3 (7) μM (anti-CP antibodies, clone 5D). (b) [CP] = 1 μM; [Ab] = 0 (1), 0.8 (2), 1.8 (3), 2.4 (4), 3.2 (5), and 4.4 (6) μM ($F(ab')_2$ of anti-CP antibodies, clone 5D) (c) [CP] = 0.033 μM; [Ab] = 0 (1), 0.06 (2), 0.12 (3), 0.24 (4), and 0.48 (5) μM (anti-CP antibodies, clone 3D). (d) [CP] = 0.033 μM; [Ab] = 0 (1), 0.06 (2), 0.12 (3), 0.24 (4), and 0.48 (5) μM (non-specific anti-insulin antibodies).

A monoclonal antibody against CP was obtained as described [2]. Antibodies were precipitated with a half-saturated aqueous ammonium sulfate solution and dialyzed against 50 mM sodium phosphate buffer (pH 8.0). Antibody concentration was estimated spectrophotometrically at 280 nm (Hitachi-557, Japan), assuming the molar absorptivity of a 1 mg/ml solution of Ab of 1.38 and molecular weight of 150 kD. Fab′-

and F(ab′)$_2$-fragments were obtained using pepsin hydrolysis [6].

Coproporphyrin I (dihydrochloride) was kindly donated by Prof. G. V. Ponomarev (Institute of Medical Chemistry, Moscow, Russia).

Micellar CP solutions were prepared by dilution of the stock CP solution (1 mM, in 0.1 M NaOH) with 0.1 M AOT solution in n-octane. The desired hydration degree of the micellar solution was achieved by addition of the appropriate volume of the aqueous buffer solution (50 mM phosphate, pH 8.0). For example, 60 μl of aqueous buffer solution was added to 2 ml of 0.1 M AOT solution in *n*-octane to obtain a reverse micellar solution at a hydration degree of 16.7. Micellar Ab-containing solutions were prepared by solubilization of aqueous solution of antibodies (0–40 μM, in 50 mM Na-phosphate, pH 8.0) in the reverse micellar solution of CP; the desired hydration degree of the micellar solution was achieved as described above. The system became optically transparent after 0.1–0.5 min shaking.

Aqueous phase (indicated below as "water") was buffered with 50 mM Na-phosphate, pH 8.0, in all experiments with the exception of pH effect study (universal buffer 0.1 M Na_2HPO_4 + 0.1 M $KHCO_3$ + 0.1 M AcONa in the presence of 0.15 M NaCl).

Fluorescence spectra were measured at room temperature (22°C) using a Perkin-Elmer Luminescence Spectrometer LS50B (λ_{ex} 395 nm).

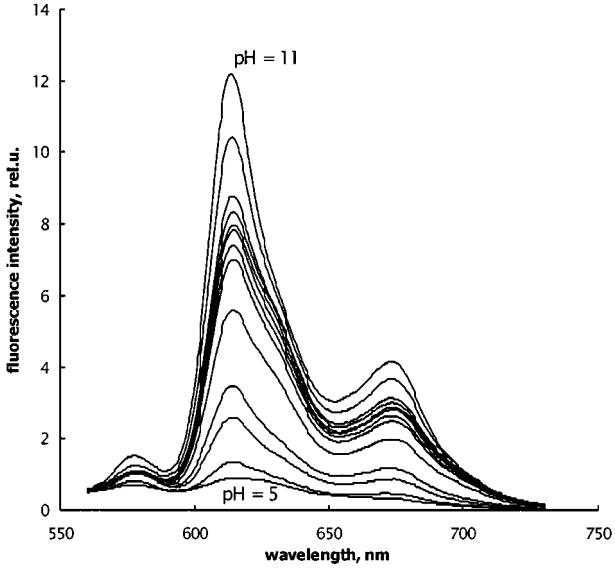
RESULTS AND DISCUSSION

CP fluorescence was strongly quenched upon addition of anti-CP antibodies in aqueous solution (Figs. 1a, 1c), quenching being accompanied by a shift of the emission maximum to the long-wavelength region, from 612 to 630 nm. The same quenching effect was observed by using Fab′ or F(ab′)$_2$ (Fig. 1b) fragments of antibodies. This effect was specific, because no quenching was observed if non-specific antibodies were used (Fig. 1d).

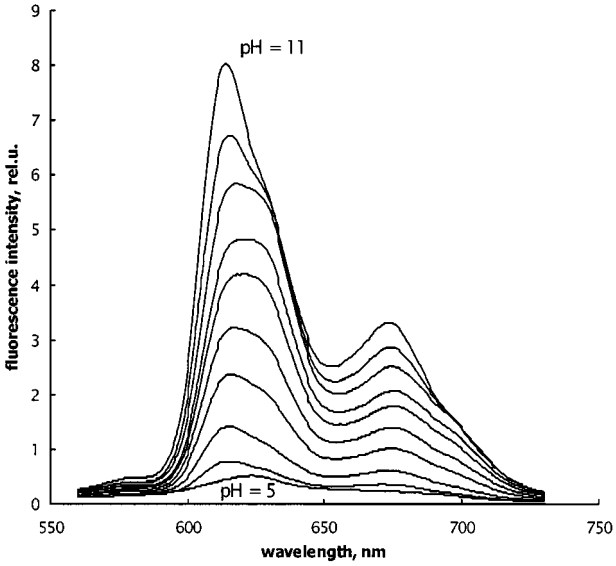
Internal Quenching by Antibody

There are at least two different possible explanations of this quenching: (1) CP-Ab binding causes the change of the protonation extent of the carboxypropionic groups of CP and the corresponding change of the quantum yield of the CP fluorescence. A similar phenomenon for porphyrins in detergent solution was described in [7,8]; (2) quenching by an amino acid residue at the binding site of coproporphyrin of the antibody moiety takes place as was described for protoporphyrin-apoperoxidase complex [9].

It is known that protonation of the carboxypropionic groups of CP and other porphyrins affects quantum yield of porphyrin fluorescence, i.e., an increase of the protonation degree of side carboxypropionic groups of porphy-



(a)



(b)

Fig. 2. The effect of pH on the CP (0.1 μM) fluorescence emission spectra in presence of 1 μM of Ab, clone D5 (a) or 1 μM of F(ab′)$_2$, clone D5 (b) in aqueous solution (universal buffer 0.1 M Na_2HPO_4 + 0.1 M $KHCO_3$ + 0.1 M AcONa in presence of 0.15 M NaCl). (a) pH was varied from 5 (lower spectrum) to 11 (upper spectrum) with the step of 0.5 pH unit. (b) pH = 5 (lower spectrum); 6; 6.5; 7; 7.5; 8; 9; 10; 10.5; 11 (upper spectrum).

Table I. CP Fluorescence Decay in Aqueous Solution and in a Solution of Triton X-100 Calculated Using the Equation $I = I_o * e^{-kt}$

		pH				
Medium	Parameters	5.0	6.0	7.0	8.0	10.0
Water	I_o*10^{-4}	1.10 ± 0.04	1.18 ± 0.02	1.98 ± 0.03	1.96 ± 0.03	1.63 ± 0.03
	$k*10^2$, ns^{-1}	7.40 ± 0.18	6.25 ± 0.12	6.05 ± 0.05	6.16 ± 0.06	5.83 ± 0.08
	τ = 1/k, ns	13.5 ± 0.35	16.0 ± 0.3	16.5 ± 0.2	16.2 ± 0.2	17.15 ± 0.25
Triton X-100	I_o*10^{-4}	0.15 ± 0.04			0.095 ± 0.01	
	$k*10^2$, ns^{-1}	4.94 ± 0.12			4.93 ± 0.13	
	τ = 1/k, ns	20.2 ± 0.3			20.4 ± 0.3	

rins decreases the quantum yield of porphyrins in aqueous solutions [7,8].

For CP decrease of the quantum yield was slight from pH 11 to pH 6.5; at pH 6.5–5.0 fluorescence decreased drastically, but the shape of the spectrum remained the same from pH 11 up to 5.0. We also have studied the kinetics of the fluorescence decay of the free CP and found that the lifetime of the CP molecule in the exited state (16 ns) does not change in the pH range from 6 to 8. Earlier it was postulated that in an aqueous solution of Triton X-100 solubilization of CP in the micelles is accompanied by the protonation of one of the carboxypropionic groups of CP [8]. We found that the lifetime of the CP molecule in the excited state, also in presence of Triton X-100, does not significantly change at different pH (see Table I).

Protonation of the CP carboxypropionic groups decreases the quantum yield of the CP free base but does not change the fluorescence spectrum shape and the lifetime of the free CP molecule in the excited state. Data described in [3] showed the change of the lifetime of the excited CP molecule in presence of anti-CP antibodies and it was shown that the kinetic decay curves were two-exponential. Data obtained from fluorescence polarization spectra allowed us to attribute the slow component (16 ns) to fast rotation of free CP. The fast component (2.5 ns), with slow rotation, could be attributed to the complex of CP with Ab [3]. Thus, the type of fluorescence quenching seen with the binding of coproporphyrin to the antibody has a dynamic origin [10], i.e., it is accompanied by the change of the lifetime of the excited molecule. From these results we can conclude that the strong quenching of CP fluorescence by binding to anti-CP antibodies cannot be explained by the protonation of the CP carboxypropionic groups.

To check the second hypothesis, quenching by amino acid residue(s) at the binding site of the antibody molecule, we have studied the effect of amino acids and their derivatives on the fluorescence of free CP. We see from Table II that only three amino acids quenched CP fluorescence, namely lysine, methionine, and tryptophan, the last one having a very high quenching constant. It is interesting that the quenching efficiency depended on the hydrophobicity of the quencher: the more hydrophobic amino acid derivatives were better quenchers compared to the corresponding amino acids. These results indicate that strong CP fluorescence quenching upon the binding to Ab can be caused by contact of CP with the tryptophan residues located near the binding site of the antibody. It is interesting to note that the tryptophan residue is a conserved amino acid in the peroxidase family and, according to X-ray data, is located in the porphyrin-binding pocket of peroxidase [11]. It is also possible that lysine and methionine are located in the antigen-binding cavity.

To determine the quenching type, we have studied the kinetics of the CP fluorescence decay in presence of varied concentrations of lysine, methionine, and tryptophan (Table III). We see from Table III that the CP lifetime

Table II. Stern-Volmer Constants for the Quenching of CP Fluorescence by α-Amino Acids Calculated Using the Equation $I_o/I = 1 + K[\text{quencher}]$

Quencher	K, M^{-1}
Phenylalanine	1.3 ± 0.6
Serine	1.4 ± 0.2
Arginine	1.1 ± 0.4
Cysteine	1.9 ± 0.5
Methionine	3.7 ± 0.6
Tyrosine	1.0 ± 0.2
Imidazole	$(9.8 \pm 0.5)*10^{-2}$
Lysine	3.3 ± 0.6
AcONa	1.1 ± 0.2
Cystine	1.2 ± 0.6
Tryptophan	47 ± 1
N-Acetyltryptophan amide	81 ± 3

Table III. Characteristics of the CP Fluorescence Kinetic Decay in Aqueous Solution (pH 7.3) in Presence of α-Amino Acids Calculated Using the Equation $I = I_o * e^{-kt}$

Amino acid	Amino acid concentration, (mM)	$I_o * 10^{-3}$	$k * 10^2$, ns^{-1}	$\tau = 1/k$, ns
Lysine	6.5	2.7 ± 0.1	6.1 ± 0.2	16.3 ± 0.4
	13	1.48 ± 0.06	5.8 ± 0.1	17.2 ± 0.3
Methionine	6.9	1.8 ± 0.1	6.2 ± 0.2	16.1 ± 0.4
	13	2.2 ± 0.1	6.25 ± 0.2	16.0 ± 0.4
Tryptophan	3.6	1.9 ± 0.1	6.22 ± 0.2	16.1 ± 0.4
	7.3	2.1 ± 0.1	6.39 ± 0.23	15.7 ± 0.5

was not changed (within experimental error) in the presence of these three amino acids (static quenching).

Thus, both model experiments, with pH and amino acids, clearly demonstrate the static type of CP fluorescence quenching in contrast to the complexing of antibodies with CP. We can explain this discrepancy only by protein dynamics. The dynamic character of the CP fluorescence quenching in the complex with antibody (a decrease of the quantum yield as a result of the accidental collisions of the fluorophore and quencher molecules) could be explained by the conformational mobility of the antibody backbone containing tryptophan residues near the binding site of coproporphyrin. Protein dynamics also can lead to local pH changes and consequently to different degrees of protonation of the CP carboxylic group.

External Quenching by Heavy Atom Ions

To get more information on the antigen location in the interior of the binding cavity we investigated the effect of external quenchers (iodide and cesium ions) on the fluorescence of the CP complex with Ab or $F(ab')_2$ in the pH range 6–11. We found that the character of CP fluorescence quenching by external quenchers was quite different in the absence and in the presence of Ab (or Fab′): iodide ions did not quench the fluorescence peak of CP at 612 nm at high concentrations of Ab or $F(ab')_2$ at all, and the quenching of the same peak by cesium ion was 3 times less effective in presence of Ab compared to the quenching of free CP. These results allowed us to propose that CP in the immunocomplex is located deep in the binding cavity of the Ab molecule and has restricted accessibility for the external quenchers. The greater inaccessibility of the iodide ion can be explained by the negative charge near the binding site. The other peak (630 nm, see below) was not quenched in the presence of Ab or $F(ab')_2$ by either ion at pH 6–8, but, unlike at 612 nm, was markedly quenched at pH 8–10. At high pH (10–11) the quenching efficiency of both peaks (612 and 630 nm) increased for both positively and negatively charged ions. This fact could be explained by a conformation change of the binding site and corresponding opening of the binding cavity.

pH Dependence of CP-Ab Fluorescence Intensity

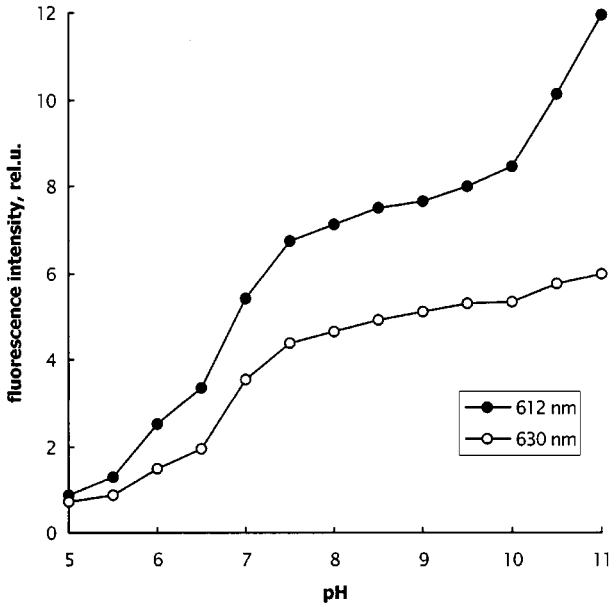
We have studied pH effect on CP fluorescence emission spectra in presence of specific antibodies (Fig. 2a) and their $F(ab')_2$ fragments (Fig. 2b). We can see from the plot of fluorescence intensity versus pH (Fig. 3), that dependence of the intensity from pH is a little different for 612 and 630 nm (two peaks prevailing at minimal and maximal Ab concentrations, respectively, see Fig. 1) at high pH, so the ratio of the CP forms corresponding to these peaks is affected by pH in the region of high pH values.

Spectra

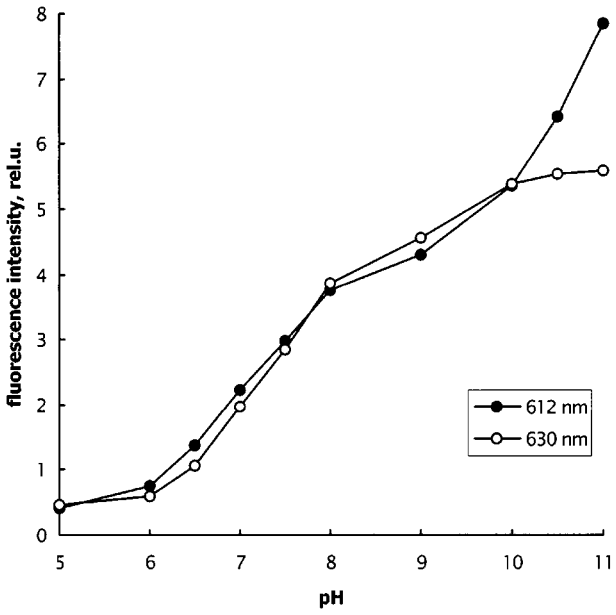
Another effect resulting from the CP-Ab binding is the shift of the maximum 612 nm to 630 nm. This shift takes place only at high Ab (or Fab′) concentrations (Fig. 1a–c). Our experiments with the external quenchers showed that the peak at 612 nm can not be attributed to the fluorescence of the remaining free CP; this peak present the properties of the CP which is bound to Ab. We propose that the 612 nm peak can be attributed to the 1:1 CP:Ab complex, and 630 nm peak to 1:2 CP:Ab complex (dimeric antibodies and one hapten). This hypothesis is based on the fact that the 630 nm peak appears at approximately the same Ab concentrations independently of different CP concentrations (10^{-8} M or 10^{-6} M, see Figs. 1a and 1c).

To verify this dimerization hypothesis we have studied the CP-Ab binding in the reverse micellar system. Reverse micellar systems are nonpolar organic solvents containing small water droplets surrounded with a monolayer of hydrated surfactant molecules.

The most important characteristic of the reverse micellar system is hydration degree, the ratio of water



(a)



(b)

Fig. 3. CP fluorescence intensity (emission at 612 and 630 nm) in presence of Ab (clone D5) (a) or F(ab′)$_2$ (clone D5) (b) versus pH (data from Fig. 2).

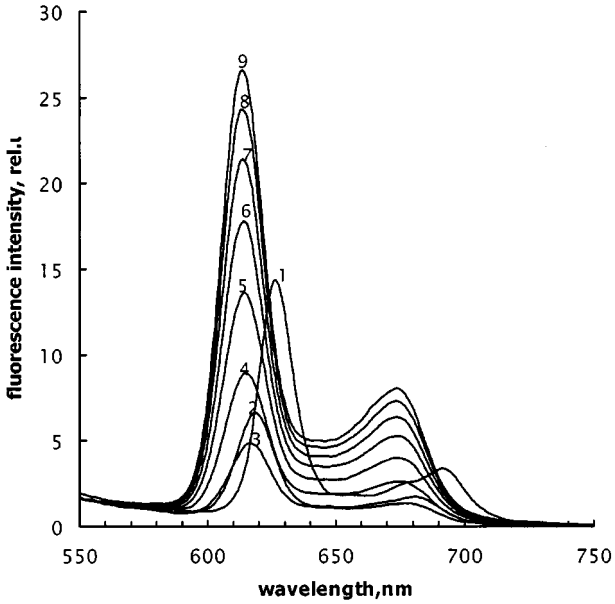


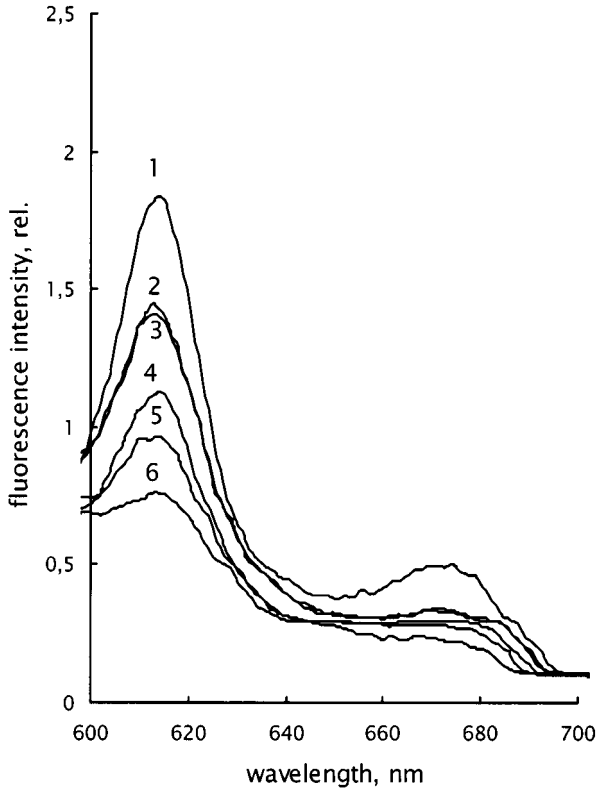
Fig. 4. CP fluorescence emission spectra in reverse micelles AOT/octane/water: effect of hydration degree: [AOT] = 0.1 M, [CP] = 0.04 μM (calculated per total volume, water pool + organic solvent), W_0 = 0.5 (1), 4.2 (2), 8.3 (3), 12.5 (4), 16.7 (5), 20.8 (6), 25 (7), 29.2 (8), and 33.4 (9).

and surfactant concentrations (W_0 = [H_2O]/[surfactant]). This parameter defines the size of the reverse micelle. Reverse micellar systems, due to their ability to form micelles of the desired size, are able to solubilize the antibody-antigen complex and it is known that antigen-antibody binding takes place in reverse micelles [12–19]. Recently it was demonstrated, that W_0 influences the affinity of anti-fluorescein antibodies to fluorescein [19].

Fluorescence spectra of CP in reverse micellar system are strongly affected by W_0: fluorescence intensity increases with the increase of W_0 (Fig. 4). If no water was added to the reverse micellar system ($W_0 < 1$),[a] the maximum of the CP fluorescence emission spectrum was at 627 nm. The maximum was shifted to 613 nm with the increase of W_0 (Fig. 4). This means that the value of W_0 had to be kept constant while studying the Ab effect

[a] Commercial surfactant (AOT) preparations usually contain small amount of water, of about 0.2–0.5 water molecules per one surfactant molecule.

Fig. 5. The effect of added anti-CP (a–c) or non-specific (d) antibodies on CP emission spectra in reverse micelles AOT/octane (W_0 = 16.7, [AOT] = 0.1 M). (a–c) [Ab] (anti-CP, clone D3) = 0 (1), 0.14 (2), 0.29 (3), 0.57 (4), 1.14 (5), and 2.29 (6) μM; [CP] = 0.04 μM (a), 0.12 μM (b), and 0.37 μM (c). (d) [Ab] (non-specific, anti-insulin) = 0 (1), 0.12 (2), 0.25 (3), 0.49 (4), and 1.0 (5) μM, [CP] = 1 μM. Ab and CP concentrations are calculated per water pool of reverse micelles.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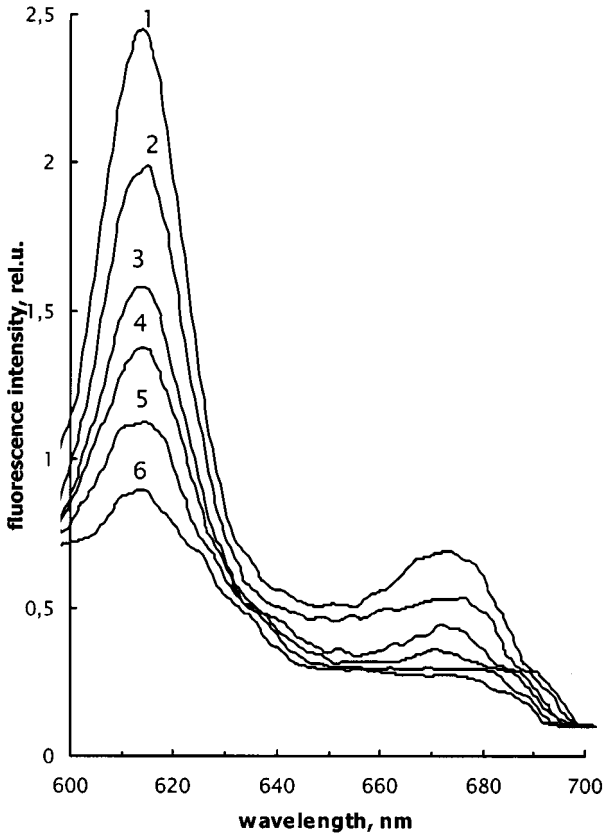
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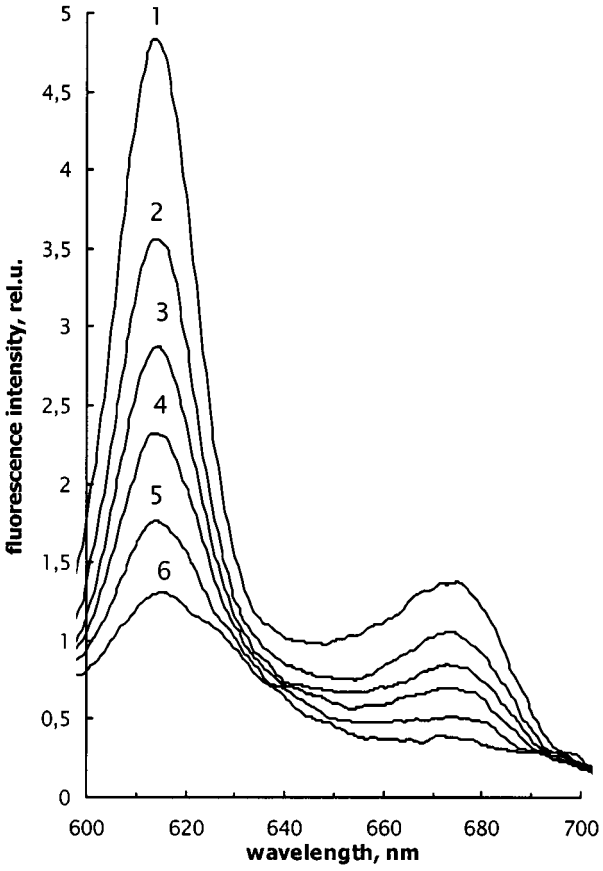
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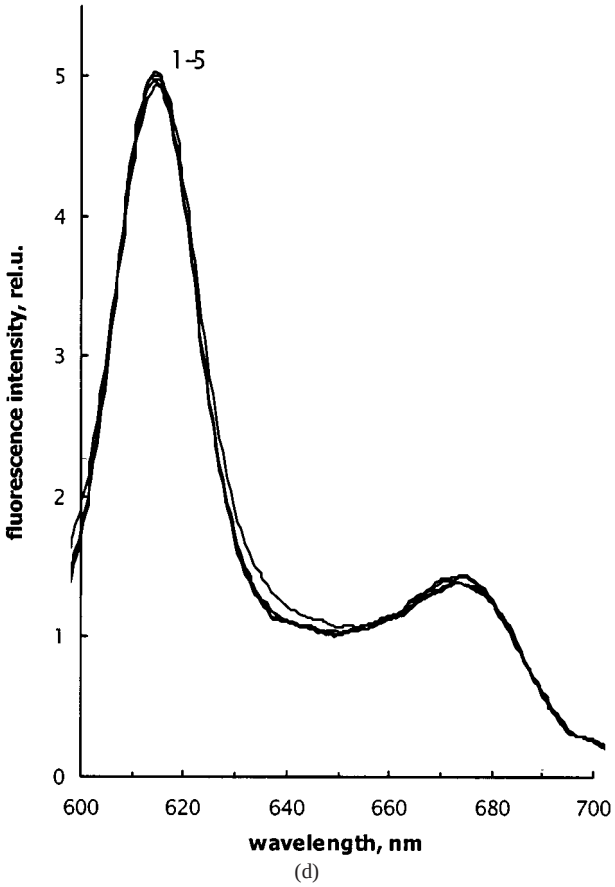
(c)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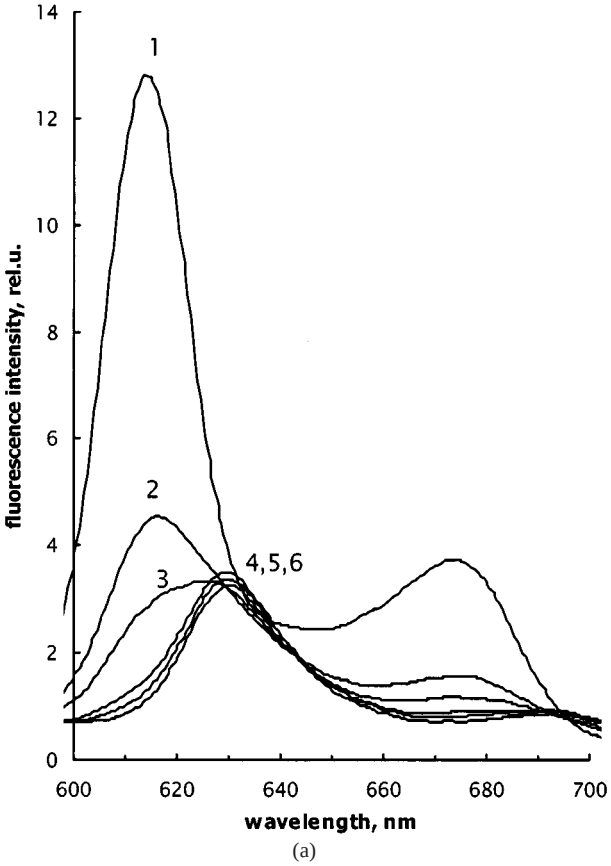
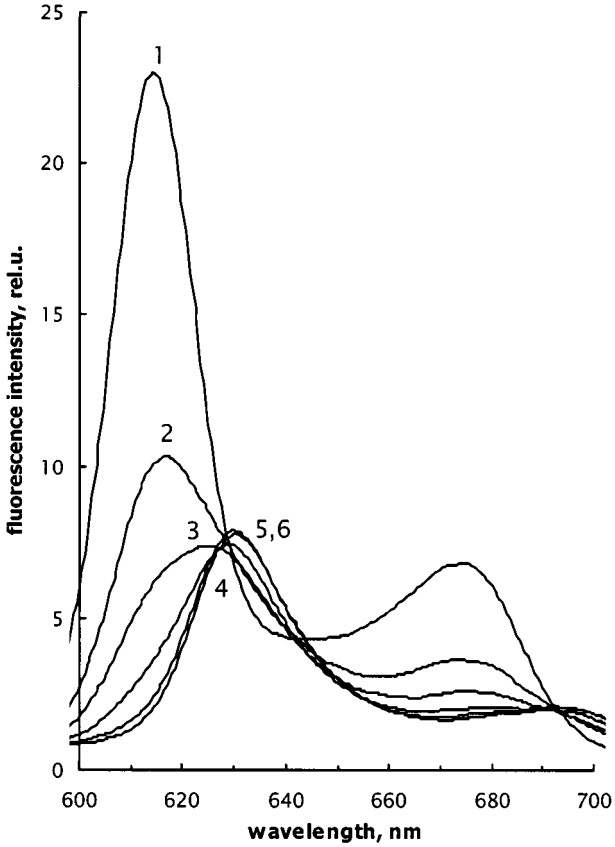
(d)



(a)



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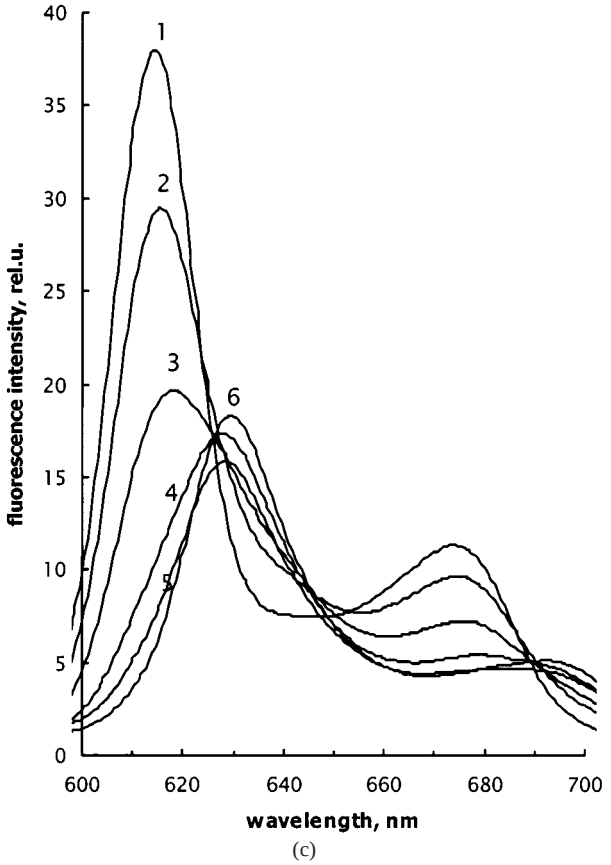

(c)

Fig. 6. High concentrations of CP and anti-CP antibodies (clone D3) in reverse micelles AOT/octane (W_0 = 16.7, [AOT] = 0.1 M). [Ab] = 0 (1), 2.4 (2), 4.8 (3), 9.5 (4), 19 (5), and 38 (6) μM; [CP] = 1.33 μM (a), 4 μM (b), and 12 μM (c); Ab and CP concentrations are calculated per water pool of reverse micelles.

on the CP fluorescence spectra. We chose the $W_0 = 16.7$ (corresponding to the water content of 30 μl per 1 ml of 0.1 M AOT in *n*-octane) because a hydration degree of 15–20 was optimal for the binding of propazine and anti-propazine antibodies in the same reverse micellar system [18].

CP fluorescence was quenched after the addition of anti-CP antibodies in the reverse micellar medium (Figs. 5a–5c) similar to results obtained in aqueous solution (Fig. 1). This quenching effect was also specific, because no quenching was observed if nonspecific antibodies were used instead of specific ones (Fig. 5d).

The effects of the concentration of added anti-CP antibodies on the CP emission spectra in reverse micelles are presented in Figures 5a–c. We investigated the same real concentrations of CP and antibodies in reverse micelles (Fig. 5a) as in water (Fig. 1c). We proposed that the both antibodies and CP are located mainly in the water pool of the reverse micelles, so "real" in this case means that we calculated the concentrations per aqueous microphase (water pool) of the reverse micelles, but not per total volume of organic bulk phase plus water microphase. In reverse micellar medium (Figs. 5a–c) we see only the quenching effect of the antibodies, but no shift of the emission maximum as was seen in water (Fig. 1c). This result confirms our dimerization hypothesis since the main difference between water and reverse micelles is that the reverse micelle is able to solubilize only one antibody molecule, so dimerization of Ab is much less possible in reverse micelles compared to water (assuming the Ab concentration is the same). The presence of the quenching effect indicated that Ab-CP binding takes place both in reverse micelles and water.

It is interesting that we observed a shift of the CP emission maximum in reverse micelles if we drastically increased Ab concentration in the water pool of reverse micelles (Fig. 6). A possible explanation for this effect could be again Ab dimerization. It is known that reverse micelles are able to solubilize large protein molecules whose size is greater than that of the water pool of the reverse micelle. So it could be possible (to a very small extent) that there are Ab dimers in AOT reverse micelles at $W_0 = 16.7$. If we increase both CP and Ab concentrations, the equilibrium Ab(monomer) ↔ Ab(dimer) ↔ [Ab(dimer)CP] is shifted, finally, to the right side, enough to detect the shift of the maximum of the fluorescence spectrum.

We propose a special kind of Ab dimerization upon the binding with CP. Fab′ fragments of the Ab are involved in this dimerization process (because the 630 nm peak was observed not only for Ab but also for separated Fab′ fragments of the Ab). Because the peak position for CP in reverse micelles at low W_0 is also red shifted, we propose that the surrounding of CP in a dimer is similar to that in reversed micelles with low level hydration degree. The same surrounding for CP also appeared in complexes of antibodies with 1:1 conjugate of CP with a large protein, soybean inhibitor of trypsin (SIT). The large SIT molecules (20 kD) prevent the direct CP interaction with the second Ab at the binding site, but the 629 nm peak still exists (Fig. 7).

CONCLUSIONS

The antigen-antibody interaction between CP and anti-CP antibodies was studied by a fluorescence method in water and a reverse micellar system, *n*-octane/Aerosol OT. We observed quenching of the CP fluorescence after binding to the antibodies. The mechanism of this quenching is static quenching, most probably, by tryptophan residues (or maybe lysine or methionine). Apparent dynamic quenching in this case arises from protein backbone motion. A new peak at 630 nm that appeared upon antibody binding to CP can be attributed to a special kind of antibody Fab′-Fab′ dimer as was confirmed in experiments in reverse micelles. We propose that the surrounding of CP in a dimer is similar to that of reversed micelles with a low level of the hydration degree.

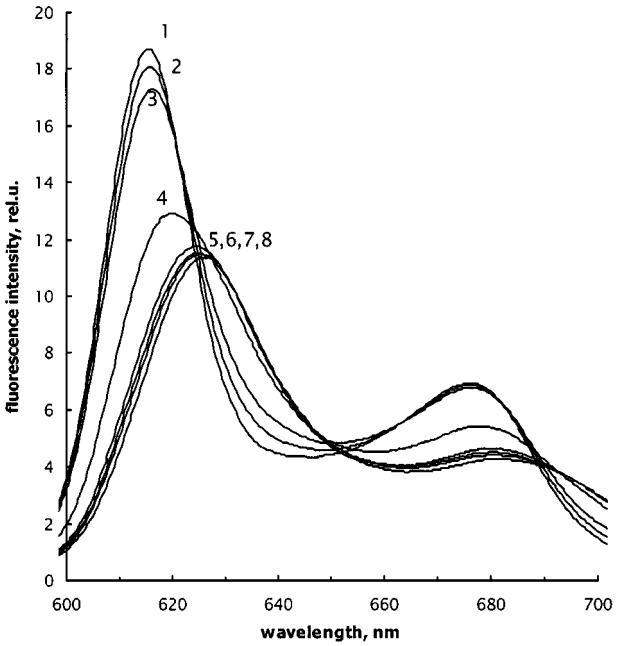

Fig. 7. Fluorescence emission spectra of the conjugate CP-SIT (1 μM) in presence of anti-CP antibodies (clone D5); [Ab] = 0 (1), 0.2 (2), 0.5 (3), 0.6 (4), 0.7 (5), 0.8 (6), 1.3 (7) and 2 (8) μM.

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