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Study of Interaction of Ceruloplasmin, Lactoferrin, and Myeloperoxidase by Photon Correlation Spectroscopy

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Abstract—In this work, the diameters of protein complexes formed upon interaction of ceruloplasmin (CP) with lactoferrin (LF) and myeloperoxidase (MPO) were determined. Gage dependence of the diameter of protein particles (myoglobin, albumin, LF, CP, MPO, aldolase, ferritin) on their molecular mass logarithm was calculated. The diameter of a complex formed upon mixing CP and LF was 8.4 nm, which is in line with the radius of gyration obtained previously when the 1CP–1LF complex was studied by small-angle X-ray scattering. The diameter of a complex formed upon interaction of CP with MPO is 9.8 nm, corresponding to the stoichiometry 2CP : 1MPO. The diameter of a complex formed when LF is added to the 2CP–1MPO complex is 10.7 nm. The latter is consistent with the notion of a pentameric structure 2LF–2CP–1MPO with molecular mass of about 585 kDa.

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Key words: ceruloplasmin, lactoferrin, myeloperoxidase, protein–protein interaction, photon correlation spectroscopy

Photon correlation spectroscopy (PCS) is widely used for evaluation of particle diameter in solution, including studies of complexes formed by proteins [1]. Studying the stoichiometry of protein–protein interactions by spectral methods is more advantaged than using chromatography as it excludes interplay between the components of the protein complexes with the surface of the chromatographic matrix. Anionic and cationic proteins can manifest a stronger interaction with chromatographic resin because part of their charge is screened within a complex, the latter having a smaller overall charge than its components taken separately. Such an effect can result in underestimation of molecular mass values obtained by gel filtration [2]. The objects of investigation in the present study, i.e. metal-containing proteins ceruloplasmin (CP), lactoferrin (LF), and myeloperoxidase (MPO), can form stable complexes under physiological conditions of pH and ionic strength in solution [3]. Leukocytic proteins LF and MPO have pronounced cationic properties (p*I* 8-9 and 9-10, respectively). Their complexes with anionic CP (p*I* 4.7) are formed due to electrostatic interactions as CP–LF and CP–MPO complexes dissociate upon increasing ionic strength or decreasing pH in solution [3]. Interaction with LF results in an increase in ferroxidase activity of CP, which is interesting because LF is able to efficiently chelate the reaction product, Fe^{3+} [4]. Direct contact of CP with the active center of MPO results in inhibition of peroxidase and chlorinating activities of the latter [5]. Studying the stoichiometry of the complexes formed by CP with LF and MPO led to ambiguous results. For instance, disc-electrophoresis demonstrated the possibility of formation of complexes CP–2LF, CP–4LF, and of those with even greater share of LF [6].

On the other hand, gel filtration revealed the presence of solely the complex with equimolar relation of partner proteins, 1CP–1LF [7]. Small-angle X-ray scattering beamed the presence of the complex 1CP–1LF in solution, along with which a study of the conditions of complex formation showed that using phosphate buffer allows formation of multi-component complexes [8]. Similarly, a number of methods (gel filtration, disc-electrophoresis) detected equimolar complexes formed upon interaction of CP with MPO [3]. However, kinetic

Abbreviations: CP, ceruloplasmin; LF, lactoferrin; MPO, myeloperoxidase; PCS, photon correlation spectroscopy.

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parameters of the enzymatic reactions changed when CP interacted with MPO, along with changes in spectral features of complexes with various ratios of partner proteins indicating that the proteins interact at 2CP : 1MPO ratio [5]. In particular, it is enough to add 2 moles of CP to 1 mole of MPO to shift the maximum of the absorption caused by the heme in MPO. Such a ratio of the partner proteins seems logical on account of the dimeric structure of MPO. Also, 1 mole of MPO added to 2 moles of CP is sufficient to launch *p*-phenylenediamine oxidation catalyzed by CP [5]. A study of the triple complex formed by CP, LF, and MPO showed that the complex is eluted from the column within the volume corresponding to the 1 : 1 : 1 ratio of the components [3]. This suggested that when LF makes contact with CP, the latter can interact only with one of the two protomers of MPO. Nevertheless, when affinity chromatography was used we found no competition between LF and MPO for interaction with CP [3]. Furthermore, LF would never impede the inhibitory activity of CP upon MPO, which rules out the displacement of one of the two CP molecules from the 2CP–MPO complex upon joining LF [9].

In this study we investigated the stoichiometry of complexes formed by CP, LF, and MPO using PCS.

MATERIALS AND METHODS

Reagents used in the study were produced by BioRad (USA), Merck (Germany), Pharmacia (Sweden), Serva (Germany), Sigma (USA), and MEDIGEN Laboratories (Russia).

Monomeric CP remaining stable upon long storage, having $A_{610}/A_{280} > 0.045$ and the prevalence (95%) of non-fragmented protein in the preparation, was obtained by affinity chromatography of human blood plasma on protamine-Sepharose [10]. Homogeneous LF was purified from breast milk using ion-exchange chromatography on CM-Sepharose and gel filtration on Sephadex G-100 superfine [6]. MPO from human leukocytes was obtained by successive affinity chromatography on heparin-Sepharose, hydrophobic chromatography on phenyl-Sepharose, and gel filtration [3]. The A_{430}/A_{280} ratio (R_z) in the resulting preparation was 0.85, which is characteristic of homogeneous MPO.

Particle diameter determination in a sample using a Beckman Coulter N5 Submicron Particle Size Analyzer is based on registering the particle diffusion rate in a fluid. It is described by the Stokes–Einstein equation: $D = k_B T/3\pi\eta d$, where $k_B = 1.38 \cdot 10^{-16}$ (erg/K) is Boltzmann's constant, T (K) is temperature, η (poise) is viscosity of the solvent, d (cm) is the diameter of a particle, for which the diffusion coefficient D is estimated. Thus, determination of D contains information about the diameter of particles. The Beckman Coulter N5 analyzer uses PCS and data elaboration by the Contin program to determine particle diameter. A laser with 632.8 nm wavelength is installed, and the range of registration is from 3 to 3000 nm. We used reference proteins with known molecular masses for which the particle diameters had been ascertained, such as myoglobin (19 kDa), albumin (67 kDa), LF (78 kDa), CP (132 kDa), MPO (145 kDa), aldolase (158 kDa), and ferritin (450 kDa). Water and TBS buffer (10 mM Tris-HCl, 150 mM NaCl, pH 7.4) used in experiments were filtered through a filter with 0.22 μm pores. Protein concentrations in solution were varied empirically from 1 to 10 mg/ml, since the intensity of light scattering controlled prior to the measurements should remain within certain limits as required by the instructions for the device. Statistical elaboration of the data was done using Microsoft Excel 2007.

RESULTS AND DISCUSSION

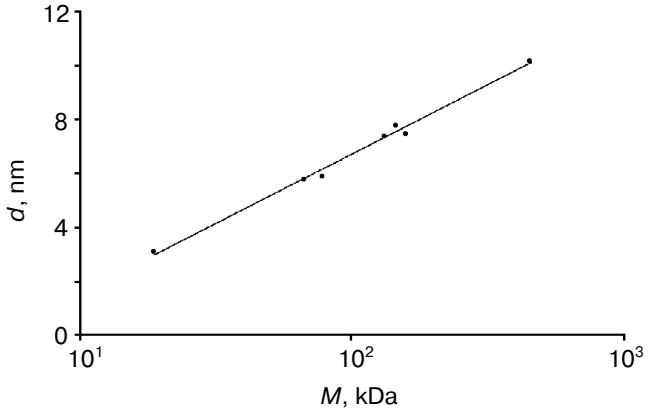
Diameter values for particles of globular proteins with known molecular masses (myoglobin, albumin, LF, CP, MPO, aldolase, ferritin) were estimated. Table 1 contains data on the correspondence of the values obtained to those resulting from crystallography and small-angle X-

Table 1. Comparison of diameters of particles estimated by PCS and those taken from the literature

Protein	d, nm (N5 Beckman Coulter)	d, nm
Myoglobin	3.1 ± 0.2	3.5 [11]
Albumin	5.8 ± 1.8	6.0 [11]
Lactoferrin	5.9 ± 1.0	6.4 [8]
Ceruloplasmin	7.4 ± 0.8	7.2 [8]
Aldolase	7.5 ± 2.3	8.0 [11]
Myeloperoxidase	7.8 ± 1.2	7.6 [12]
Ferritin	10.2 ± 3.3	12.2 [11]

Table 2. Diameters of complexes (d) as measured by Beckman Coulter N5 instrument and molecular mass (M) values (measured and calculated) obtained upon mixing CP with LF and MPO

Complex (number of determinations)	d, nm	M_{mes}, kDa	M_{calc}, kDa
1CP–1LF (15)	8.4 ± 1.2	209 ± 30	210
1MPO–2CP (12)	9.8 ± 1.4	386 ± 55	409
1MPO–2CP–2LF (16)	10.7 ± 0.8	585 ± 44	565



Calibration dependence of molecular mass (logarithmic scale) on protein particle diameter

ray scattering (SAXS). The Pearson correlation coefficient between experimental data and the diameter of protein particles (d) presented in the literature was estimated as 0.97. A calibrating graph (figure) reflecting the dependence of particle diameter (d, nm) on the logarithm of molecular mass (M, kDa) can be described as the equation $d = 5.19\log M - 3.66$ and characterized by coefficient of determination $R^2 = 0.99$.

Particle diameter values and the respective molecular mass values for the complexes of CP with LF and MPO are summarized in Table 2. When diameter of particles formed upon mixing CP and LF was measured, values having low polydispersity index were obtained only for the 1CP–1LF mixture. When LF was added to CP in amounts surpassing the equimolar relation, particle diameter values decreased while the mean square deviation grew. This spoke in favor of accumulation in the mixture of free LF along with the 1CP–1LF complex as PCS allows discrimination between particles that have diameters differing at least 2-fold; otherwise, diameters are averaged and the standard deviation increases. The diameter 8.4 nm obtained for the CP–LF complex is close to the value we had obtained previously by SAXS, i.e. 8.6 nm [8].

Upon adding CP to MPO, particle diameter values with low polydispersity index were obtained for the mixture with molar relation 1MPO–2CP. The molecular mass value of this complex (386 ± 55 kDa) confirms correctness of the conclusion of such a molar ratio. Adding to the 1MPO–2CP complex the two-molar equivalent of LF resulted in growth of the complex size. This questions a possibility of complex formation with 1MPO–1CP–1LF stoichiometry that should have a smaller diameter than 1MPO–2CP. Judging by the molecular mass value obtained (585 ± 44 kDa), the complex 1MPO–2CP–2LF most probably was formed. Further addition of LF resulted in a decrease in particle diameter and in an increase in standard square deviation.

Summarizing these data, two binding sites for CP on a dimeric MPO molecule can be postulated. This seems highly probable on account that an inhibitory molecule of CP should contact with each of the two active centers in MPO. This conclusion is supported by the studies on the effect of MPO on CP activity towards *p*-phenylenediamine, when 1 mole of MPO was enough to activate 2 moles of CP [5]. Upon adding LF to the 1MPO–2CP complex, each CP molecule interacting with monomers of MPO is able to bind LF. This results in formation of a pentameric structure: LF–CP–MPO–CP–LF.

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